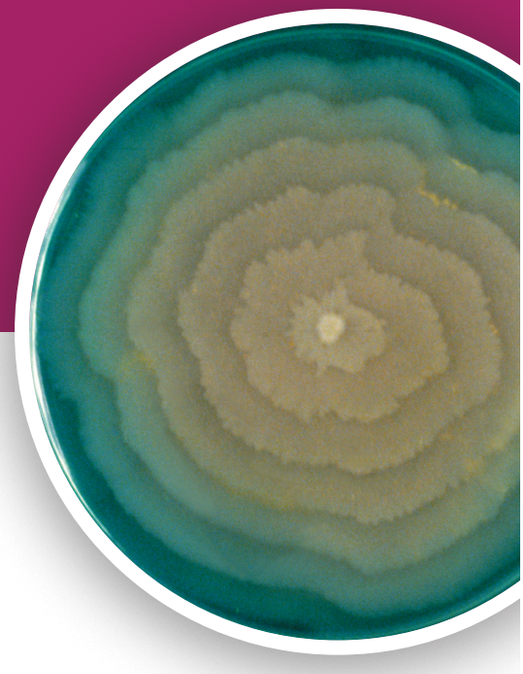
Second Edition

MICROBIOLOGY

Laboratory Theory & Application

ESSENTIALS



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This book is dedicated to the more than 3,000 U.S. health-care workers who lost their lives to COVID-19 during the 2020–2021 pandemic.

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PREFACE

Revision Reflections

The bulk of this second edition of *Microbiology: Laboratory Theory & Application, Essentials*, was developed under the specter of COVID-19. Given this timing, I often reflected on the word *essential*—a word that echoed in the media as we heard about essential workers, essential new vaccines, and essential treatments and transmission precautions. The fact that this book has been distilled to essentials from more comprehensive progenitors of the *Microbiology: Laboratory Theory & Application* series (*MLTA* series) was not lost on me, and it reminded me that most of the students taking an introductory microbiology course are interested in pursuing a career in health care-related fields. Indeed, it was a driving force for envisioning how to further refine the book to meet the training needs of our essential health-care workforce of tomorrow—or, as I refer to them, my students, many of whom are actively engaged in countermanding the pandemic.

My colleagues and now-retired peer authors, Mike Leboffe and Burt Pierce, spent the better part of three decades shaping the *MLTA* series and it has been my distinct honor to become the steward and new voice of the series. As Mike has done in previous revisions that he spearheaded upon Burt retiring, I maintained the "we" in the revision as a recognition of this work as an evolution of a longer standing and already warmly received text. My intentions in this revision were not to supplant these talented and experienced voices, but to augment them in a way that rounds out the series with an eye on molecular methods and medical applications, both of which are areas within my expertise as a medical scientist.

To that end, this second edition revision is a comprehensive reflection on the student laboratory learning experience and what is essential and employable for students interested in becoming health-care workers or pursuing medical microbiology, a laboratory sciences credential, or another medically focused discipline. The revision also considers those students who take a microbiology course to enter public health, epidemiology, or some other career where an introduction to microbiology laboratory curriculum is useful. Ultimately, at some point in our lives we are all patients, so a generalist non-major would also find this content useful. In short, this revision considers the student who is new to microbiology and may not yet have substantive prerequisite knowledge.

Synopsis of Changes from *MLTA Essentials,* 1e, to *MLTA Essentials,* 2e

1. The lab exercise order has been reorganized to more closely align with the order of a typical microbiology lecture/lab sequence.
2. Learning outcomes have been added to all exercises.
3. Content has been partitioned under more subheadings to decrease cognitive load.
4. Lab Technique reference sheets have been added to allow students to easily refer to them when performing these techniques in later labs.
5. The Background and Clinical Application sections were modified to better meet students where they are coming from and explain to them where they need to go and what they need to know to be successful in a clinical setting.
6. Wording in figure legends has been reduced and details have been layered directly into the art where appropriate.
7. Procedural diagrams now follow a linear format for easier comprehension.
8. Lab related questions have been diversified to include a more stratified representation of Bloom's learning taxonomy. In addition to the "in lab" documentation of findings, students now have access to multiple choice, true/false, labeling, short answer/fill in the blank, and critical thinking questions to support their review of content. These questions can be used as pre-lab and/or post-lab work.
9. Many references have been removed to save space. References have been added to footnotes only for material that is cutting edge and may not yet be widely known, for topics that are not general knowledge and that must really be investigated, and for hotly debated topics.

Exercise 1

- This exercise is divided into parts A, B, and C to help faculty more easily direct students to the parts of the lab they plan to cover.
- A specific exercise for using oil immersion was added.
- The discussion of how to calculate the limit of resolution was omitted.

- The discussion of numerical aperture also was omitted.
- A review of real versus virtual images and the optics details therein was deleted.

Exercise 2

- This exercise is divided into parts A, B, and C to help faculty more easily direct students to the parts of the lab they plan to cover.
- Materials for each section are cross-referenced for easier set-up.
- The "measuring organisms" aspect of the lab was removed, because the vast majority of reviewers stated that calibrating the micrometer is not a lab they deem essential, and many said they omit it. Also, quantitative assessment of microbial dimensions is not usually a learning outcome in an allied health microbiology course.
- The review of eukaryotic organisms by superkingdoms was eliminated to more closely mirror what students would be doing in the lecture portion of the course (the three domain and six kingdom model). In medicine, we still refer to protists and specifically to protozoans. This is important terminology for students to effectively tackle the pharmacology and pathophysiology aspects of their training. That said, the figure on the seven superkingdoms is retained and this taxonomic model's existence is acknowledged.
- A new figure on the superkingdoms was added.
- A revised figure on taxonomic domains and kingdoms also was added.

Exercise 3

- This exercise is divided into parts A, B, C, and D to help faculty more easily direct students to the parts of the lab they plan to cover.
- The procedural diagrams were completely overhauled to make the exercise easier for a novice to follow and perform.
- This lab now employs a highly modularized format in the form of Lab Techniques that give faculty the instructional flexibility they want and need.
- Inoculation of agar deeps was added. A high percentage of adopters use later labs that employ use of agar deeps and thus may appreciate an earlier exposure to how to handle agar deeps.
- Content on complex versus enriched media was added because we build on this concept in the biochemical test media section.
- A review of inoculating loops and needles and the commentary on aseptic transfer and its goals was incorporated into the lab instead of having it as a sidebar feature box before the lab.
- References to moving the tube versus the inoculating tool were removed. That approach is awkward in terms of dexterity maximization and anything that reduces manual dexterity is a risk in the lab.

Exercise 4

- This exercise is divided into parts A, B, and C to help faculty more easily direct students to the parts of the lab they plan to cover.
- The content was revised to make it more modularized.
- Additional content was added discussing environmental sampling with a strong cautionary statement on safety.
- Quadrant plate inoculation steps were combined into a single schematic (Fig. 4.3) and the text was modified to emphasize sterilization and cooling of the loop between plate sections, an essential step that students often overlook.
- The protocol was updated to include safer practices (e.g., keeping the lid on instead of exposing the sample to air).

Exercise 5

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 6

- The art layout was changed to a field guide format to highlight the beautiful photography.

Exercise 7

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 8

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 9

- A safety warning based on the CDC's safety recommendations for undergraduate teaching labs was added.

Exercise 10

- In this exercise, the explanations were simplified and calculations were broken down more systematically.
- Inherent limitations of the standard plate count method were included.

Exercise 11

- The photos were organized into a mini Instructional Atlas for Staining so that it can be highlighted as a key feature instead of relegated to a superfluous, between-the-exercise space.
- The lab was shifted to be a review of simple staining and bacterial cell morphology.
- The morphology content was incorporated into the lab proper to reinforce the message that the material is not optional or ancillary but is required to achieve the lab's teaching goal.
- Cell surface area content, which is more appropriate for a lecture, was removed.
- Staining screen was removed from the materials list, because it is not used in most teaching labs and isn't needed in the procedure.

Exercise 12

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 13

- The Background content for Gram-stain chemistry was clarified.
- A common misconception that students hold (i.e., that the Gram-staining procedure "makes" bacteria Gram positive or Gram negative) was addressed.
- The Gram-variability information was clarified.

Exercise 14

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 15

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 16

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 17

- The exercise title was modified to draw more attention to the exercise content because it is an important topic for introductory students.

Exercise 18

- Additional information on obligate anaerobes and also what to look for in the middle of the tube in terms of oxygen gas levels was added.

Exercise 19

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 20

- A new figure based on using 25 broth tubes instead of 21 was added (Fig. 20.3 broth and plate sample organization and inoculation process).

Exercise 21

- Terminology was revised to use the more prevalent term *neutralophile* to prevent student confusion between neutrophile and neutrophil.

Exercise 22

- A BSL-1 agent was added as a suggested replacement for the BSL-2 agent.
- Spot inoculation was omitted and replaced with zigzag inoculation because it is more successful in showing the desired effect.
- Table 22.1 was updated to remove common misconceptions.

Exercise 23

- The Background section was greatly expanded to explain what coliforms are and why we would want to be able to detect them in a sample.
- Updated information on neutral red dye was added to be more accurate.
- Spot inoculation was omitted and replaced with zigzag inoculation because it is more successful in showing the desired effect.
- Table 23.1 was updated to remove common misconceptions.

Exercise 24

- Spot inoculation was omitted and replaced with zigzag inoculation because it is more successful in showing the desired effect.
- Table 24.1 was updated to remove common misconceptions.

Exercise 25

- A safer option was added for professors who want to use stock cultures instead of throat swabs.
- For those using throat swabs, additional safety guidelines are provided.
- A correction was added regarding methemoglobin, which is oxidized, not reduced.
- A streak-stab technique for known stock cultures was included in the procedure to better align Background content with the procedure.

Exercise 26

- The Background section was entirely rewritten for clarity, including foundational details on the two metabolic processes mentioned. Electron tower content was removed.
- Content on ascertaining motility was removed because O-F media is not typically used for motility determination.

Exercise 27

- A key has been added to better explain fermentation broth results.
- Clarifying information has been added to Table 27.1 on fermentation broth test results and interpretations.

Exercise 28

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 29

- Extraneous content on how aerobic respiration generates toxic oxygen intermediates has been removed for a tighter alignment with lecture textbooks.
- Tables and figures have been more seamlessly interwoven into the Background section.

Exercise 30

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 31

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 32

- More information has been added about citrate, and corrections have been made to the mechanism involving citrate permease (citrate lyase acts before citrate permease; in addition, citrate permease is distinct from oxaloacetate decarboxylase).

Exercise 33

- A reminder to students to inoculate negative controls has been added.

Exercise 34

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 35

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 36

- Exercise 5-11 SIM Medium and Exercise 5-16 Motility Agar from the previous edition have been combined into one exercise. This was done because the stand-alone motility agar is usually only used when the bacterium reduces sulfur and thereby obscures the motility observations in SIM agar.

Exercise 37

- The content related to interpretations of the test results has been broken up to promote student comprehension.
- The explanation for the "reversion process" has been reworked to make it clearer for students who struggle with this topic.

Exercise 38

- The Application section has been greatly expanded to showcase why it is important to differentiate *S. aureus* from other less virulent *Staphylococcus* species and the problems that *S. aureus* can cause in the human body.

Exercise 39

- This is a new lab to the second edition of this book. It was added to showcase the importance of this test in identifying important pathogens such as *S. aureus, S. epidermidis*, *B. anthracis, B. cereus, C. tetani, C. perfringens, C. difficile,* and others.

Exercise 40

- A fuller definition and explanation of hand washing has been added to this lab since it is such a foundational tool for soon-to-be health-care providers.
- The procedure has been clarified so students know when to wear gloves and when not to.

Exercise 41

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 42

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 43

- The description of the zone of inhibition has been clarified, and students are more thoroughly instructed on how to develop conclusions about the test bacterium's susceptibility to the antibacterial drug(s) being tested.

Exercise 44

- Students are now encouraged to compare these tests to the Kirby-Bauer test performed in Exercise 42. In so doing, they will see that bacitracin, novobiocin, and optochin susceptibility testing is *far* less rigorous in terms of standardization than the Kirby-Bauer test. This is because bacitracin, novobiocin, and optochin susceptibility tests are not used to guide decisions about which drugs would be most effective against an infectious agent afflicting a patient. Instead, these susceptibility tests are for bacterial identification. The previous edition did not make this important point clear.
- This edition emphasizes to students that the drugs tested in this exercise are dangerous if ingested or injected and therefore, aside for certain topical applications, they have limited (and in some cases zero) approved applications in human medicine.

Exercise 45

- No major changes were made to this exercise besides those listed in the Synopsis of Changes, page iii.

Exercise 46

- This epidemic simulation lab now has two versions: the first, as in the previous edition, uses *S. marcescens* as the transmissible agent; the second uses Glo Germ powder or gel as the transmissible agent. This will be a better option for schools with more stringent safety standards.

Exercises 47 and 48: Unknown Laboratory Exercise

The first edition included five unknown exercises. For this edition, these exercises were condensed down to two unknown labs (Ex. 47 and Ex. 48) and a variety of resources for aiding students as they perform the unknown exercises.

- Exercise 47: This dry lab helps students explore the process of identifying an unknown in the context of a clinical case. Because it is a dry lab it can be done in class or at home as either a preparation for the hands-on unknown lab exercise (Ex. 48) or as an augmentation to the wet-lab aspect of the course. The question series is deigned to be fully scorable and is carefully designed as a problem-based exploration to encourage students to carefully reflect upon and apply what they have learned.
- Exercise 48: The flowcharts from the previous edition still exist in an appendix. However, the content has been modified to better express to students that time is of the essence in clinical situations and that consequently a variety of biochemical tests are typically performed simultaneously to reduce time to definitive bacterial identification.

Acknowledgments

The first time I met Mike Leboffe in person was at an ASMCUE (American Society for Microbiology, Conference for Undergraduate Educators) meeting in Washington, DC, in 2019. Ahead of this in-person encounter, Mike and I mainly knew of one another through our respective authoring endeavors and through our conversations with David Ferguson, President and CEO of Morton Publishing. Knowing that Mike was ready to follow Burt into retirement, David brokered a year-long episode of the Dating Game to bring us together and map a path to *MLTA*'s continued legacy and evolution.

Having labored on developing and updating my own textbook, I can fully appreciate how, after so many years of attentive care, it would be difficult to entrust one's "book baby" to someone else. After all, a book used to teach *thousands* of people undergoes a considerable amount of scrutiny, and putting one's name alongside another's on the cover takes a tremendous amount of trust and vulnerability tolerance. As such, I sincerely thank Mike and Burt for their vote of confidence that equates to entrusting me to be the new captain of the *MLTA* ship they built and launched. I promise you both that I'll carefully circumvent icebergs when charting new waters, keep the rigging in good order, and not fall prey to the sirens of complacency as the discipline and teaching practices evolve.

As the top executive at Morton Publishing, David would have some difficult questions to answer if he selected the wrong person to assume one of Morton's flagship enterprises, so I am honored that he entrusted the task to me. All this to say, being the steward and new voice of the career work of others has a weighty significance that I am proud to undertake and do not take lightly. It's a tangible compliment be deemed "the right person."

For my part, taking on the *MLTA* series was not something I ventured into without serious reflection. Being an author is *a lot* of work and most academic authors, this one included, have numerous irons in the

proverbial fire. I had to consider how and if I could give the *MLTA* series the attention it requires while balancing my other career and familial obligations. The task wasn't without its challenges, but thanks to Marta Pentecost (Senior Acquisitions Editor) and Rayna Bailey (Editorial Project Manager), those challenges were made conquerable. These brilliant and impressive women made working closely while socially distancing as natural as breathing. Of course, the editorial effort would never have wings without the production team in our star lineup. I want to thank Will Kelley (Production Manager) and Joanne Saliger (Production Assistant) for their careful work on paging, implementing art and design revisions, and all other things production related.

I also extend my gratitude to our faculty reviewers, adopters, and students who have provided invaluable feedback to direct the revision process. You are the "why" behind it all. To Erin Amerman, my dear friend and fellow authoress, I owe a special debt of gratitude for her unflagging friendship, encouragement, and vote of confidence. I have not encountered many people who are as secure and grounded as Erin and who have the generosity of spirit that she has.

Lastly, I thank my much-loved family for their support as I spent *many* late nights and weekends entrenched in edits and page proofs. To my two cherished daughters, Lourdes Catherine and Delia, thank you for teaching me how to love unconditionally. And, to my dear husband, Andrew, you are the glue that keeps me together. ●

Lourdes Norman-McKay, Ph.D.
April 2021

About the Author



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Dr. Norman-McKay earned her B.S. in microbiology and cell science from the University of Florida and her Ph.D. in biochemistry and molecular biology from the Pennsylvania State University College of Medicine. She completed a postdoctoral fellowship in microbiology and immunology at Penn State College of Medicine, where she was awarded a National Research Service Award from the National Institutes of Health to study the role of viruses in cancer. She has 19 years of experience teaching a wide range of general sciences such as biology, virology, biochemistry, microbiology, anatomy and physiology, and clinical genetics to students at all levels from freshman to postgraduates. She has been recognized by her peers and students with the Outstanding Faculty Award at Florida State College at Jacksonville, where she is currently a full-time professor. Dr. Norman-McKay has also served as an associate dean over science programs, developed curricula for STEM programs (to include a baccalaureate degree program in biomedical sciences and various STEM workforce certification programs), and served as a curriculum designer and subject matter expert for the Florida Space Research Institute. She is actively engaged as a section editor for the American Society for Microbiology's *Journal of Microbiology and Biology Education* and has been a central contributor to diverse STEM education outreach initiatives through ASM, to include drafting new learning outcomes for Microbiology in Nursing and Allied Health. She is also a speaker for the U.S. Department of State's Bureau of International Information Programs, through which she promotes innovation in STEM education and women in STEM. In addition to being the new lead author of the *Microbiology: Laboratory Theory and Application* series, she is also the author of *Microbiology: Basic and Clinical Principles*. In her free time, she enjoys traveling, reading, spending time with family, and exploring the great outdoors in Vermont. She lives in St. Johns, Florida, with her two daughters and husband.

Check out her TEDx talk, *The Love Affair with Hating Science*.



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— Lab Notes —



Health Hazard (Long-term)



Flammable



Irritant



Compressed Gas



Corrosive



Explosive



Oxidizer



Environmental Hazard



Toxic

Introduction: Safety in the Microbiology Laboratory

In This Section

Safety and Laboratory Guidelines 2

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Safety and Laboratory Guidelines

We hope that you find microbiology lab to be an interesting and exciting experience, but at the outset you must be made aware of some potential hazards. Improper handling of chemicals, equipment, and/or microbial cultures is dangerous and can result in injury or infection. Safety with lab equipment will be addressed when you first use that specific piece of equipment, as will specific examples of chemical safety. Our main concern here is to introduce you to safe handling and disposal of microbes.[1]

Because microorganisms present varying degrees of risk to laboratory personnel (oneself, other students, technicians, and faculty), people outside the laboratory, and the environment, microbial cultures must be handled safely. Classifying microbes into four biosafety levels (BSLs) provides a set of minimum standards for laboratory practices, facilities, and equipment to be used when handling organisms at each level. These biosafety levels, defined in the U.S. government publication, *Biosafety in Microbiological and Biomedical Laboratories,* 6th edition (2020), are summarized here and in Table S.1. For complete information, readers are referred to the original document.

[1] Your instructor may augment or revise these guidelines to fit the conventions of your laboratory.

BSL-1: Organisms do not typically cause disease in healthy individuals and present a minimal threat to the environment and lab personnel. Standard microbiological practices are adequate. These microbes may be handled in the open, and no special containment equipment is required. Examples include *Bacillus subtilis*, *Escherichia coli* (most strains), *Rhodospirillum rubrum*, and *Lactobacillus acidophilus*.

BSL-2: Organisms are commonly encountered in the community and present a moderate environmental and/or health hazard. These organisms are associated with a variety of human diseases, most of which can be successfully treated if identified in a timely manner. The infection routes of primary concern are ingestion, inhalation, or penetration of the skin (percutaneous). Individuals performing work prone to splashes or aerosol generation (even though these organisms are not generally known to be transmitted by aerosols) should work in a biological safety cabinet (BSC, Fig. S.1). Otherwise, laboratory work may be done using standard microbiological practices. Examples include *Salmonella*, *Staphylococcus aureus*, *Clostridioides difficile* (formerly *Clotsridium difficile*), and *Borrelia burgdorferi*.

Biosafety Cabinets

Biosafety cabinets are classified into three categories, all of which draw air into the cabinet to minimize microbial contamination back into the room.

Class I BSCs resemble chemical fume hoods with a protective glass in the front, but have a HEPA filter along the exhaust path to prevent microbes from entering the environment.

Class II BSCs use laminar airflow, which minimizes turbulence, and HEPA filters to protect the user and materials within the cabinet from contamination. The exhaust passes through a HEPA filter before its release into the environment.

Class III BSCs are completely sealed and gas tight with a fixed viewing window. Incoming air passes through a HEPA filter, whereas materials to be handled are passed through a double-door system where the intermediate compartment is an autoclave. (Other methods may also be used, but accomplish the same purpose.) Exhaust air passes through two HEPA filters or a HEPA filter and an air incinerator. Materials are handled with arm-length, heavy-duty gloves built into the wall of the cabinet.

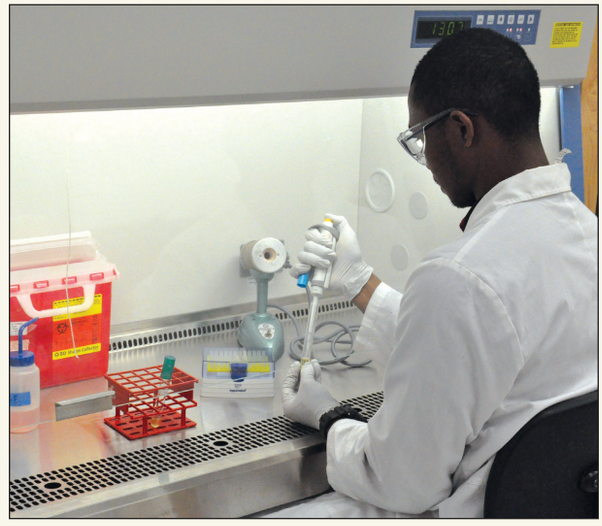


FIGURE **S.1 Biological Safety Cabinet (BSC) in a Teaching Laboratory** In this Class II BSC, air is drawn in from the room and is passed through a HEPA filter prior to release into the environment. This airflow pattern is designed to keep aerosolized microbes from escaping from the cabinet. The microbiologist is pipetting a culture. When the BSC is not in use at the end of the day, an ultraviolet light is turned on to sterilize the air and the work surface.

BSL-3: Organisms are of local or exotic origin and are associated with respiratory transmission and serious or lethal diseases where treatment and/or vaccines may or may not be available. Special ventilation systems are used to prevent aerosol transmission out of the laboratory, and access to the lab is restricted. Specially trained personnel handle microbes in a Class II or III BSC, not on the open bench. Examples include *Bacillus anthracis*, *Mycobacterium tuberculosis*, and West Nile virus.

BSL-4: Organisms have a great potential for lethal infection. Inhalation of infectious aerosols, exposure to infectious droplets, and autoinoculation are of primary concern. The lab is isolated from other facilities, and access is strictly controlled. Ventilation and waste management are under rigid control to prevent release of the microbial agents to the environment. Specially trained personnel perform transfers in Class III BSCs. Class II BSCs may be used as long as personnel wear positive pressure, one-piece body suits with a life-support system. Examples include agents causing hemorrhagic diseases, such as Ebola, Marburg, and Lassa fever viruses.

The microorganisms used in introductory microbiology courses depend on the institution, objectives of the course, and student preparation. Most introductory courses use organisms that may be handled at BSL-1 and BSL-2 levels, so we have followed that practice in designing this set of exercises.

Following are general safety rules to reduce the chance of injury or infection to you and to others, both inside and outside the laboratory. Although both BSL-1 and BSL-2 guidelines are listed, we believe it is best to err on the side of caution and that students should learn and practice the safest level of standards (relative to the organisms they are likely to encounter) at all times. Please follow these and any other safety guidelines required by your college.

Chemical safety is also important in a microbiology laboratory. Be aware of the hazards presented by the chemicals you are handling. Containers should be labeled with a standard set of precautions as seen in Figure S.2. Numbers are assigned to the degree of health, fire, and reactivity hazard posed by the chemical. On stickers placed by the laboratory, there also is a space to enter specific hazards, such as acid, corrosive, and radioactivity. Pictograms, shown at the beginning of this section, are also used.

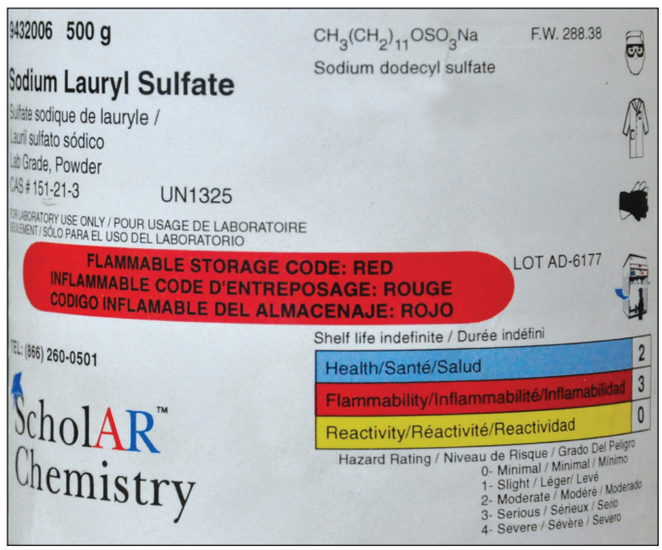


FIGURE **S.2** **Chemical Hazard Label** • The blue, red, and yellow boxes on this label indicate the health, flammability, and reactivity levels, respectively, of the compound sodium dodecyl sulfate (SDS). The colors are standardized (regardless of the chemical) and each is assigned a number between "0" (minimal) and "4" (severe) as shown in the legend at the lower right. SDS presents a moderate health hazard, is very flammable, but minimally reactive. Also notice the icons in the upper right, which indicate (from top to bottom) that this compound should be handled with eye protection, a lab coat, gloves, and adequate ventilation. Be aware of these warnings and comply with the associated cautions for all chemicals you handle in lab.

TABLE **S.1** Summary of Recommended Biosafety Levels for Infectious Agents

BSL	Agents	Practices	Safety Equipment (primary barriers)	Facilities (secondary barriers)
1	■ Not known to consistently cause disease in healthy adults ■ e.g., *Lactobacillus casei*, *Bacillus subtilis*, *Rhizobium leguminosarum*	■ Standard microbiological practices	■ Primary barriers: none required ■ Personal Protective Equipment (PPE): laboratory coats and gloves; eye, face protection as needed	■ Laboratory bench practices and sink required
2	■ Agents associated with human disease; treatments and/or vaccines are usually available ■ Routes of transmission include percutaneous injury, ingestion, and mucous membrane exposure ■ e.g., *Staphylococcus aureus*, *Salmonella enterica*, measles virus	**BSL-1 practices plus:** ■ Limited lab access ■ Biohazard warning signs ■ "Sharps" precautions ■ Biosafety manual defining any needed waste decontamination	■ Primary barriers: Class I or II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials ■ PPEs: laboratory coats, gloves, face protection as needed	**BSL-1 plus:** Autoclave available
3	■ Indigenous or exotic agents with potential for aerosol transmission ■ Disease may have serious or lethal consequences; treatments and/or vaccines may be available ■ e.g., *Yersinia pestis*, *Mycobacterium tuberculosis*, rabies virus	**BSL-2 practices plus:** ■ Controlled lab access ■ Decontamination of all waste ■ Decontamination of all lab clothing before laundering ■ Baseline serum	■ Primary barriers: Class I or II BSCs or other physical containment devices used for all manipulations of agents ■ PPEs: protective lab clothing, gloves, respiratory protection as needed	**BSL-2 plus:** ■ Physical separation from access corridors ■ Access to self-closing double door ■ Exhausted air not recirculated ■ Negative airflow into laboratory
4	■ Dangerous/exotic agents that pose high risk of life-threatening disease, aerosol-transmitted lab infections, or related agents with unknown risk of transmission; treatment and vaccines are unavailable ■ e.g., Ebola virus, Lassa virus	**BSL-3 practices plus:** ■ Clothing change before entering ■ Shower on exit ■ All material decontaminated on exit from facility	■ Primary barriers: all procedures conducted in Class III BSCs or Class I or II BSCs *in combination with* full-body, air-supplied, positive pressure personnel suit	**BSL-3 plus:** ■ Separate building or isolated zone ■ Dedicated supply and exhaust vacuum, and decon system ■ Other requirements outlined in the text

Source: Adapted from *Biosafety in Microbiological and Biomedical Laboratories*, 6th edition (Washington, DC: U.S. Government Printing Office, 2020).

Student Conduct

- To reduce the risk of infection, do not smoke, eat, drink, or bring food or drinks into the laboratory room, even if lab work is not being done at the time.
- Do not apply cosmetics or handle contact lenses in the laboratory.
- Wash your hands *thoroughly* with soap and water after handling living microbes and before leaving the laboratory each day. Also, wash your hands after removing gloves.
- Do not remove any organisms or chemicals from the laboratory.
- Lab time is precious, so come to lab prepared for that day's work. Figuring out what to do as you go is likely to produce confusion and accidents.
- Work carefully and methodically. Do not hurry through any laboratory procedure.

Basic Laboratory Safety

- Wear protective clothing (i.e., a lab coat) in the laboratory when handling microbes. Remove the coat prior to leaving the lab and autoclave it regularly (Fig. S.3). Do not take it home for washing until it has been autoclaved.
- Wear only closed-toe shoes in the laboratory.
- Wear eye protection whenever you are handling microbes or chemicals (especially during heating) even if you wear glasses or contacts (Fig. S.3).
- Turn off your Bunsen burner when it is not in use. In addition to being a fire and safety hazard, it is an unnecessary source of heat in the room.
- Tie back long hair, because it is a potential source of contamination as well as a likely target for fire.
- If you are feeling ill, go home. A microbiology laboratory is not a safe place if you are ill.
- If you are pregnant, immune compromised, or are taking immunosuppressant drugs, please see the instructor. It may be in your best long-term interests to postpone taking this class. Discuss your options with your instructor.
- If it is your lab's practice to wear disposable gloves while handling microorganisms (and this is becoming the norm), be sure to remove them each time you leave the laboratory. The proper method for removal is with the thumb under the cuff of the other hand's glove and turning it inside out without snapping it (Fig. S.4). Gloves should then be disposed of in the container for contaminated materials. Finally, wash your hands.
- Wear disposable gloves while staining microbes and handling blood products—plasma, serum, antiserum, or whole blood. Handling blood can be hazardous, even if you are wearing gloves. Consult your instructor before attempting to work with any blood products.
- Use an antiseptic (e.g., Betadine) on your skin if it is exposed to a spill containing microorganisms. Your instructor will tell you which antiseptic you will be using. Have a lab partner turn on the water for you if your hands have been contaminated.
- Never pipette by mouth. Always use a mechanical pipettor (Fig. C.1, Appendix C).
- Dispose of broken glass or any other item that could puncture an autoclave bag (contaminated or not) in an appropriate sharps or broken-glass container (Fig. S.5).
- Use a fume hood to perform any work involving highly volatile chemicals or stains that need to be heated.
- Find the first-aid kit, and make a mental note of its location.
- Find the fire blanket, shower, and fire extinguisher, note their locations, and develop a plan for how to access them in an emergency.
- Find the eyewash basin, learn how to operate it, and remember its location.

Reducing Contamination of Self, Others, Cultures, and the Environment

- Wipe the desktop with a disinfectant (e.g., Coverage or 10% chlorine bleach) before *and* after each lab period. Never assume that the class before you disinfected the work area. An appropriate disinfectant will be supplied. Allow the disinfectant to evaporate; do not wipe it dry.



FIGURE **S.3** **Safety First** • This student is prepared to work safely with microorganisms. The lab area is uncluttered, tubes are upright in a test-tube rack, and the flame is accessible but not in the way. The student is wearing a protective lab coat, gloves, and goggles, all of which are to be removed prior to leaving the laboratory. Not all procedures require gloves and eye protection. Your instructor will advise you as to the standards in your laboratory.

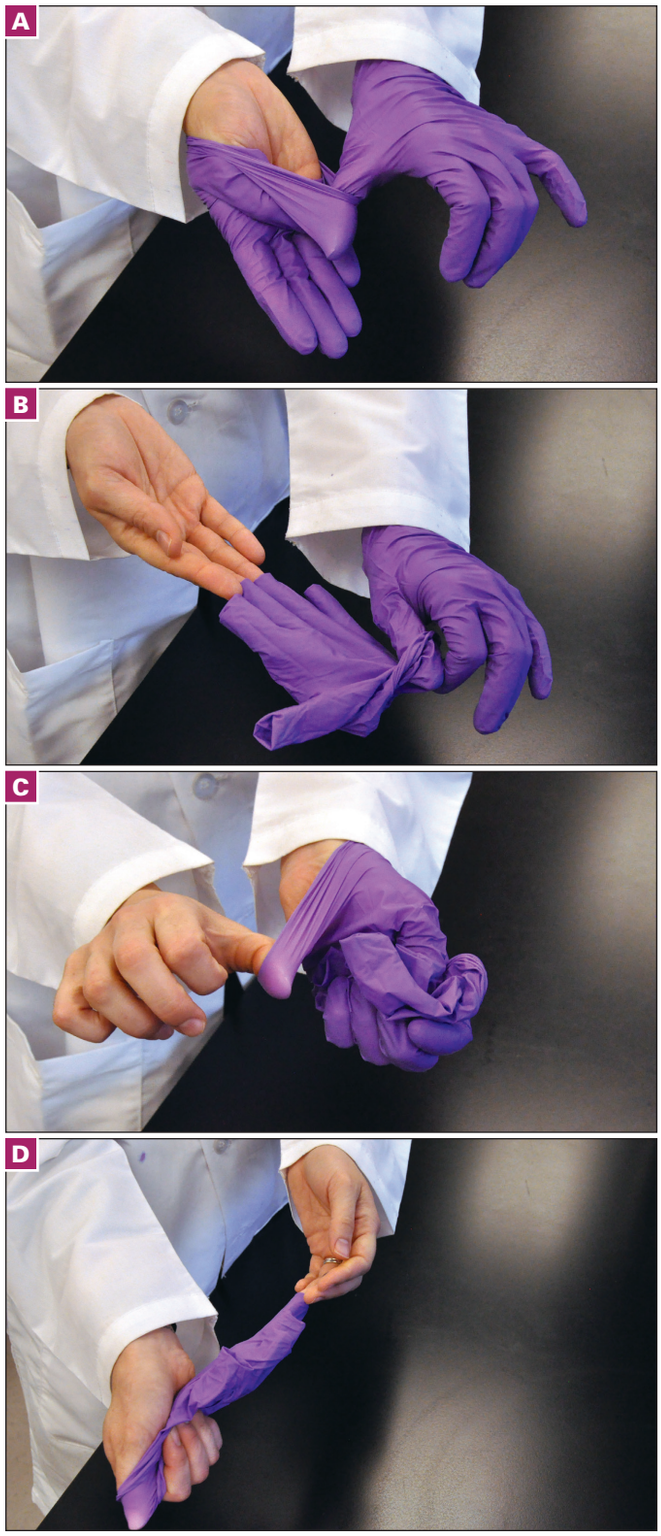


FIGURE **S.4** **Proper Glove Removal** ● Protective gloves fit tightly around the hands and must be removed in such a way that limits their snapping, which produces aerosols. (**A**) Begin by hooking the thumb of one hand under the cuff of the glove on the other hand. (**B**) Next, roll the glove off the hand so that it turns inside out. (**C**) Take the removed glove in the hand that still has a glove on it and repeat the process. (**D**) As you roll the second glove off your hand, the first glove will end up inside the second glove, which also ends up inside out. Upon completion, all contaminated surfaces are inside the second glove.

- Never lay culture tubes on the table; they always should remain upright in a tube holder (Fig. S.3). Even solid media tubes contain moisture or condensation that may leak out and contaminate everything it contacts.
- Cover any culture spills with paper towels. Soak the towels immediately with disinfectant and allow them to stand for 20 minutes. Report the spill to your instructor. When you are finished, place the towels in the container designated for autoclaving.
  - If the culture spills on you, remain where you are, do not touch anything, and have your lab partner notify the instructor. Your instructor will advise your group on how to handle the spill.
  - If you get a microbial culture in your eyes, IMMEDIATELY have a lab partner lead you to the eyewash basin and rinse your eyes for at least 15 minutes. Time is of the essence for eye contamination. Your lab partners must notify the instructor of the spill and your situation, and follow their instructions for handling your care and the spill.
- Keep all nonessential books and papers off the desk. A cluttered lab table is an invitation for an accident that may contaminate your expensive school supplies. Your instructor will advise you on where to store these items.
  - Cell phones, tablets, computers, and other electronic devices must never be on the lab table when working with microbes. Contamination of these items can be a health hazard as well as expensive.



FIGURE **S.5** **Sharps Container** ● Needles, glass, and other contaminated items that can penetrate the skin or an autoclave bag should be disposed of in a sharps container. Do not fill above the dashed black line. Notice the autoclave tape in the lower left. The white stripes will turn black after proper autoclaving. Above the autoclave tape is the address of the institution that produced the biohazardous waste.

- When pipetting microbial cultures, place a disinfectant-soaked towel on the work area. This reduces contamination and possible aerosols if a drop escapes from the pipette and hits the tabletop.

Guidelines Governing Handling of BSL-2 Organisms

Following is a list of precautions for handling BSL-2 organisms. A number of these were covered previously, but are repeated here along with the new ones and now are considered "highly recommended." It is also highly recommended that before students are allowed to handle BSL-2 organisms, they should demonstrate skill in handling BSL-1 organisms safely.

Your college may have other guidelines for handling BSL-2 organisms and other standards of practice may be adopted during the lifetime of this book, so pay attention to any announcements your lab instructor may give. And, as always, it is the instructor's responsibility to choose organisms and lab exercises appropriate to the skill level of their students.

- The lab instructor should announce to the class when BSL-2 precautions are in effect. Your college may also institute a mechanism for identifying BSL-2 cultures, such as using red caps on culture tubes. Be aware of any conventions used by your lab.
- Access to the lab must be limited once work with the BSL-2 organisms has begun. Doors and windows must be closed.
- All unnecessary materials (books, backpacks, etc., in other words, anything you plan on taking home) must be kept off the work surface and safe from aerial contamination and spills. Your lab will have a designated place for their storage.
- Disinfect your lab tabletop before and after the activity. Allow the disinfectant to air-dry.
- A lab coat, gloves, and eye protection must be worn throughout the handling of BSL-2 organisms. Do not touch any items not directly involved in the lab activity. Properly remove and dispose of the gloves when finished. (See Fig. S.4 for proper glove removal.)
- Writing utensils must be "lab dedicated," that is, they do not leave the lab until they have been disinfected. It is best to wipe them down with disinfectant after use but keep them in your lab drawer for the entire semester. There is no sense in risking contamination of multiple pens and pencils by using different ones each day.
- Electronic devices (e.g., laptops, tablets, cell phones) must be kept off the work surface and stored in an area protected from spills and aerial contamination.
- Use page covers to protect your lab exercise pages while performing the activity. Once your work is completed, disinfect the page covers, wash your hands, and remove the pages. When dry, the page covers can be stored in your locker and reused. The lab book pages can be taken home.
- It is best to use a photocopy of your data sheet to record results. Once you have decontaminated the area, it can be photographed or scanned with a dedicated document scanner in the lab and the results can be transcribed to the original data sheet. Dispose of the photocopy in an appropriate biohazard container. It must not leave the lab.
- Minimizing aerosol production during the transfer of microorganisms is important and the instructions in Section 1 address methods for doing so. However, here is a summary of recommended practices specific to BSL-2 organisms.
  - Wire loops and needles should be incinerated in an enclosed electric incinerator (refer to Section 3, Fig. 3.3A, p. 51). Sterilization with an open flame should be avoided because of its potential for aerosol production.
  - An alternative to the electric incinerator is to perform transfers with sterile disposable loops or sterile wooden sticks. Both will work for transferring from solid or liquid media. After each use, they should be put in a disposal container (such as a can) contaminated end down, submerged in about 2 cm of disinfectant. When finished with the exercise, the can should be put in a bin for autoclaving, or any other disposal receptacle your lab uses. Alternatively, sticks and disposable loops can be put in a sharps container if that is your lab's practice.
  - Pipetting must be done in such a way that the last drop is *not* "blown out" unless a petri plate lid is covering a plate. Appendices C and D give specific instructions for pipetting BSL-2 organisms.
- Clean up after the activity.
  - For incubation, place tubed cultures in a rack. For plate cultures, it is best to tape the lids on each plate individually by taping around the edge of the plate rather than taping around the base and lid (Fig. S.6). This allows viewing of the agar surface without having to remove the tape.
  - Wipe down the work surface, writing utensils, and page covers (if any) with disinfectant and let them air-dry. Only after the disinfectant has dried is it okay to place items to be removed from lab on your table.

- Remove your gloves, eye protection, and lab coat and store them according to your lab's conventions.
- Wash your hands thoroughly.

- Photograph or scan your data sheet and store or dispose of it properly.

Disposing of Contaminated Materials

In most instances, the preferred method of decontaminating microbiological waste and reusable equipment is the autoclave (Fig. S.7).

- Remove all labels from tube cultures and other contaminated *reusable* items and place them in the designated autoclave container. This will likely be an open autoclave pan to enable cleaning the tubes and other items following sterilization.
- Dispose of plate cultures (if plastic petri plates are used) and other contaminated non-sharp *disposable* items in the designated autoclave container, such as an autoclave bag (Fig. S.8). Petri plates should be taped closed. (***Note:*** Autoclave containers are designed to be autoclaved, permanently closed, and discarded. Therefore, do not place reusable and nonreusable items in the same container.)



FIGURE **S.6** **Taping Plates** ● It is a good idea to tape the lid of a petri plate to its base when incubating or storing it, especially if it contains a BSL-2 organism or a culture of unknown microbes. (**A**) One method is to wrap a length of tape around the lid and then use your thumb to press it firmly to the base. (**B**) When finished, the entire agar surface is visible and not obscured by any tape, making lid removal unnecessary to view colonies.

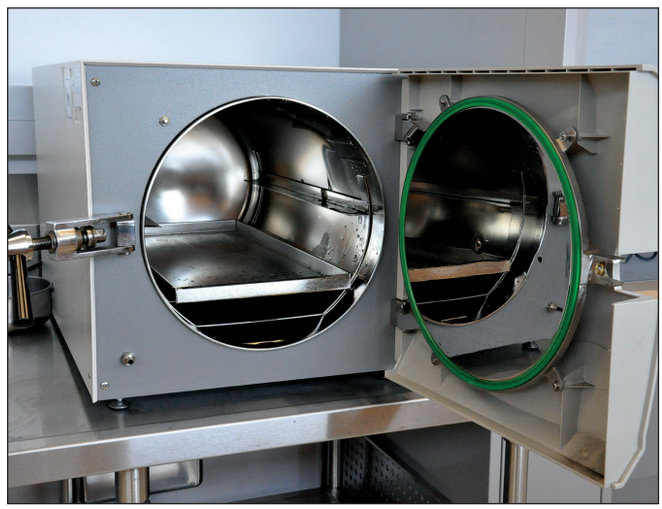


FIGURE **S.7** **An Autoclave** ● Media, cultures, and equipment to be sterilized are placed in the basket of the autoclave. Steam heat at a temperature of 121°C (produced at atmospheric pressure plus 15 psi) for 15 minutes is effective at killing even bacterial spores. Some items that cannot withstand the heat, or have irregular surfaces that prevent uniform contact with the steam, are sterilized by other means.



FIGURE **S.8** **An Autoclave Bag** ● Nonreusable items (such as plastic petri plates) are placed in an autoclave bag for decontamination. petri plates should be taped closed. Do not overfill or place sharp objects in the bag. Notice the autoclave tape at the middle right. The white stripes will turn black after proper autoclaving. At the lower right is the address of the institution that produced the biohazardous waste.

- Dispose of all blood product samples and disposable gloves in the container designated for autoclaving.
- Place used microscope slides of bacteria in a sharps container designated for autoclaving, or soak them in disinfectant solution for at least 30 minutes before cleaning or discarding them. Follow your laboratory guidelines for disposing of glass.
- Place contaminated broken glass and other sharp objects (anything likely to puncture an autoclave bag) in a sharps container designated for autoclaving (Fig. S.5). Uncontaminated broken glass does not need to be autoclaved, but should be disposed of in a specialized, broken-glass container.

Student Compliance with Laboratory Safety Regulations

Your institution may augment the safety regulations outlined above or compile a list tailored to your laboratory. In either case, these constitute a Laboratory Safety Statement. It is highly recommended that students verify that they have read and understand these safety regulations, agree to comply with them, and are aware of noncompliance consequences by signing a statement to that effect. The American Society for Microbiology has produced a publication (Emmert et al., 2012) that includes a sample Laboratory Safety Statement and a student signature page. The signature portion in a slightly modified form is reproduced on page 13. Your instructor may have you sign it or sign their own version of it.

Reference

Meechan, P. J. and J. Potts. U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories,* 6th ed. Washington, DC: U.S. Government Printing Office, 2020.

A Word About Experimental Design

Like most sciences, microbiology has descriptive and experimental components. Here we are concerned with the latter. Science is a philosophical approach to finding answers to questions. Despite what you may have been taught in grade school about *THE* "Scientific Method," science can approach problems in many ways, rather than in any *single* way. The nature of the problem, personality of the scientist, intellectual environment at the time, and good, old-fashioned luck all play a role in determining which approach is taken. Nevertheless, in experimental science, one component that is always present is a **control** (or controls).

A controlled experiment is one in which all **variables** except one—the **experimental variable**—are maintained without change. Frequently, we are looking for the cause of some phenomenon. By limiting variables to only one, we can draw a provisional conclusion about whether or not that variable causes the phenomenon.

If changing the variable causes a change in the phenomenon, then we can provisionally conclude that the variable and the phenomenon are causally related; that is, we have demonstrated a **cause and effect relationship** between the phenomenon and the experimental variable. Alternatively, if there is no observed change, we can eliminate the experimental variable from involvement with the phenomenon.

For example, if we want to determine the effect of increasing temperature on microbial growth rate, we could grow the same microorganism in two test tubes containing the same nutrient source at two different temperatures. One would be grown at "normal" temperature (whatever it is for that organism) and the other would be grown at a higher temperature. Everything but temperature would be the same, so if we see a difference in growth rate we can attribute it to the experimental variable, temperature.

But how would we know there is a difference in growth rate? Simple, by comparing the growth rate at the higher temperature to the growth rate at the "normal" temperature. The "normal" temperature tube is the control; it provides a baseline growth rate against which the experimental growth rate is compared. Growth rate in the experimental tube will be faster, slower, or the same as the control.

Without the control we would only be able to measure growth rate in the experimental tube, but we couldn't answer the question about the effect of higher temperature on growth rate. Had we grown different microorganisms with different nutrient sources at different temperatures and we saw a difference, we wouldn't know if the difference was due to the organism, the nutrients available, and/or the temperature. This is why we test one variable at a time.

Controls are an essential and integral part of all experiments, but there are many types. Two commonly used controls are **positive** and **negative controls.** A positive control is one that is set up to produce a positive result. Alternatively, a negative control is set up to demonstrate a negative result or no change.

As you work your way through the exercises in this book, pay attention to the various ways positive, negative, and other controls are used to improve the **validity** (effectiveness) of the experiment and the **reliability** (accuracy) of the results. In fact, there are many questions on the data sheets that ask some form of the question: "What is the role of the control in this experiment?" We are pretty sure that your professor won't be satisfied with the answer, "It's a control."

Microbiological experimentation often involves tests that determine the ability of an organism to use or produce some chemical, or to determine the presence or absence of a specific organism in a sample. Ideally, a positive result in the test indicates that the microbe has the ability or is present in the sample, and a negative result indicates a lack of that ability or absence in the sample (Fig. S.9).

The tests we run, however, have limitations and occasionally may give **false-positive** or **false-negative** results. An inability to detect small amounts of the chemical or organism in question would yield a false-negative result and would be the result of inadequate **sensitivity** of the test (Fig. S.9). An inability to discriminate between the chemical or organism in question and similar chemicals or organisms would yield false-positive results when the similar chemical or organism was being tested and would be the result of inadequate **specificity** of the test (Fig. S.9). Sensitivity and specificity can be quantified using the following equations:

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

The closer sensitivity and specificity are to a value of one, the more useful the test. As you perform the tests in this book, be mindful of each test's limitations, and be open to the possibility of false-positive and false-negative results.

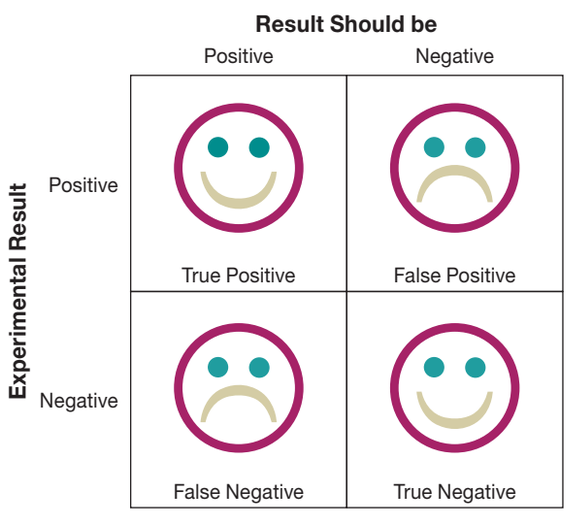


FIGURE **S.9** **Limitations of Experimental Tests** • Ideally, tests should give a positive result for specimens that are positive, and a negative result for specimens that are negative. False-positive and false-negative results do occur, however, and these are attributed to inadequate specificity and inadequate sensitivity, respectively, of the test system.

Data Presentation: Tables and Graphs

In microbiology, we perform experiments and collect data, but it is often difficult to know what the data mean without some method of organization. Tables and graphs allow us to summarize data in a way that makes interpretation easier.

Tables

A table is often used as a preliminary means of organizing data. As an example, Table S.2 shows the winning times for each male and female age division in a half-marathon race. Again, the aim of a table is to provide information to the reader. Notice the meaningful title, the column labels, and the appropriate measurement units. Without these, the reader cannot completely understand the table and your work will go unappreciated! Data tables are provided for you on the data sheets for each exercise in this book, but you may be required to fill-in certain components (units, labels, etc.) in addition to the data.

TABLE **S.2** **Winning Half-Marathon Times by Sex and Age Division** •

Male Runners		Female Runners	
Winner's Age (years)	Winning Time (minutes)	Winner's Age (years)	Winning Time (minutes)
15	73	15	88
25	67	24	82
31	67	30	82
35	71	39	84
40	71	42	85
52	78	50	109
62	95	62	108
70	123	70	126

Graphs

Table S.2 does give the information, but what it is telling us may not be entirely clear. It appears that the half-marathon times increase as the runners get older, but we have difficulty determining if this is truly a pattern. That is why data also are presented in graphic form at times; a graph usually shows the relationship between variables better than a table of numbers.

X–Y Scatter Plot The type of graph you will be using in this manual is an "*X*–*Y* Scatter Plot," in which two variables are graphed against each other. Figure S.10A shows the same data as Table S.2, but in an *X*–*Y* Scatter Plot form.

Notice the following important features of the graph in Figure S.10A:

- *Title:* The graph has a meaningful title, which should tell the reader what the graph is about. A title of "Age vs. Winning Time" is vague and inadequate.
- *Dependent and independent variables:* The graph is read from left to right. In our example, we might say for the male runners, "As runners get older, winning times get longer." *Winning time* depends on *age*, so winning time is the *dependent* variable and age is the *independent* variable. (Age does *not* depend on the winning time!) By convention, the independent variable is plotted on the *x*-axis and the dependent variable is plotted on the *y*-axis. By way of comparison, notice the consequence of plotting age on the *y*-axis and winning time on the *x*-axis: "As runners get slower, they get older," which doesn't reflect the actual relationship between the variables and worse yet, is nonsense.
- *Axis labels:* Each axis is labeled, including the appropriate units of measure. "Age" without units is meaningless. Does the scale represent months? Years? Centuries?
- *Axis scale:* The scale on each axis is uniform. The distance between marks on the axis is always the same and represents the same amount of that variable. (But increments on the *x*-axis don't have to equal those on the *y*-axis, as shown.) The size of each increment is up to the person making the graph and is dictated by the magnitude and range of the data. Most of the time, we choose a length for the axis that fills the available graphing space.
- *Axis range:* The scale for an axis does not have to begin with "0." Use a scale that best presents the data. In this case, the smallest *y*-value was 67 minutes, so the scale begins at 60 minutes.
- *Multiple data sets and the legend:* The two data series (male and female times) are plotted on the same set of axes, but with different symbols that are defined in the legend at the right. The symbols shown differ in color *and* shape, but one of these is adequate.
- *Best-fit line:* If a line is to be drawn at all, it should be an average line for the data points, not one that "connects the dots" (Fig. S.10B). Notice that the points are not necessarily *on* the line. The purpose of a best-fit line is to illustrate the general trend of the data, not the specifics of the individual data points. (Be assured that most graphs in your textbooks where a smooth line is shown were experimentally determined and the lines are derived from points scattered around the line.) There is a mathematical formula that allows one to compute the slope and *y*-intercept of the **trend line** if the relationship is linear, or a **best-fit line** if the relationship is nonlinear (as in the half-marathon times example), but this is beyond our needs. For our purposes, a hand-drawn trend line that looks good is good enough. (If you use a computer graphing program, then it will produce the trend line without you doing any of the math—the best of all situations!)



FIGURE **S.10** **Sample X-Y Scatter Plot** • A graph often shows the relationship between variables better than a table of numbers. Examine this sample and identify the essential components of a quality graph (see text). (**A**) Presentation of data without a best-fit line is acceptable if there are not enough data points to justify illustrating a trend. (**B**) Shown here are the same data but with a trend line. Notice that the points do not fall directly on the line but, rather, that the line gives the general trend of the data. "Connecting the dots" is not appropriate.

Bar Graphs

Bar graphs are used to illustrate one variable. Often, the variable is considered to be categorical. That is, the data are in distinct groups (rather than continuous, where the breaks are arbitrary). Using a bar graph to show the relationship between winning times and ages is inappropriate. Examine Figure S.11A. Notice that the space each bar fills is meaningless; that is, the only important part of the bar is the top, which is the value used in the *X*–*Y* scatter plot. Also notice that the *x*-axis scale is not uniform—the gaps between bars range from 4 years (31 to 35) to 12 years (40 to 52). An appropriate use of a bar graph would be the distribution of student performance on an exam, even though exam scores are not categorical (Fig. S.11B). Notice that the space each bar fills has meaning. Each student in a particular group adds height to the bar.

Data Presentation: Be Creative, But Complete

There is no single correct way to produce a graph for a particular data set. Actually, most people working independently would graph the same data set in different ways (e.g., different scales, colors, wording of the title, and axis labels), but the essential components listed would have to be there. You will be asked to graph some of the data you collect. Be sure your graphs tell a complete and clear story of what you have done.

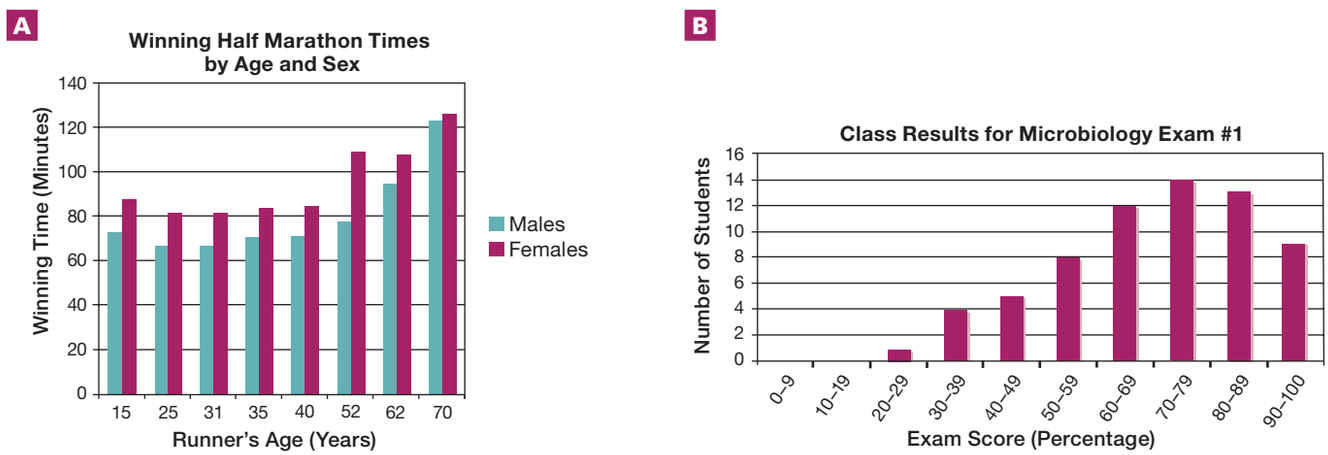


FIGURE **S.11** **Bar Graphs** • A bar graph is appropriate to present data involving a single variable, especially if that variable is easily divided into distinct categories or groups. (**A**) Plotting the winning half-marathon times from Table S.2 using a bar graph is inappropriate because the only meaningful point is at the top. (**B**) A bar graph is useful in presenting data of a single variable, such as the number of students earning a specific score on their microbiology exam.

STUDENT SAFETY CONTRACT

Student Agreement on Laboratory Safety

I have read the Laboratory Safety Statement as written in *Microbiology: Laboratory Theory & Application, Essentials*, 2nd edition, and/or the equivalent document supplied by the Department of Biological Sciences,

_____ ,

(Institution Name)

and I understand its content. I agree to abide by all laboratory rules set forth by the instructor. I understand that my safety is entirely my own responsibility and that I may be putting myself and others in danger if I do not abide by all the rules set forth by the instructor.

COURSE: _____

STUDENT NAME (PRINT): _____

STUDENT SIGNATURE: _____

DATE: _____



SECTION

1

Microscopy

In This Section

EXERCISE 1 Introduction to Light Microscopy **17**

EXERCISE 2 Microscopic Examination of Eukaryotic Microbes **29**

— Lab Notes —

1

EXERCISE 1

Introduction to Light Microscopy

After doing this exercise, you should be able to:

1. Identify and describe the functions of the compound light microscope's parts.
2. Perform bright-field microscopy to view specimens.
3. Use the high-power oil-immersion lens to view prepared specimens.
4. Calculate final magnification.
5. Name three other examples of light microscopy aside from bright-field microscopy.
6. Discuss light microscopy's clinical applications.

Background

There are many forms of microscopy, but **bright-field microscopy** is the simplest and most common way we observe microscopic organisms in the microbiology laboratory. Typically, we use a compound microscope for bright-field microscopy (Fig. 1.1). In bright-field microscopy, the specimen appears as a darker object set against a bright background—hence the technique's name (Fig. 1.2).

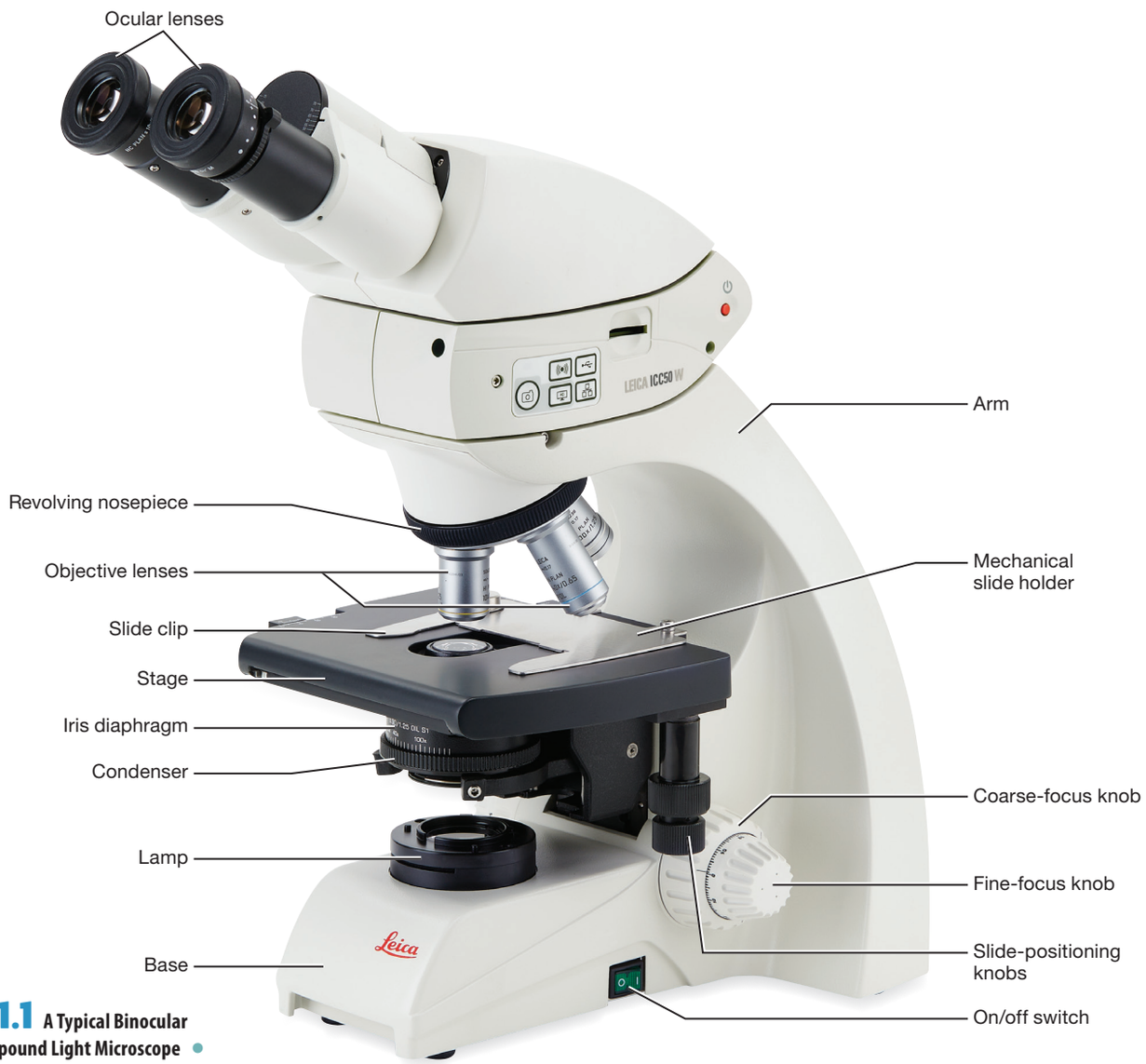


FIGURE **1.1** **A Typical Binocular Compound Light Microscope**

Compound light microscopes provide two levels of magnification, a design feature that *compounds* (increases) how enlarged the final image appears. The objective lens provides the first layer of magnification and the ocular lens provides the second. So, to determine the final image magnification, we multiply the magnification each of these lenses provides. Each lens has its magnification level marked on the lens barrel (Fig. 1.3).

$$\text{Final magnification} = (\text{Objective lens magnification}) \times (\text{Ocular lens magnification})$$

Most compound microscopes provide a maximum final magnification of 1000× (the "×" after this number indicates that this is a magnification level; you should apply this formatting when recording your magnification information for the lab exercise). Although higher magnifications are possible, increasing magnification beyond about 1300× yields a blurry image. This is because image **resolution** (image sharpness) tends to decrease as magnification increases. The **limit of resolution** (or **resolving power**) is a measurement of how far apart two points must be for us to see the points as separate (Fig. 1.4). Resolution *increases* (improves) as the limit of resolution *decreases*. Even the best light microscope will have a resolving power maximum of about 0.2 µm. That means even the best equipment can only allow us to see two points as distinct from one another if they are *at least* 0.2 µm apart.

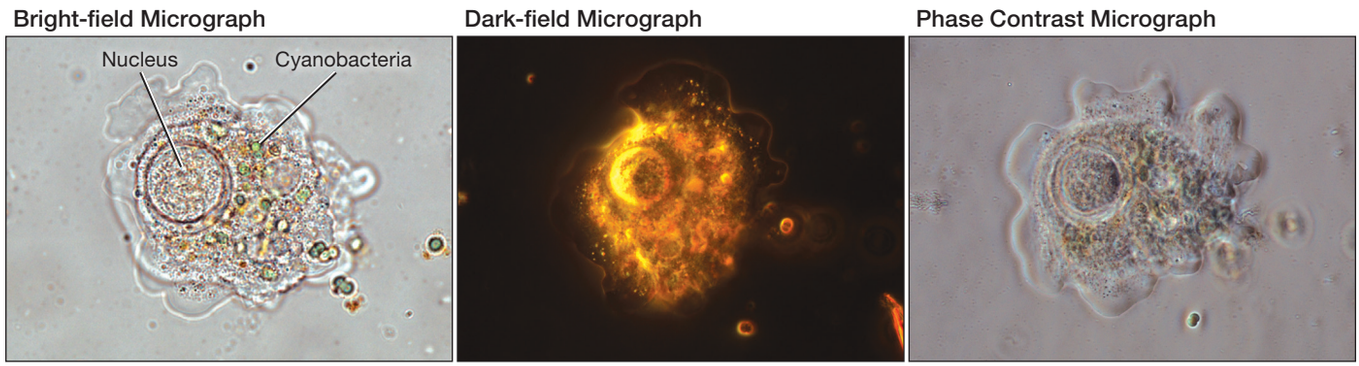


FIGURE **1.2** **Three Types of Light Microscopy to View an Unstained Amoeba**

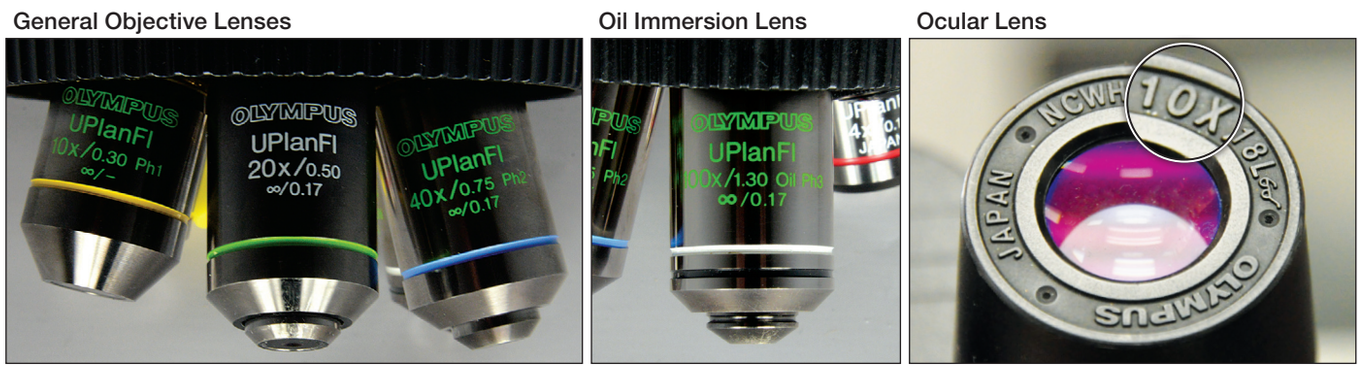


FIGURE **1.3** **Lens Magnification Markings** • The markings on an objective lens barrel describe the lens' optical features. Here we focus on magnification markings. **Objective Lenses:** The yellow-banded lens provides a 10× magnification; the green-banded lens provides a 20× magnification; and the blue-banded lens provides a 40× magnification. These color bands are standard for quick magnification reference. Many microscopes also have a 4× lens, which would have a red band (not shown). **Oil-Immersion Lens:** This lens has two bands; the white band indicates the lens provides 100× magnification; and the black band indicates this lens must be used with immersion oil to obtain the sharpest image. Note that the word *oil* is also etched on the lens' barrel. Immersion oil can damage lenses that do not have an "oil" specification. **Ocular Lens:** The lens shown here provides a 10× magnification. Some ocular lenses are not 10×, so check the specifics before calculating final magnification.

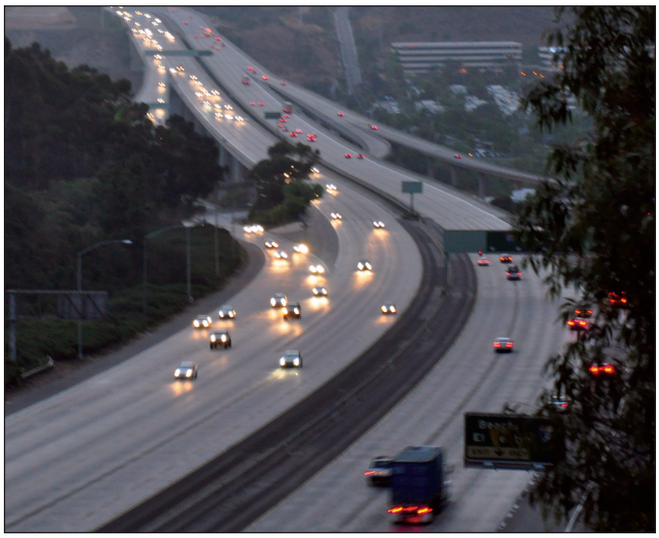


FIGURE **1.4** **Limit of Resolution** • Most automobile headlights are about 1.5 m apart. Looking at the cars in the photo's foreground, it's easy to see a car's headlights as separate. The more distant cars appear smaller (but really aren't) as does the apparent distance between the headlights. When the apparent distance between the headlights reaches about 0.1 mm, they become indistinguishable as separate lights. This is because 0.1 mm is the human eye's limit of resolution.

Light's Journey through the Compound Light Microscope

The image we see in light microscopy results from how light interacts with the specimen and what light eventually makes its way to our eyes. Light's journey through the compound microscope starts at the **lamp** (or light source) located at the base of the microscope. From the lamp, light then passes through the **condenser**, a lens that focuses and aims the light onto the specimen. The condenser does not provide magnification, but it is important for improving image resolution.

The **iris diaphragm** is located just above the condenser. It allows you to control how much light illuminates the specimen; the more open the iris diaphragm, the more light aimed at the specimen. Microscopy may be many things, but it shouldn't be physically painful! If your eyes hurt because the view is too bright, you can reduce the brightness by adjusting the iris diaphragm so less light is shining onto the sample or you can dim the light using the **light-intensity knob**. Similarly, if you are straining to see the sample because it's too dark, open the iris diaphragm and/or increase the source lamp's intensity.

After light passes through the iris diaphragm, it passes through the glass slide and the sample mounted on the slide. Light that passes through the specimen and the glass slide then makes its way to the **objective lens**, the lens on the microscope's revolving nosepiece. This is the first lens that provides image magnification. From there, light makes its way to the **ocular lenses**, where the second and final image magnification occurs.

Oil Immersion in Bright-Field Microscopy

Most microbiology lab microscopes have a high-power oil-immersion lens. This lens has the word *oil* and 100× etched on the lens' barrel. To achieve the best image with such a lens, we must place oil on the slide and submerge the tip of the lens into the immersion oil.

But how can a tiny drop of oil make such a big difference in image resolution? The answer lies in the fact that light experiences **refraction** (bends) when it passes from one medium to a different medium, such as when it passes from water into air. Because air and water bend (refract) light differently, an underwater object appears blurry when you view it from above the water line.

By using immersion oil, we reduce the blurring that refraction produces. This is because immersion oil has a refractive index that is extremely close to that of glass. Therefore, by adding a drop of immersion oil to the surface of the slide and then submerging the tip of the oil lens into that oil, we effectively eliminate the air-glass interface that would otherwise have led to a blurry image.

Other Forms of Light Microscopy

Due to the light microscope's limited magnification and resolution potential, it's difficult to *directly* view things smaller than about 500 nm. This means light microscopy is not ideal to directly see microbes like *Mycoplasma*, a genera of bacteria that are about 150–350 nm in size and include pathogens such as *Mycoplasma pneumoniae* (the causative agent of walking pneumonia). Similarly, we cannot use light microscopy to directly view most viruses. That said, we can *indirectly* view *Mycoplasma* and viruses using a specialized form of light microscopy called **fluorescence microscopy** (Fig. 1.5). Although we rarely perform fluorescence microscopy in an introductory microbiology teaching lab, it's extremely useful in clinical microbiology labs.

As compared to fluorescence microscopy, which requires a highly specialized microscope, two other forms of light microscopy—**dark-field microscopy** and **phase-contrast microscopy**—are easily performed using a modified compound light microscope. Both phase-contrast and dark-field microscopy manipulate light waves and alter how light interacts with the specimen. They do so in such a way that they improve specimen contrast without needing to stain the specimen. Avoiding specimen staining can be desirable since most staining procedures distort and kill the specimen. For examples of dark-field and phase-contrast micrographs, refer to Figure 1.2.

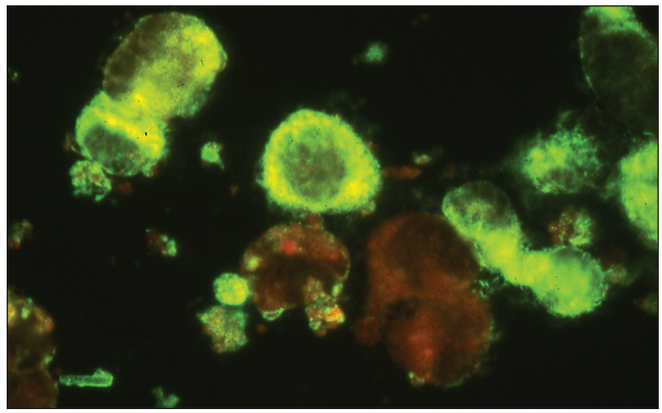


FIGURE **1.5** **Fluorescence Microscopy** • Fluorescence micrograph of *Mycobacterium kansasii*. The apple-green color is characteristic of fluorescence microscopy.

Clinical Applications

Light microscopes help health-care providers and lab personnel identify microbes in patient samples. As you may suspect, a compound microscope is indispensable in any microbiology lab, but did you know that many primary care providers and specialists (e.g., gynecology, dermatology, etc.) also have a compound light microscope available to help them perform more accurate point-of-care diagnosis? It's cheap, fast, and easy to use a compound light microscope to detect pinworm infections, urinary tract infections, trichomoniasis (a common sexually transmitted infection caused by the protozoan *Trichomonas vaginalis*), fungal skin infections, vaginal yeast infections, and bacterial vaginosis (vaginal inflammation caused by the overgrowth of certain bacteria that are normally present in the vagina).

When combined with staining techniques (which later lab activities cover), light microscopy is an indispensable diagnostic tool. In some cases, seeing the causative pathogen in a patient sample remains a "gold standard" for diagnosis. For example, seeing acid-fast bacteria in a patient's sputum (coughed-up mucus) is still a mainstay for diagnosing tuberculosis.

MATERIALS

- ❑ Compound light microscope
- ❑ Lens paper
- ❑ Lens-cleaning solution or 95% ethanol
- ❑ Immersion oil
- ❑ Letter "e" slide (Part A)
- ❑ Colored-threads slide (Part B)
- ❑ Prepared slides of bacteria and/or other microbes (instructor's choice) (Part C)

Using a Compound Light Microscope

These are generalized instructions that are useful for this lab and that you may also refer to whenever you are performing microscopy during the course. Your instructor may supplement the following with specifics related to your lab's equipment.

Getting the Microscope Ready for Use

1. If you must move the microscope, do so with both hands. Place one hand on the microscope's arm and the other under the base.
2. Carefully place the microscope on the table.
3. Unwind the power cord and plug in the microscope.
4. With gentle pressure, clean the microscope's ocular and objective lenses with lens paper and lens cleaner. Improper cleaning will scratch the lenses; follow your instructor's directions and preferences on this.
5. Turn on the lamp and adjust the lamp intensity to its highest level.
6. Slowly and gently turn the rotating nosepiece so that the scanning objective (4× objective lens) "clicks" into place over the slide. If your microscope lacks a 4× lens, position the low-power objective (10×) into place over the stage. Do not rotate the nosepiece by the lenses, this can damage the lenses and cause them to unscrew from the nosepiece.
7. Use the coarse-focus knob (the larger knob on the body of the microscope) to bring the stage to its highest position.

Focusing the Microscope Without Immersion Oil

1. Check that your microscope's stage is in the highest position and that the 4× objective is clicked into place over the stage.
2. Use the slide clip to secure the slide you want to view.
3. Center your specimen over the light. If your microscope has a mechanical slide holder, use the slide-positioning knobs to do this; otherwise, use your hand to gently position the slide.
4. With your eyes relaxed and slightly above the oculars, look into the microscope. If the light is too bright, dim the lamp and/or close the iris diaphragm. If using a binocular microscope, adjust the ocular lenses' spacing for your eyes so you see a single full circle of light.
5. While looking through the ocular lenses, slowly lower the stage by rotating the *coarse-focus* knob. Do this until you achieve the sharpest image possible. From this point on, you should no longer need to use the coarse-focus knob.
6. Now, while looking through the microscope, use the *fine-focus* knob to sharpen the image.
7. Scan the specimen and position it in the center of the field of view so you do not "lose" it upon advancing to a higher magnification.
8. Now, advance to a higher magnification by clicking the next objective into place over the specimen. Do not move your slide or the stage, just click the next objective lens into place. If you started with the 4× lens, you should now have the 10× objective in place.
9. Look into the microscope and, if necessary, slightly adjust the fine focus to sharpen the image. You can also adjust the image brightness if necessary. Some iris diaphragms have markings to show which setting works best for a specific objective lens. If your microscope has this feature, match the iris diaphragm setting with the objective lens you have in place.
10. Rotate the 40× objective into place and adjust the fine focus to sharpen the image.

Focusing the Microscope Using Immersion Oil

When viewing tiny cells such as bacteria or the details of a blood smear, the oil-immersion lens usually provides the best view.

1. Perform each step described in the "Focusing the Microscope Without Immersion Oil" section.
2. Once your specimen is in focus under the 40× objective, gently rotate the nosepiece to a position midway between the 40× lens and the oil-immersion lens (100× lens).
3. Do *not* move your stage down before adding the immersion oil or you will "lose" your focused image. Instead, as you "float" between the 40× and 100× objectives, place a drop of immersion oil onto the specimen. ***Do not drip oil on the microscope, especially its non-oil lenses. If you do, immediately clean off the oil with lens cleaner and lens paper.***
4. Now, slowly rotate the 100× lens so its tip is submerged in the oil drop, pass through it, and then return the lens into the oil. This minimizes the occurrence of air bubbles. ***Note:*** It may look as though the lens will not clear the slide. Provided you have followed all directions, it should click into place and the tip of the lens will be submerged in the oil. If the lens will not easily rotate into place, do not force it into place, you will break the slide and damage the oil lens. Instead, stop and ask for help.
5. ***Caution!*** This is another step where students tend to break slides. Use the fine-focus knob to *slowly* sharpen the image. If you encounter any resistance from the instrument's focus knob, stop and ask for help. Do not force a knob to turn; you will break the slide.
6. If the image appears dark, refer to Step 5 in the "Focusing the Microscope Without Immersion Oil" section.

Storing the Microscope

Follow these steps when you finish using your microscope.

1. Use the coarse-focus knob to completely lower the microscope's stage (or completely raise the objectives).
2. Remove the slide from the microscope's stage.
3. If you used immersion oil, use lens paper with lens cleaner to gently remove oil from any permanent slides you viewed before you return them to storage.
4. If you used immersion oil, use lens paper and lens cleaner to gently wipe any excess oil off the 100× lens.
5. "Click" the scanning objective (4× lens) into position over the stage.
6. Return the iris diaphragm to the fully opened state.
7. Turn off the lamp.
8. Unplug your microscope.
9. Wrap the electrical cord according to your instructor's preference.
10. If available, cover the microscope with a dust cover.
11. Return the microscope to its appropriate storage place.

PROCEDURE

Your instructor may have you do some or all parts of the following lab. Pay attention to their instructions and proceed in the order they direct you.

PART A: Viewing a Letter "e"

In this part of the lab, you'll view a macroscopic specimen (a letter "e"). This exercise helps to acquaint you with the microscope's parts and with focusing the microscope on a sample *without* using immersion oil. You'll also see that the images we observe are not always what they appear to be.

1. Follow each step in the "Getting the Microscope Ready for Use" section.
2. Obtain a letter "e" slide and examine it *without* the microscope. Record the orientation of the letter with the slide facing up and the label to the left. Sketch the orientation of the letter "e" on your data sheet.
3. Place the slide on the stage in the same position as you examined it with your naked eyes (label facing upward and positioned to your left).
4. Now, follow Steps 1–7 as described in the "Focusing the Microscope without Immersion Oil" section. For this exercise, you will only observe the letter "e" slide under the 4× objective.
5. Once you have the letter in focus, sketch the apparent orientation of the letter "e" on your data sheet. How, if at all, is the letter's orientation different from what you expected? Record your answer on your data sheet.
6. Now move the *slide* to the right. On your data sheet, record the direction the *image* moves.
7. Reposition the "e" in the center of the field. Move the *slide* toward you and on your data sheet, record the direction the *image* moves.
8. Remove the slide from the microscope and return it to its proper storage location.

PART B: Viewing Colored Threads

In this exercise, you'll view a macroscopic specimen (colored threads). This exercise provides additional practice with focusing the microscope on a sample *without* using immersion oil. You'll also experience the challenge of differentiating layers of detail in a specimen and you'll practice viewing a sample in different focal planes (positions where one layer of the sample appears in focus while the other layers appear blurry).

1. Obtain a colored-threads slide and view it with your naked eye (Fig. 1.6). Try to determine where in the stack each color resides. Is the red thread on the top, bottom, or middle? Do the same for the yellow and blue threads.
2. Now, follow Steps 1–7 as described in the "Focusing the Microscope without Immersion Oil" section.
3. Observe the slide with the 4× objective and try to determine the order of the threads. Record your observations on your data sheet. It may be helpful to observe the threads at various focal planes; you can use the fine-focus knob to do this.

4. Observe the slide with the 10× objective and try to determine the order of the threads. Record your observations on your data sheet. Observe the threads at various focal planes.
5. Now, click the 40× objective into place and observe the threads. Record what you see on the data sheet. Again, observe the threads at various focal planes.
6. Remove the slide from the microscope and return it to its proper storage location.

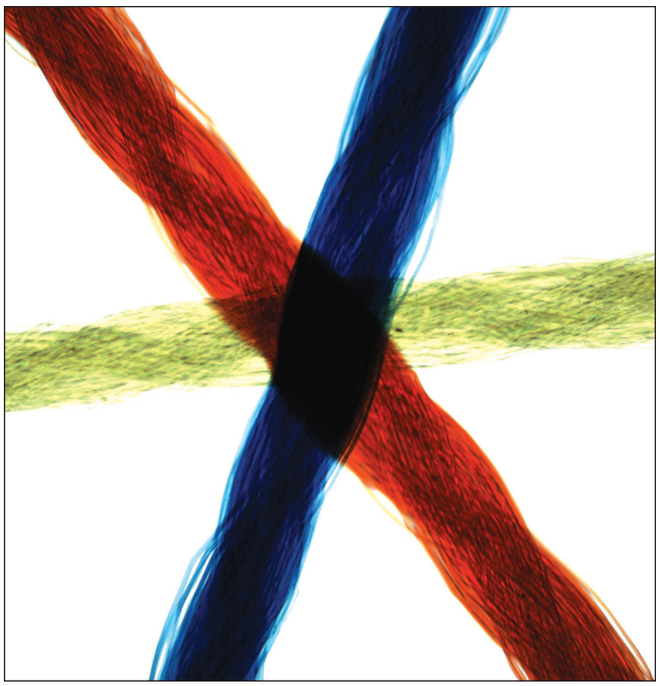


FIGURE 1.6 **Which Thread is on Top?** • Even with the microscope, determining the order of threads from top to bottom is a challenge. This will require patience and use of the fine focus! Making it worse, not all the slides will be the same. Good luck!

PART C: Using Oil Immersion to View a Microscopic Specimen

In this exercise, you'll view at least one microscopic specimen selected by your instructor (e.g., bacteria, a blood smear, etc.). This exercise helps familiarize you with the parts of the microscope and with focusing the microscope on a sample using immersion oil.

1. Your instructor will tell you which slides to view for this exercise.
2. Clean the slide with lens paper and lens cleaner before you start.
3. Follow the steps as described in the "Focusing the Microscope Using Immersion Oil" section.
4. Document your observations on your data sheet.
5. When finished, follow the steps in the "Storing the Microscope" section.

— Lab Notes —

1

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 1

DATA SHEET

Introduction to Light Microscopy

During the Lab

1 Based on the microscope you used, calculate the final magnification at which you viewed the letter "e" in Part A.

2 From Part A, sketch your letter "e" observations in the table provided. Be sure the slide is right-side up with the label oriented to your left.

Appearance of the "e" with the naked eye	While looking at the "e" under the microscope, how (if at all) is the letter's orientation different from what you expected?	When the stage moves to the right, the image moves...	When the stage moves toward you, the image moves...

3 Record your observations of the colored threads for each scenario in the table provided.

Naked eye	Top:	Middle:	Bottom:
4× Objective lens	Top:	Middle:	Bottom:
10× Objective lens	Top:	Middle:	Bottom:
40× Objective lens	Top:	Middle:	Bottom:

4 In the sketch circle provided, draw your observations for *at least* one slide that you viewed with oil immersion. (Your instructor will designate what slides you should view.)

Specimen Label: _____

Image's final magnification (show your calculation below): _____

Lab Related Questions

1 Color the microscope parts as indicated.

Coarse-focus knob (orange)

Fine-focus knob (purple)

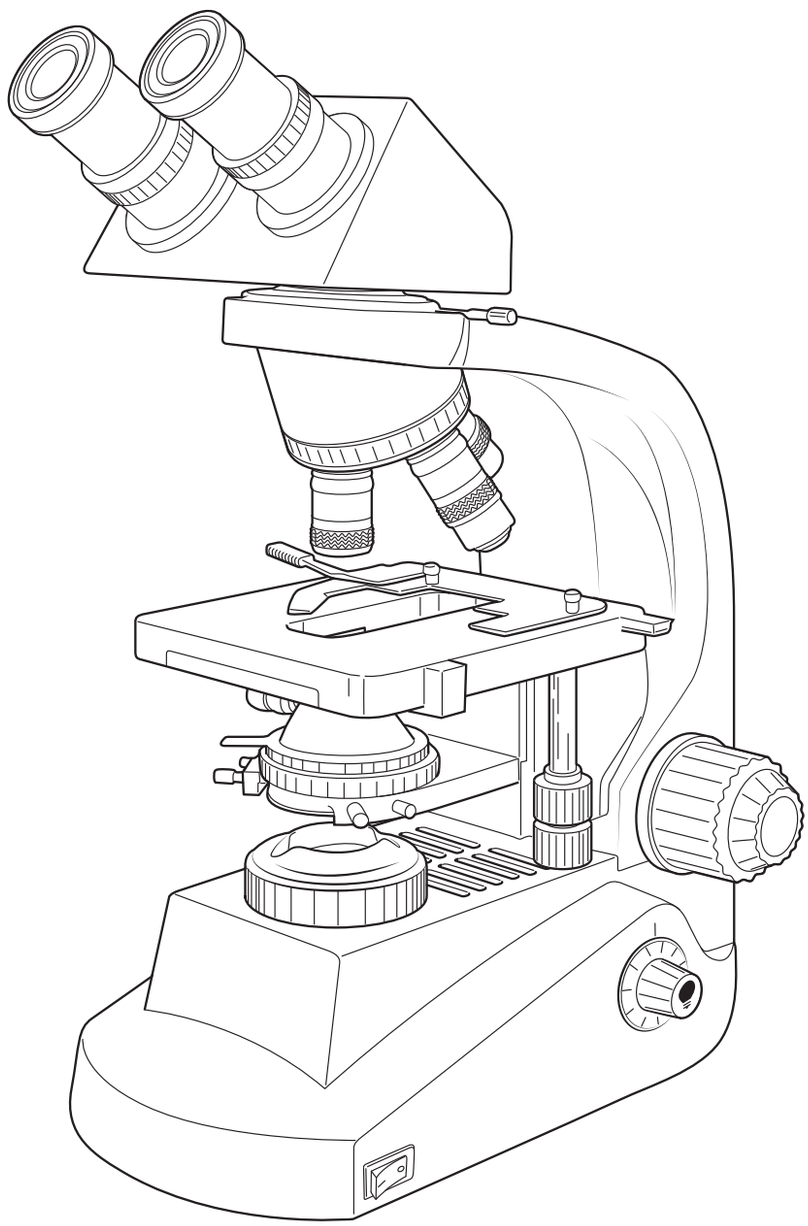
Slide-positioning knobs (yellow)

Objective lenses (red)

Ocular lenses (blue)

Revolving nosepiece (black)

Slide clip (green)



Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 1
DATA SHEET
(continued)

2 Assume you view a specimen at a final magnification of 55×. If the objective lens was 5×, then what was the microscope's ocular lens magnification rating?

a. 5×
b. 10×
c. 11×
d. 55×
e. Not enough information

3 Which of the following could help you detect a virus in a patient sputum sample?

a. Bright-field microscopy
b. Dark-field microscopy
c. Phase-contrast microscopy
d. Fluorescence microscopy
e. None of the above

4 Some steps listed should *not* be used if you want to successfully view a specimen via oil immersion. First, draw a line through the steps you would NOT use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Place oil on the slide.

_____ Use the coarse-focus knob to focus while viewing the sample with the 100× lens.

_____ Use the fine-focus knob to focus the specimen under 10× magnification.

_____ "Float" between the 40× and 100× objective lenses.

_____ Focus the specimen under 40× magnification.

_____ Lower the stage so you may more easily apply oil onto the slide.

_____ Place your slide on the stage.

_____ Rotate the 100× lens into place.

5 As magnification increases, _____ tends to decrease.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Light microscopy can help us directly view viruses.

_____ A microscope with a resolving power of 0.2 µm would be described as having a lower resolving power than a microscope with resolving power of 0.3 µm.

_____ Immersion oil improves image resolution by reducing light refraction.

_____ The 40× objective lens is also known as the scanning objective.

_____ The final magnification of an image is determined by multiplying the level of magnification provided by the ocular lens by the magnification provided by the objective lens.

7 Name the microscope part:

_____ Opens and closes to modulate how much light is aimed at the specimen.

_____ The part of the microscope that focuses the light aimed at the specimen.

_____ The only objective lens that should encounter immersion oil.

_____ The lens that provides the second and final image magnification.

8 Resolving power is best described as the ability to _____.

a. keep two points separated
b. differentiate between two distinct points
c. merge two distinct points
d. magnify a specimen
e. refract light

Critical Thinking

1 Why is wavelength the main limiting factor for resolution in light microscopy?

2 Would you expect water to work as well as oil for oil-immersion microscopy? Explain your reasoning—*be specific*.

3 If you do not adjust the lamp intensity or the iris diaphragm, you will notice that your specimen appears darker when viewed via the 100× lens as compared to its appearance when viewed via the 40× lens. Explain why this normal optical effect occurs.

EXERCISE 2

Microscopic Examination of Eukaryotic Microbes

After doing this exercise, you should be able to:

1. Describe the basic differences between prokaryotes and eukaryotes.
2. Name the three domains and six kingdoms of taxonomy.
3. List the taxonomic hierarchy categories from broadest to most specific.
4. Correctly write a scientific name.
5. List several features of kingdom Fungi and name some representative members.
6. List several features of kingdom Protista and name some representative members.
7. Define the term *protozoan* and name the four main groups of protozoans.
8. State what feature is primarily used to group protozoans into their four main categories.
9. Describe the features of select fungi viewed on premade and/or wet-mount slides.
10. Describe the features of select protists viewed on premade and/or wet-mount slides.

Background

Cells are described as being either prokaryotic or eukaryotic. **Prokaryotic** cells are unicellular (single-celled) microbes that have ribosomes, but lack a nucleus and other membrane-bound organelles (e.g., mitochondria, Golgi apparatus, endoplasmic reticulum, etc.). In general, prokaryotes are smaller and genetically simpler than their eukaryotic cousins (Fig. 2.1). In contrast, **eukaryotic** cells have a nucleus along with other membrane-bound organelles. Among the eukaryotes, we find unicellular and multicellular organisms. Although most of this book focuses on prokaryotes, here we'll focus on the eukaryotes with a specific emphasis on fungi and protists.

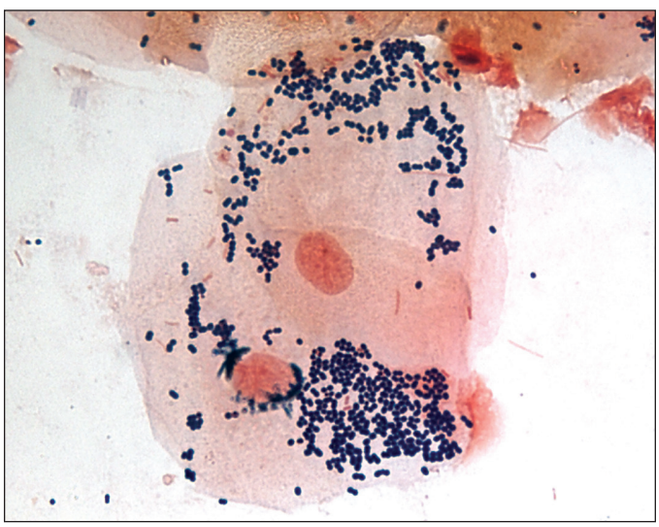


FIGURE **2.1** **Bacteria and Eukaryotic Cells** • This Gram-stained specimen was taken from just below the gum line in the mouth. The large, pink cells are human epithelial cells; notice the prominent nucleus in each cell. The small, purple and pink cells are bacteria, which are typically smaller than eukaryotic cells, roughly 1 µm to 5 µm in length versus 10 µm to 100 µm for eukaryotic cells.

Taxonomy and Eukaryotic Organisms

Taxonomy is a cataloging system that arranges living things into groups. The broadest taxonomic category is called a **domain** while the most specific is the **species** level. There are currently three taxonomic domains: Archaea, Bacteria, and Eukarya. Prokaryotes fall within the first two domains (Archaea and Bacteria) while eukaryotes are grouped within domain Eukarya. Based on genetic evidence, domain Eukarya shares a common evolutionary ancestor with the prokaryotes (Fig. 2.2).

Classifying organisms beyond the domain level has been a taxonomic battleground. The latest development in taxonomy was to divide domain Eukarya into seven **superkingdoms** (or supergroups). This newer classification model eliminates the more widely known kingdom model (Fig. 2.3).

While the superkingdom model more accurately reflects how eukaryotic organisms are genetically related, it adds complexity to learning the basic observable features of microorganisms. Because our main goal is to explore basic microbial features as opposed to genetics, in this lab we'll use the older kingdom model.

The number of kingdoms in the taxonomic hierarchy has varied over the years with the total number ranging from five to eight. For our purposes, we'll apply the six-kingdom model. In this model, prokaryotes are spread across **kingdom Bacteria** and **kingdom Archaea** while eukaryotes span four kingdoms: **Animalia**, **Plantae**, **Fungi**, and **Protista**. Regardless of how many kingdoms we acknowledge, each is further subdivided into more precise groupings as follows: phylum, class, order, family, genus, and species, with species being the most specific grouping.

An organism's scientific name includes its genus and species designation. All scientific names are written with the genus name capitalized and the species name lowercased. The entire scientific name is then either underlined or italicized. When writing a scientific name, you should first write the full name. In later references, you can abbreviate the name by writing the first letter of the genus as an uppercase letter followed by a period and the full species name in lowercase letters. As with a full scientific name, abbreviated names should be either underlined or italicized (Fig 2.4).

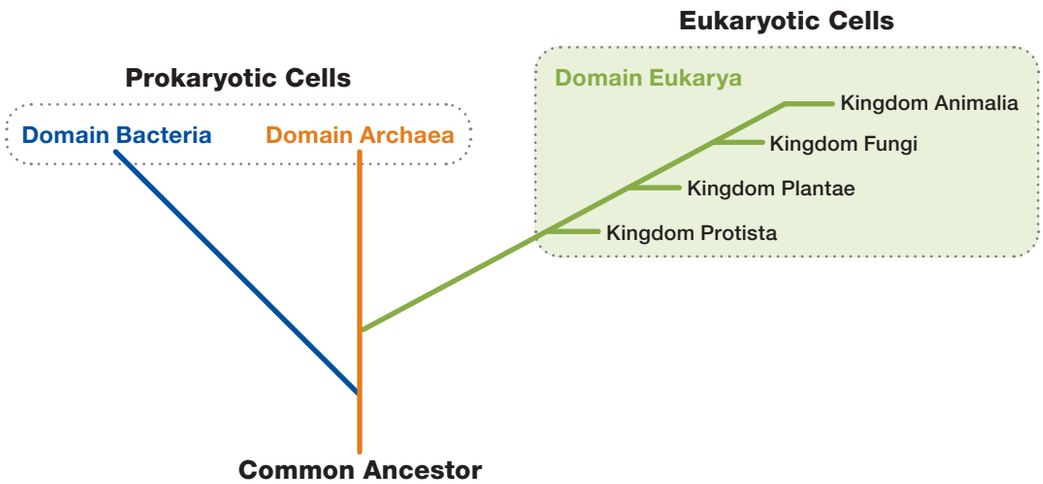


FIGURE **2.2** **The Three Taxonomic Domains of Life** ●

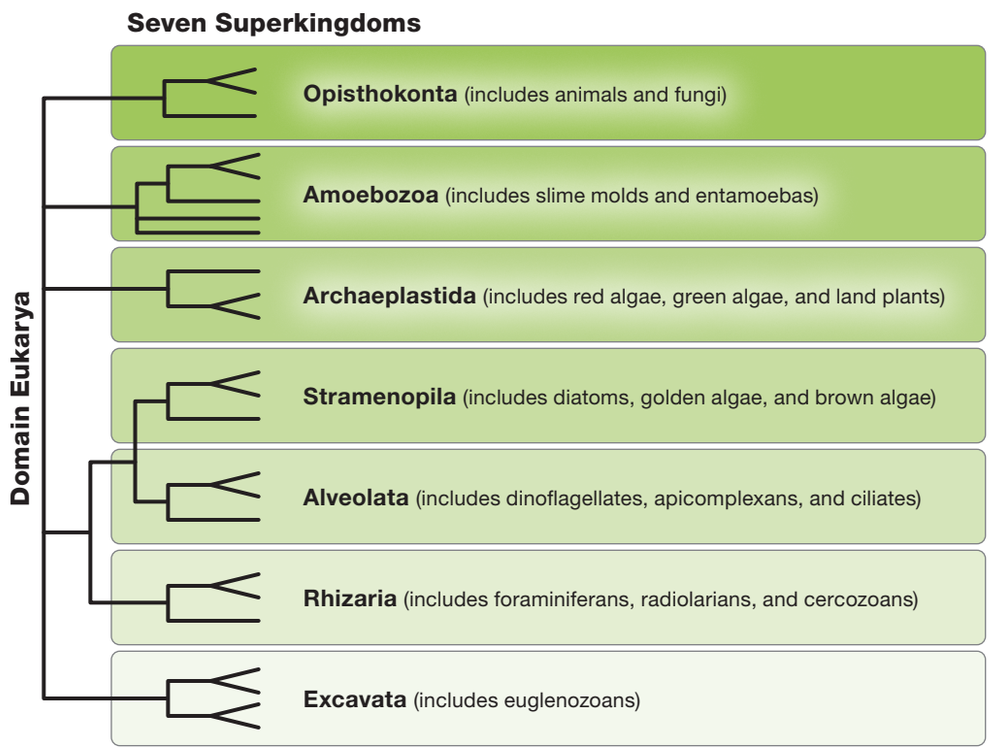
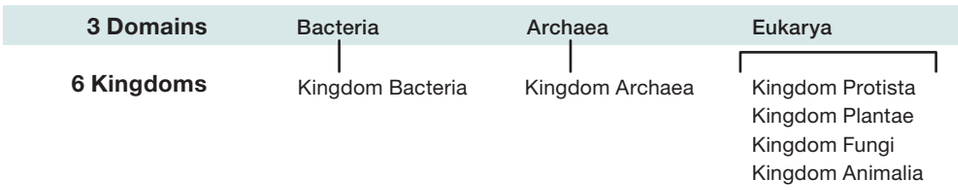


FIGURE **2.3** **Domain Eukarya's Seven Superkingdoms** ● Genetic analyses have helped scientists organize domain Eukarya into superkingdoms. The superkingdom model's details are still being debated. When organisms are grouped based on their genetics, it's easier for us to see that diatoms are closely related to certain algae while fungi are more closely related to animals than to slime molds.



Broadest category

Most specific category

Example	
Domain	Eukarya
Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	Penicillium
Species	Chrysogenum
Full scientific name: *Penicillium chrysogenum* Abbreviation: *P. chrysogenum*	

FIGURE **2.4** **Taxonomic Hierarchy and Scientific Names** • You can remember the taxonomic hierarchy order with the following mnemonic device: **D**ear **K**ing **P**hilip **c**ame **o**ver **f**or **g**reat **s**paghetti.

Exploring Kingdom Fungi

Fungi (singular: fungus) are nonmotile, non-photosynthetic eukaryotes. Their cell wall is usually made of the polysaccharide **chitin**, not cellulose as in plants. Aside from pathogenic fungi, most fungi are **saprobes**, meaning they get their nutrients from decomposing organic matter. Based on their appearance, fungi are informally divided into **filamentous molds** and **unicellular yeasts**.

Molds

Molds grow as filaments of cells. These filaments are called **hyphae** (singular: hypha) and collectively form a **mycelium**. Molds produce a variety of sexual and asexual reproductive spores, which are used as a basis for classification. Three genera of molds will be covered here: *Rhizopus*, *Aspergillus*, and *Penicillium*.

- ***Rhizopus***

  The dozen or so *Rhizopus* species are fast-growing molds that produce white or grayish, cottony growth. The mycelium becomes darker with age as sporangia form, giving it a "salt and pepper" appearance (Fig. 2.5A). *Rhizopus* species produce broad surface and aerial hyphae with irregular diameters. Anchoring **rhizoids** form where the surface hyphae (**stolons**) join the bases of the long, unbranched **sporangiophores** (Fig. 2.5B).

  Rhizopus's life cycle has both sexual and asexual phases. Asexual **sporangiospores** are produced by large, circular sporangia borne at the ends of the elevated sporangiophores (Fig. 2.5C). A hemispherical **columella** supports the sporangium. After release, the sporangiospores develop into hyphae identical to those that produced them.

  On occasion, sexual reproduction occurs when hyphae of different mating types (designated + and – strains) make contact. Initially, **progametangia** extend from each hypha and nuclei and cytoplasm flow into them (Fig. 2.5D). As a consequence, they enlarge and contact one another. Upon contact, a septum separates the end of each progametangium into a **gametangium** and a **suspensor** (Fig. 2.5E).

  The walls between the two gametangia dissolve, and a thick-walled **zygosporangium** develops (Fig. 2.5F). Fusion of nuclei occurs within the zygosporangium, resulting in multiple diploid zygote nuclei. After a dormant period, zygote meiosis occurs and produces haploid **zygospores** which, when released, develop into new hyphae and the life cycle is completed (Fig. 2.5G).

  Two *Rhizopus* species (*R. oryzae* and *R. microsporus*) are primarily responsible for producing **zygomycosis** (also called mucormycosis). This infection is found most frequently in diabetics, organ transplant recipients, and other immunocompromised patients. Portal of entry and overall patient health are critical factors in determining the location and extent of infection. If untreated, the mortality rate is high, between 47–84% worldwide. On a less severe note, most species are harmless saprophytes. *R. stolonifer* is the common bread mold.

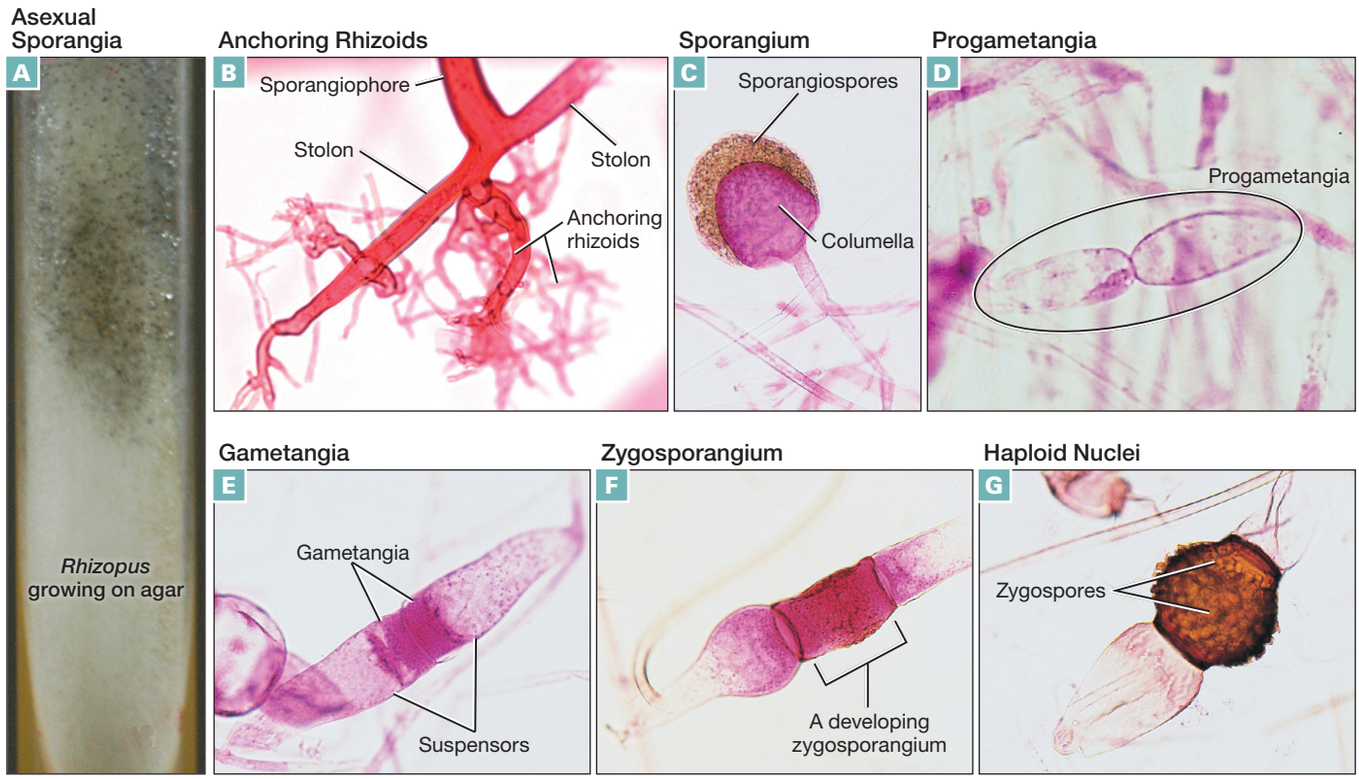


FIGURE **2.5** **Mold *Rhizopus*** • (A) **Asexual Sporangia:** The black asexual sporangia of the bread mold Rhizopus stolonifer give the mold a "salt and pepper" appearance. (B) **Anchoring Rhizoids:** Anchoring rhizoids form at the junction of each sporangiophore and the stolon. Note the absence of the septa—divisions between cells. (C) **Sporangium:** The sporangium is found at the end of a long, unbranched, and aseptate sporangiophore. The haploid asexual sporangiospores in the sporangium cover the surface of the columella, which has a flattened base. (D) **Progametangia:** The circled progametangia developed from different hyphae and will eventually fuse to form a gamete. (E) **Gametangia:** Gametangia and suspensors are shown. Gametangia contain one or more haploid nuclei from each mating type. (F) **Zygosporangium:** A zygosporangium forms when the cytoplasm from the two mating strains fuse. (G) **Haploid Nuclei:** Haploid nuclei from each strain fuse within the zygosporangium to produce many diploid zygote nuclei. (Commercially prepared slides.)

- ***Aspergillus***

Aspergillus species are commonly found in soil and on plants. Roughly 180 species have been identified. They form green to yellow or brown granular colonies with a white edge. One species, *A. niger*, produces distinctive black colonies. The *Aspergillus* "fruiting body" is distinctive, with chains of spores (called conidia) arising from one or two rows of cells attached to a swollen **vesicle** at the end of an unbranched stalk (Fig. 2.6A). Fruiting body structure and size, and conidia color are useful in species identification. It used to be thought that *Aspergillus* species were unable to sexually reproduce, but evidence is mounting that under the right environmental conditions sexual spores, called **ascospores**, are made.

A. fumigatus and other species are opportunistic pathogens that cause aspergillosis, an umbrella term covering many diseases. Immunocompromised patients have the highest risk of infection. Allergic aspergillosis may occur in individuals who are in frequent contact with the spores and become sensitized to them. Subsequent contact produces symptoms similar to asthma. Aspergilloma (fungus ball) involves colonization of the paranasal sinuses or lungs, resulting in abscess formation. It is frequently asymptomatic and resolves without treatment, but in serious cases it can be fatal if not treated.

Some *Aspergillus* species are commercially important. *A. oryzae* is used in the production of sake (made from rice), and soy paste (made from soybeans), which may be further fermented with *A. oryzae* and *A. soyae* into soy sauce. Aspergilli are also used in commercial production of citric acid, various enzymes, and many other products. Further, *A. niger* and *A. oryzae* have been designated as GRAS (generally regarded as safe) by the Food and Drug Administration and World Health Organization, which has opened the door for using them as hosts in biotechnological applications.

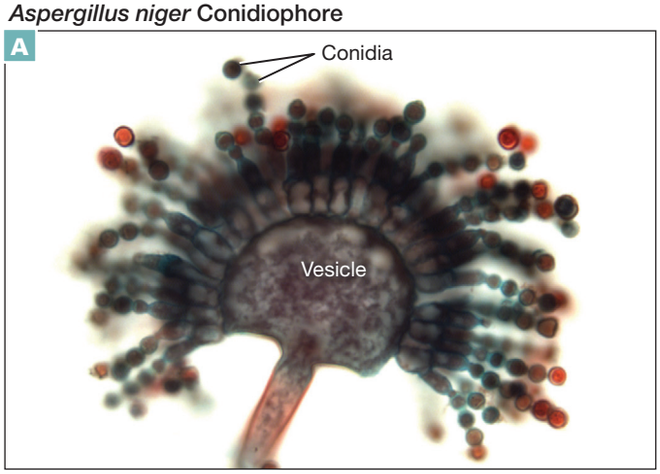
- ***Penicillium***

Members of the genus *Penicillium* are everywhere, being found in the air, soil, and decaying organic matter worldwide. Over 200 species have been

identified. They produce distinctive green, powdery, radially furrowed colonies with a white edge. *Penicillium* fruiting bodies are distinctive, looking like a brush (penicillus means "paintbrush") at the end of an elevated stalk (Fig. 2.6B).

Penicillium is best known for making the antibiotic penicillin (discovered by Alexander Fleming in 1928), but it is also a known **opportunistic pathogen** (an agent that mainly afflicts a weakened host). For example, *P. marneffei*, is endemic to Asia and is responsible for opportunistic infections of the lungs, liver, and skin in immunosuppressed and immunocompromised patients. *P. chrysogenum* is common in damp, indoor habitats and their spores can cause allergic reactions. Other species are important because they cause fruit and vegetable spoilage. Some *Penicillium* species are commercially important for making cheeses, *P. roquefortii* helps make Roquefort cheese and *P. camembertii* is used to make Camembert and Brie cheeses.

Aspergillus niger Conidiophore



Penicillium's "Brush-like" Conidiophore

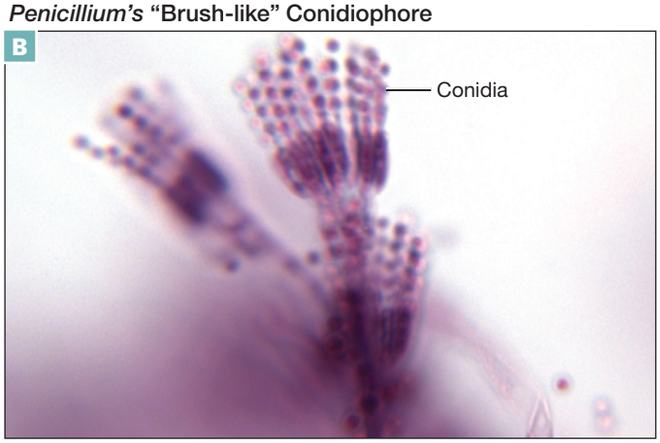


FIGURE **2.6** **Examples of Conidiophores: Molds *Aspergillus* and *Penicillium*** (A) ***Aspergillus:*** Note the chains of spores (conidia) extending from the surface of the vesicle. (B) ***Penicillium:*** *Penicillium* species produce a characteristic brush-shaped fruiting body with chains of conidia. (Commercially prepared slides.)

Yeasts

Yeasts are the only unicellular fungi, but they can arrange themselves into short chains of cells. Two types of yeast are covered here: *Saccharomyces cerevisiae* and *Candida albicans*.

- ***Saccharomyces cerevisiae***
  Saccharomyces cerevisiae is used to make bread, wine, and beer but it is not an important human pathogen. It does not form a mycelium but, rather, produces a colony similar to bacteria (Fig. 2.7A). Vegetative cells are generally oval to round in shape, and asexual reproduction occurs by budding (Fig. 2.7B). Short **pseudohyphae** are sometimes produced when the budding cells fail to separate. In an *S. cerevisiae* population, haploid and diploid cells are indistinguishable from one another and both reproduce asexually by budding. Diploid cells occasionally undergo meiosis and produce one to four haploid **ascospores**. When released, ascospores bud to produce more haploid cells. Occasionally, haploid cells of opposite mating types combine to create a new diploid cell, thus acting like gametes.
- ***Candida albicans***
  Candida albicans is part of the normal upper respiratory, skin, gastrointestinal, and urogenital microbiota of men and women. It's also the most common fungal opportunistic pathogen (Fig. 2.7C). It is the causative agent of thrush in the oral cavity, vulvovaginitis of the female genitalia, and cutaneous candidiasis of the skin. Individuals most susceptible to *Candida* infections are diabetics, those with immunodeficiency (e.g., AIDS), catheterized patients, and individuals taking antimicrobial medications that disrupt the normal microbiota balance. Despite common belief, men can develop a yeast infection of the penis, although yeast infections are far more common in women.

  Candida albicans are round to oval cells that reproduce by budding and are 3–6 µm in diameter. Sometimes the cells separate after budding, but other times they remain attached, forming branched chains of two types: **pseudohyphae**, which are narrower at their junctions, and true hyphae which are not. Both types are asexual spores. The ability to switch between pseudohyphae and true hyphae may increase virulence. The sexual life cycle is similar to *S. cerevisiae*.

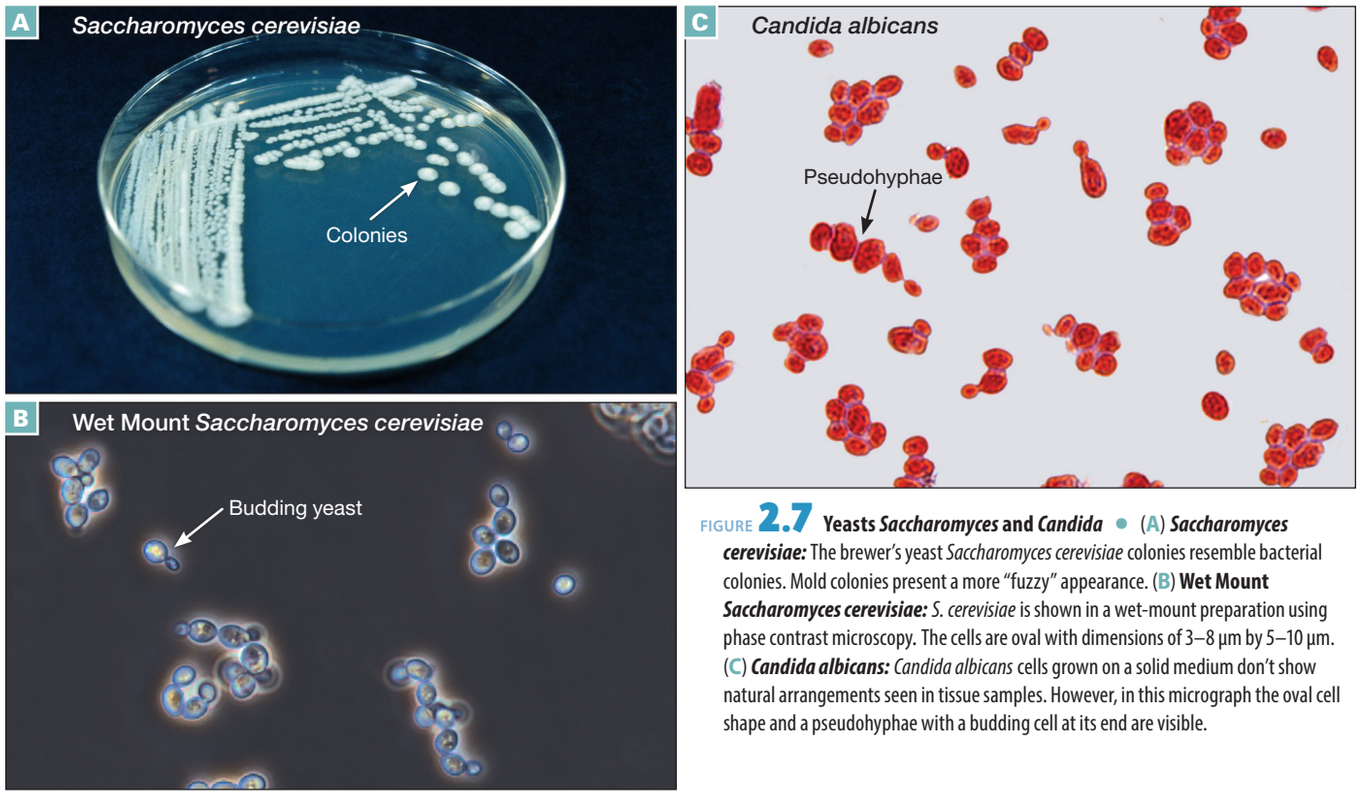


FIGURE **2.7** **Yeasts *Saccharomyces* and *Candida*** • (A) ***Saccharomyces cerevisiae:*** The brewer's yeast *Saccharomyces cerevisiae* colonies resemble bacterial colonies. Mold colonies present a more "fuzzy" appearance. (B) **Wet Mount *Saccharomyces cerevisiae:*** *S. cerevisiae* is shown in a wet-mount preparation using phase contrast microscopy. The cells are oval with dimensions of 3–8 µm by 5–10 µm. (C) ***Candida albicans:*** *Candida albicans* cells grown on a solid medium don't show natural arrangements seen in tissue samples. However, in this micrograph the oval cell shape and a pseudohyphae with a budding cell at its end are visible.

Exploring Kingdom Protista

In the six-kingdom model, kingdom Protista is a miscellaneous category encompassing organisms not assigned to the other three eukaryotic kingdoms: Animalia, Plantae, or Fungi. Protists can be described as plant-like, fungi-like, and animal-like. Some protists are photosynthetic, while others are not.

Plant-like Protists

Algae are examples of plant-like protists. These organisms are photosynthetic, typically have cellulose cell walls, and they often grow in fresh water. Most algae grow as unicellular, filamentous, or colonial species. Some unicellular algae, such as *Chlamydomonas*, use flagella for motility (Fig. 2.8A). *Volvox* is an example of a colonial species of green algae (Fig. 2.8B). *Volvox* colonies grow as spherical aggregates that contain about 50,000 cells. These algae can undergo sexual and asexual reproduction. *Spirogyra* is an example of a filamentous algae, it grows as long strands, which is why it's sometimes commonly known as "mermaid's tresses" or "water silk" (Fig. 2.9).

Diatoms are another example of a plant-like protist. These organisms, which are often referred to as microalgae, can exists as single-celled or colonial photosynthetic autotrophs (Fig. 2.10). They have a distinctive golden-brown color due to the abundance of the pigment

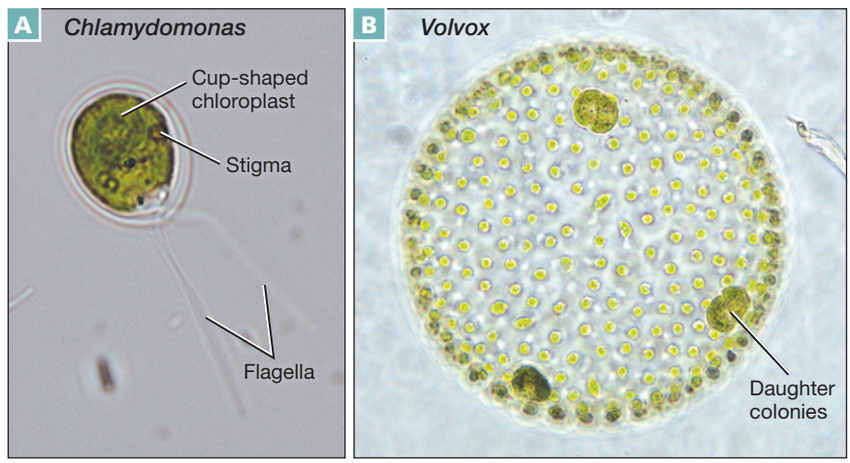


FIGURE **2.8** **Plant-like Protists: Green Algae** • (A) ***Chlamydomonas:*** *Chlamydomonas* is a unicellular green alga with a single, cup-shaped chloroplast. A red-orange stigma is found within the chloroplast and is involved in phototaxis (attraction to light). It also has two flagella for locomotion. Cells are approximately 30 µm long and 20 µm wide. (Wet mount of an environmental sample.) (B) ***Volvox:*** *Volvox* is a spherical colonial green alga made of cells similar in shape to *Chlamydomonas*. Daughter colonies form asexually from special cells in the parent colony. Release of a daughter colony involves its eversion as it exits through an enzymatically produced pore. Mature colonies can reach a size visible to the naked eye. (Wet mounts from culture.)

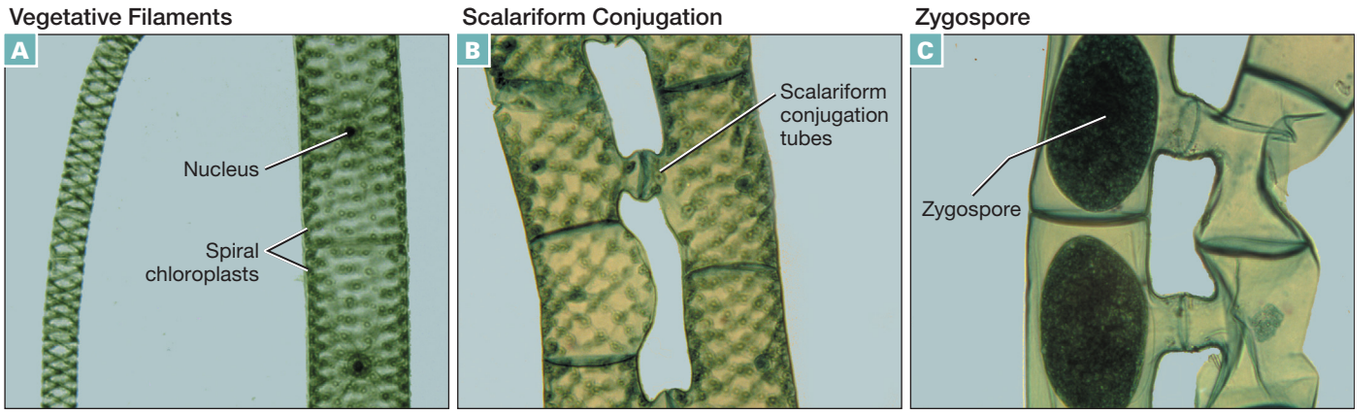


FIGURE **2.9** **Plant-like Protists: *Spirogyra*** • *Spirogyra* is a filamentous algae with distinctive spiral chloroplasts. The cells are haploid, that is, they have only one complete set of chromosomes. (A) **Vegetative Filaments:** Two vegetative filaments are shown here. Also note the nuclei in the cells on the right. (B) **Scalariform Conjugation:** These two filaments have begun scalariform conjugation, a sexual process that occurs between different filaments. The protrusions are called conjugation tubes and serve as a connection through which the cytoplasm of one cell (acting as a gamete) can move and join with the cytoplasm of a cell in the other filament. (C) **Zygospore:** The cells and their two haploid nuclei fuse to form a diploid zygospore and conjugation is complete. When conditions are favorable, the zygospore germinates by undergoing meiosis. Only one haploid nucleus survives and the zygospore cell develops into a new vegetative filament. Note the absence of spiral chloroplasts in the empty cells and in the zygospores. (Commercially prepared slides.)

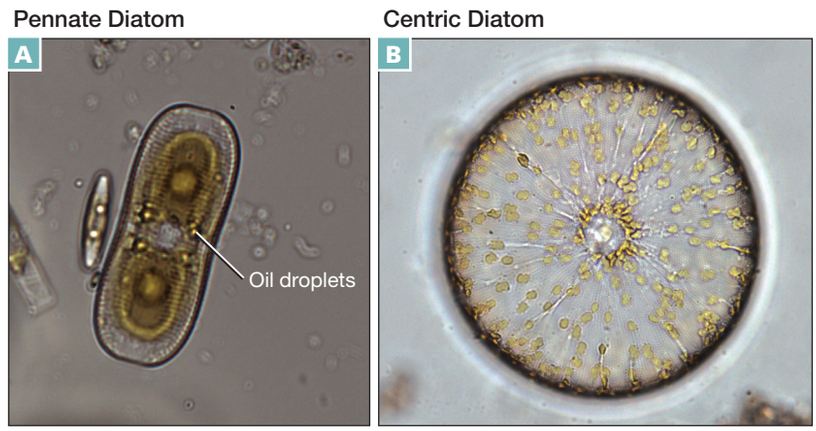


FIGURE **2.10** **Plant-like Protists: Diatoms** • Diatoms are composed of silica and organic material. They have a cell wall that is divided into two halves, one of which fits into the other like the lid and base of a petri dish, and they are divided into two groups based on their symmetry. (A) **Bilateral symmetry:** Diatoms with bilateral symmetry are described as pennate. (B) **Radial Symmetry:** Diatoms with radial symmetry are described as centric. Notice the distinctive golden chloroplasts, the cell wall ornamentation, and the oil droplets. (Wet-mount preparations of environmental samples.)

fucoxanthin. Their highly ornamented cell wall is made of silica embedded in an organic matrix and consists of two halves with one half overlapping the other in the same way the lid of a petri dish overlaps its base. **Centric diatoms** have a round shape, whereas **pennate diatoms** are bilaterally symmetrical. Oil droplets are frequently visible inside diatoms. None of the plant-like protists are known to cause diseases in humans or other animals.

Fungi-like Protists

Some protists have more in common with fungi than they do plants or animals. These fungi-like protists are non-photosynthetic and instead live as saprobes. An example includes **slime molds**. Slime molds are unicellular organisms, but they can aggregate into a multicellular state to reproduce. If you've ever looked at a decaying tree on the forest floor or a wood-mulched flower bed and seen a yellow blob that looks like yellow dog-vomit, you've likely seen a slime mold. None of the fungi-like protists are known to cause diseases in humans or other animals.

Animal-like Protists

The animal-like protists can be free living or parasitic. They include a wide range of pathogens that afflict humans and other animals. Sometimes called **protozoans** (or protozoa) these organisms are unicellular, lack a true cell wall, exhibit asexual and sexual reproduction, and are typically non-photosynthetic, although some, like *Euglena*, are photosynthetic. Protozoans are organized into four groups based on their means of motility in their adult form.

- **Amoeboid Protozoans (Sarcodina)**
  All amoebas move by forming cytoplasmic extensions called **pseudopods,** which are also used to engulf their food. Sarcodines reproduce sexually by syngamy (fusion of two gametes) and asexually by division

or budding, they do not have a cell wall, and they can be free-living or parasitic. Classical amoebas are free-living in marine, freshwater, and soil environments (Fig. 2.11A). Another group of amoebas are the **entamoebas,** some of which are parasitic (Fig. 2.11B).

- **Flagellated Protozoans (Mastigophora)**
These protozoans include free-living microbes like *Euglena* and parasitic microbes such as *Trypanosoma* species, and *Trichomonas vaginalis*.

Euglena live as photosynthetic autotrophs when light is available and when light is not available, they can live as heterotrophs (ingest and breakdown an external organic carbon source for energy). They usually have two flagella, or more rarely only one. The red "eyespot" is a distinctive characteristic that is easily seen with the light microscope (Fig. 2.12A).

Trypanosoma species are heterotrophic and include important human pathogens. Two species (*T. brucei gambiense* and *T. brucei rhodesiense*) are pathogens that cause West African trypanosomiasis and East African trypanosomiasis, respectively (Fig. 2.12B). The former is generally a mild, chronic disease that may last for years before the nervous system is affected, whereas the latter is more acute.

Trypanosomes have a complex life cycle involving the tsetse fly (genus *Glossina*) as an intermediate host, which transmits the pathogen to the human host during a blood meal. The symptoms of sleeping sickness—sleepiness, emaciation, and unconsciousness—begin when the central nervous system becomes infected. Depending on the infecting strain, the disease may last for months or years, but without drug treatment the mortality rate is high.

Trichomonas vaginalis is the causative agent of trichomoniasis in humans (Fig. 2.12C). It is the most common pathogenic protozoan infection in people who live in industrialized nations. Indeed, it is the most common sexually transmitted eukaryotic infection. The parasite can cause inflammation of genitourinary mucosal surfaces. About 70% of cases are asymptomatic, but when symptoms do develop patients may experience itching in the penis or vagina, burning upon urination, and a discharge from the penis or vagina. An infection during pregnancy has been linked to preterm labor and lower birth weight babies. Trichomoniasis can be effectively treated with antiparasitic drugs.

- **Ciliated Protozoans (Ciliophora)**
Ciliates have hair-like appendages called **cilia** covering their outer surface. Cilia provide motility when they sweep back-and-forth. And because they line the **oral groove,** they also sweep food particles inward toward the **cytostome** for ingestion. Ciliates are common in aquatic environments and include diverse species from free-living *Paramecium* species and *Stentor* to parasites like *Balantidium coli*, the only ciliated protozoan known to cause human disease (Fig. 2.13).

Paramecium and *Stentor* are non-pathogenic ciliates that feed on organic matter in their environments. *Paramecium* species are found in marine and freshwater environments and are active swimmers as they search for food. *Stentor* species are found in freshwater and, although they can swim, they spend most of their time anchored to a surface as they feed.

Balantidium coli causes a rare form of dysentery called balantidiasis. This disease is found worldwide, but it's most prevalent in tropical and subtropical

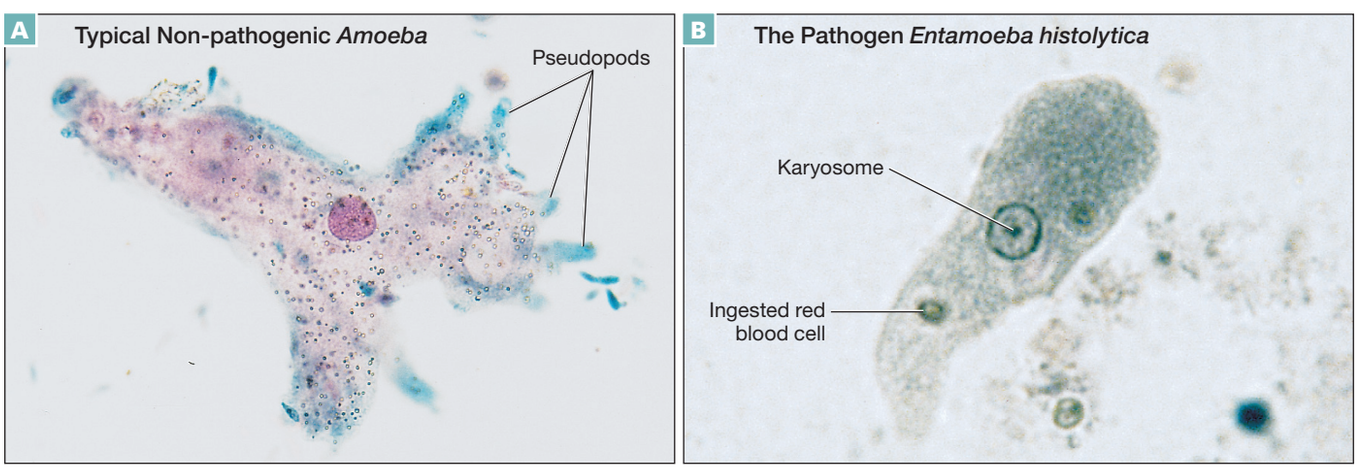


FIGURE **2.11** **Animal-like Protists: Amoeboid Protozoans** • (A) **Typical Non-pathogenic *Amoeba:*** This is a typical non-pathogenic *Amoeba*; here you can see numerous pseudopods, which are used for movement as well as engulfing prey. (Commercially prepared slide.) (B) ***Entamoeba histolytica:*** *Entamoeba histolytica* is the causative agent of amebic dysentery. Trophozoites range in size from 15 to 50 µm. Notice the small, central karyosome, the dark-staining, beaded chromatin at the periphery of the nucleus, and the ingested red blood cell, all of which are characteristic of *E. histolytica.* (Iron hematoxylin stain of fecal smear.)

developing countries. The largest risk factor seems to be contact with pigs, the suspected natural reservoir for this parasite.

B. coli exists in two forms: a vegetative trophozoite and a cyst. Infection develops upon ingesting the cyst stage, typically by consuming sewage

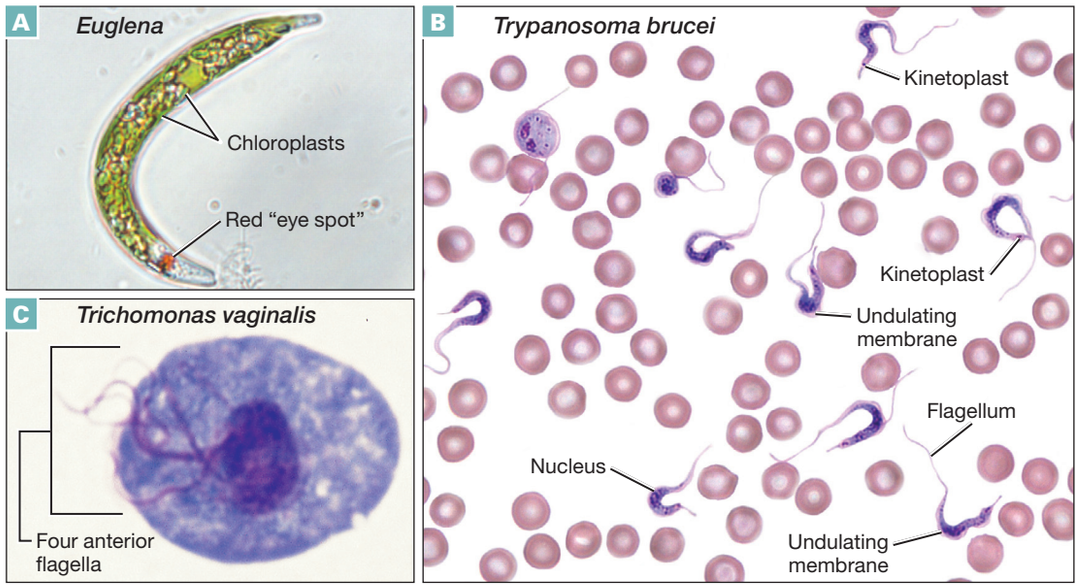


FIGURE **2.12** **Animal-like Protists: Flagellated Protozoans** ● (A) ***Euglena:*** *Euglena* is a large genus of mixotrophic flagellates. Most species have chloroplasts and a red "eyespot" located in the colorless anterior of the cell. In this cell, a single flagellum also emerges from the anterior. (Wet mount from an environmental sample.) (B) ***Trypanosoma brucei:*** The central nucleus, posterior kinetoplast, and undulating membrane are visible in this commercially prepared blood smear of *Trypanosoma brucei.* (C) ***Trichomonas vaginalis:*** The *Trichomonas vaginalis* trophozoite is the only stage of the protist's life cycle and is 7–23 µm long by 5–15 µm wide. The four anterior flagella are visible here. (Commercially prepared slide.)

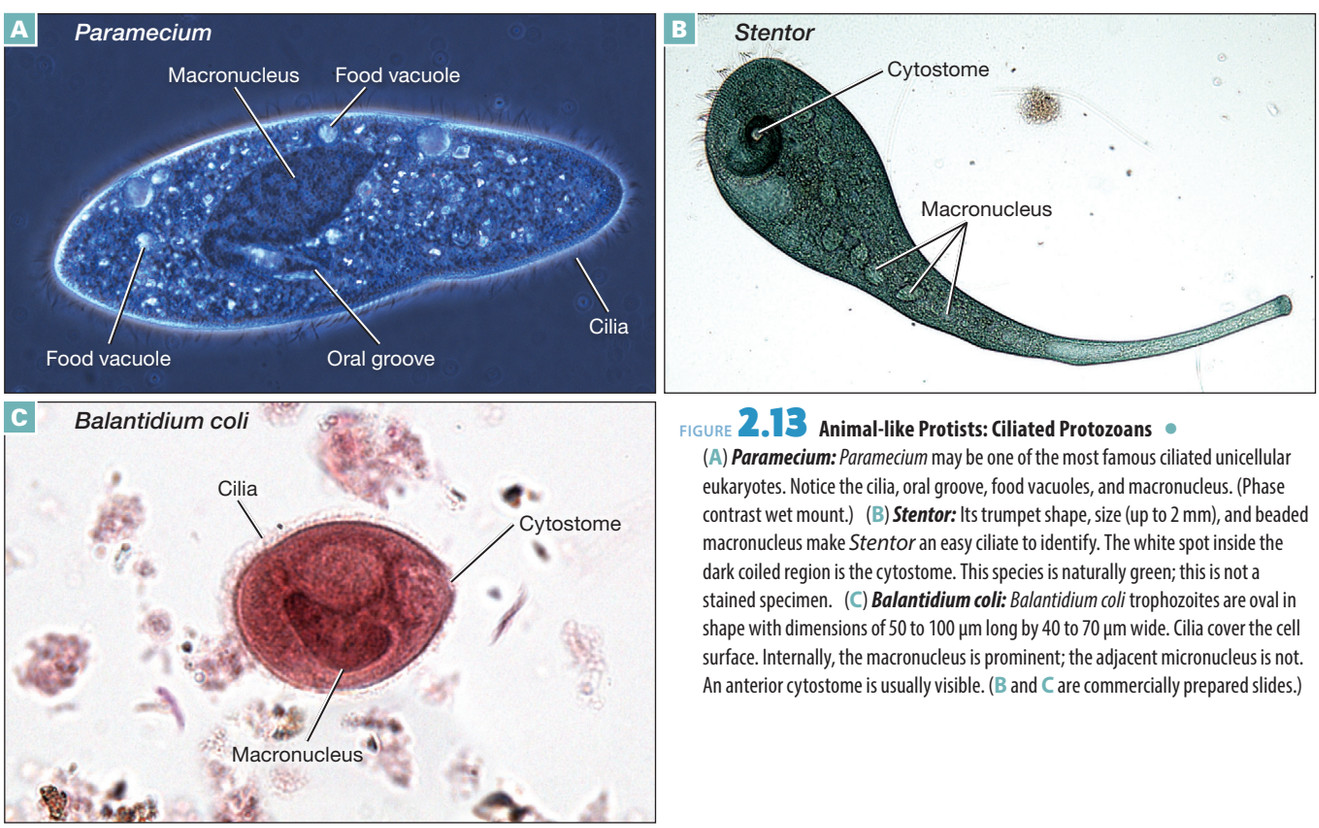


FIGURE **2.13** **Animal-like Protists: Ciliated Protozoans** ● (A) ***Paramecium:*** *Paramecium* may be one of the most famous ciliated unicellular eukaryotes. Notice the cilia, oral groove, food vacuoles, and macronucleus. (Phase contrast wet mount.) (B) ***Stentor:*** Its trumpet shape, size (up to 2 mm), and beaded macronucleus make *Stentor* an easy ciliate to identify. The white spot inside the dark coiled region is the cytostome. This species is naturally green; this is not a stained specimen. (C) ***Balantidium coli:*** *Balantidium coli* trophozoites are oval in shape with dimensions of 50 to 100 µm long by 40 to 70 µm wide. Cilia cover the cell surface. Internally, the macronucleus is prominent; the adjacent micronucleus is not. An anterior cytostome is usually visible. (B and C are commercially prepared slides.)

contaminated food or water. The cysts make their way to the host's colon where they mature into trophozoites. Laboratory diagnosis involves identifying either cysts or the trophozoite in the patient's feces. Although most infections are asymptomatic, acute infection symptoms can include bloody and mucoid feces, nausea, vomiting, and abdominal tenderness. Diarrhea alternating with constipation may occur in chronic infections. The infection is treated with a combination drug therapy.

- **Spore-Forming Protozoans (Apicomplexa)**
  In their mature form, apicomplexans lack flagella, cilia, and pseudopodia; they instead move by gliding. Most apicomplexans are obligate intracellular parasites that have a complex life cycle involving more than one host and multiple tissues. For instance, *Plasmodium* species (Fig. 2.14) cause malaria and infect both liver and red blood cells of humans, and the gut and salivary glands of the insect vector, the female *Anopheles* mosquito. Another noteworthy apicomplexan is *Toxoplasma gondi*, which is often spread by cats and causes toxoplasmosis.

Plasmodium falciparum

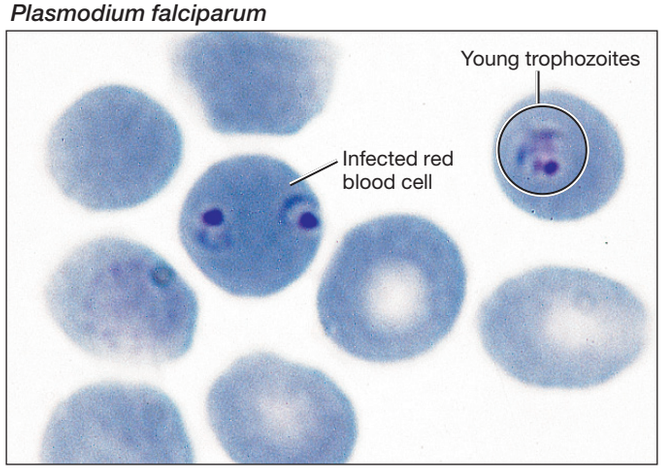


FIGURE **2.14** **Animal-like Protists: Apicomplexans** ● *Plasmodium falciparum* is a causative agent of malaria. Shown here are young trophozoites, called "ring forms," within red blood cells (RBCs). One RBC has been infected by two trophozoites, a common occurrence with *P. falciparum*. (Commercially prepared slide.)

Clinical Applications

Medically speaking, aside from the kingdom Plantae, we find infectious agents among all the eukaryotic kingdoms. A basic understanding of eukaryotic cells, especially eukaryotic pathogens, is important for anyone entering a medically related field.

In the animal kingdom, we find **helminths** (parasitic roundworms and flatworms), which are typically transmitted to their host in a microscopic form (e.g., as larvae or eggs). In the kingdom Protista, we find a miscellaneous assortment of infectious agents that cause diseases ranging from the common sexually transmitted disease trichomoniasis to rare forms of **encephalitis** (inflammation of the brain) and malaria.

Among the fungi, we find select organisms that infect humans and other animals as either **true pathogens** (cause disease in normally healthy hosts) and/or as opportunistic pathogens. Fungal infections are called **mycoses**. These infections can range from a mere discomfort, such as athlete's foot (a type of "ringworm" infection), to potentially deadly forms of encephalitis caused by a variety of fungi such as *Cryptococcus*, *Coccidioides*, *Histoplasma*, and *Blastomyces*.

MATERIALS

- ❑ Compound light microscope
- ❑ Lens paper
- ❑ Lens-cleaning solution or 95% ethanol
- ❑ Immersion oil
- ❑ Lab coat and safety glasses if performing Part C
- ❑ Prepared slides (as available) of:
  - ☐ *Aspergillus* conidiophore (Part A)
  - ☐ *Candida albicans* (Part A)
  - ☐ *Penicillium* conidiophore (Part A)
  - ☐ *Rhizopus* sporangia and gametangia (Part A)
  - ☐ *Entamoeba histolytica* trophozoites (Part B)
  - ☐ *Trypanosoma* species trypomastigotes in blood (Part B)
  - ☐ *Trichomonas vaginalis* (Part B)
  - ☐ *Balantidium coli* trophozoites (Part B)
  - ☐ *Plasmodium* species (thick blood smear) (Part B)
  - ☐ Diatoms (centric and pennate) (Part B)

(continues)

MATERIALS *(continued)*

- ❑ Clean glass slides and cover glasses (Part C)
- ❑ Cytological stains (e.g., methylene blue, Lugol's iodine) (Part C)
- ❑ Cotton swabs (Part C)
- ❑ Methyl cellulose (Part C)
- ❑ Living cultures (as available) (Part C)

Protists:

- ☐ *Amoeba*
- ☐ *Chlamydomonas*
- ☐ *Euglena*
- ☐ *Paramecium*
- ☐ *Spirogyra*
- ☐ *Stentor*
- ☐ *Volvox*

Fungi:

- ☐ *Saccharomyces cerevisiae*

PROCEDURE

There are three parts in this lab. Parts A and B involve viewing commercially prepared slides of fungi and protists. Part C involves preparing and viewing wet mounts of living organisms. You may be asked to perform all or some of these parts. Your instructor will advise you as to which ones to do and which slides/specimens you should view.

PART A: Viewing Premade Slides of Fungi

For Steps 1–5 in this lab, use the premade slides in the following list or slides specified by your instructor.

- *Aspergillus* conidiophore: identify hyphae, conidiophores, and chains of conidia.
- *Candida albicans*: identify vegetative cells and budding cells.
- *Penicillium* conidiophore: identify hyphae, conidiophores, and chains of conidia.
- *Rhizopus* (sporangia and gametangia)
  - Sporangia: identify sproangiophores, sporangia, and spores.
  - Gametangia: identify progametangia, gametangia, young zygosporangia, and mature zygosporangia.

1. Obtain a microscope and place it on the table. Check that the stage is all the way down and that the scanning objective (4× objective) is in place.
2. Begin with a prepared slide. Clean it with a tissue if it is dirty, and then place it on the microscope stage. Center the specimen under the scanning objective.
3. Follow the instructions from the Lab Technique in Exercise 1, page 21, to bring the specimen into focus.
4. Scan the specimen until you're satisfied that you have seen everything in the list provided, or as specified by your instructor. Sketch what you see in the table provided in the data sheet at the end of this lab.
5. Repeat this process with as many slides as you have time for. Use the appropriate figures in this exercise to help you identify the organisms and their structures. ***Note:*** You may have to look at more than one slide to find the listed features for each organism. Your instructor will advise you of their expectations for this.

PART B: Viewing Premade Slides of Protists

Repeat the process described in Part A, but use the slides in the following list or slides your instructor specifies.

- *Balantidium coli* trophozoites: identify cilia, macronucleus, micronucleus (maybe), and cytostome.
- Diatoms: identify centric and pennate shapes, chloroplasts, oil droplets, and cell walls with sculpturing.
- *Entamoeba histolytica* trophozoites: identify pseudopods; nucleus, central karyosome, and ingested red blood cells.
- *Plasmodium falciparum* (or other species): identify red blood cells and ring stage of trophozoites.
- *Trichomonas vaginalis:* identify nucleus and flagella.
- *Trypanosoma* in blood: identify trypomastigotes with flagella, undulating membrane, kinetoplast, nucleus, and red blood cells.

PART C: Making Wet-Mount Slides of Select Eukaryotes

1. Prepare wet mounts of available specimens by following the procedural diagram in Figure 2.15. A drop of methyl cellulose may be added to the wet mount if you have fast swimmers.
2. Sketch what you see and record cellular dimensions in the table provided on the data sheet at the end of the exercise.
3. Repeat with as many specimens as time permits.

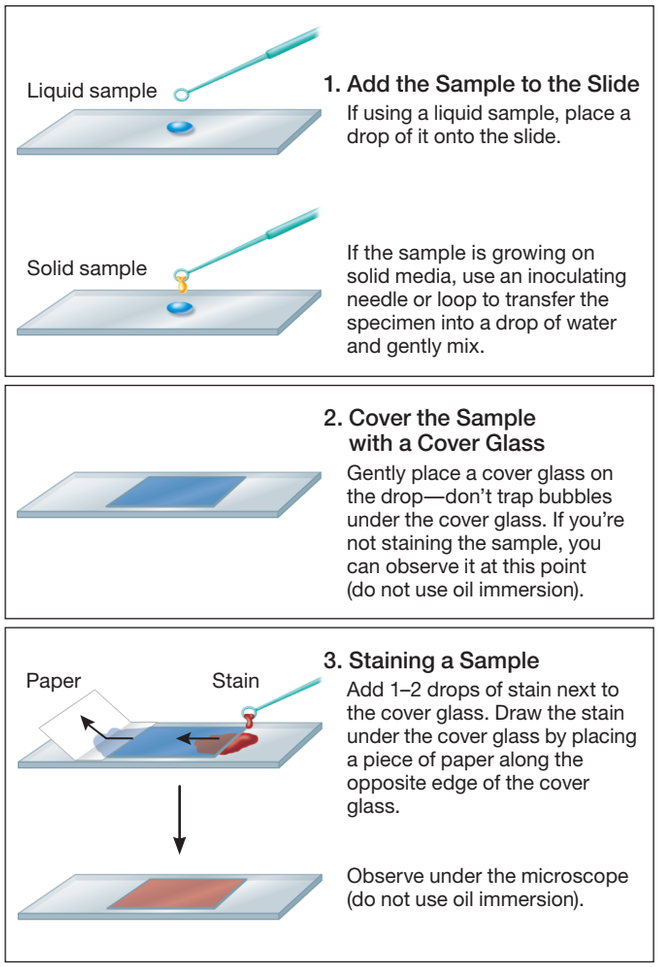


FIGURE **2.15** **Preparing a Wet-Mount Slide** • The preparation may be stained or not, but staining will eventually kill the organisms. Of course, so will drying. You can expect a wet mount to last only 15–20 minutes.

Specimens that will not be stained:

- *Amoeba*: identify nucleus, pseudopods, and ingested material.
- *Chlamydomonas*: identify chloroplast, flagella, and stigma.
- *Euglena*: identify chloroplast, flagellum, eyespot.
- *Paramecium*: identify macronucleus, cilia, oral groove, and contractile vacuole.
- *Spirogyra*: identify nuclei, spiral chloroplasts, and cell wall.
- *Stentor*: identify cilia, beaded macronucleus, and cytostome.
- *Volvox*: identify colony and daughter colonies.

The following sample requires staining with methylene blue or Lugol's iodine:

- *Saccharomyces cerevisiae*: identify vegetative cells, budding cells, and nuclei.

PART D: Clean Up

1. When you are finished observing the specimens, use lens paper to remove oil from the oil-immersion lens (if used). Also, check the 40× lens for oil and clean it if necessary.
2. Return all lenses and adjustments to their storage positions before putting the microscope away.
3. Return prepared slides to their proper storage box. Dispose of wet-mount slides as directed by your instructor.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 2

DATA SHEET

Microscopic Examination of Eukaryotic Microbes

During the Lab

1 Fill in the table for each eukaryotic microbe you observe.

Organism (indicate if wet-mount or prepared slide)	Sketch (include magnification and stain, if any)	Identifying Characteristics (list only those you observed)

(continues)

Organism (indicate if wet-mount or prepared slide)	Sketch (include magnification and stain, if any)	Identifying Characteristics (list only those you observed)

2 What features did the cells you observed have in common? How were they different?

3 If you observed the same organism on a prepared slide and a wet mount, how did the images compare?

Lab Related Questions

1 Which of the following could **not** apply to a eukaryotic organism that is unicellular, non-photosynthetic, and ciliated?

a. Protists
b. Protozoan
c. *Balantidium coli*
d. Parasite
e. Mastigophora

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 2
DATA SHEET
(continued)

2 Which features apply to fungi? (Select all that apply.)

- **a.** Saprobes
- **b.** Include multicellular organisms
- **c.** Include unicellular organisms
- **d.** Photosynthetic
- **e.** Includes pathogens
- **f.** Includes prokaryotes
- **g.** Can reproduce sexually and/or asexually
- **h.** Includes protists

3 The most common sexually transmitted eukaryotic infection is _____.

- **a.** Toxoplasmosis
- **b.** Trichomoniasis
- **c.** Giardiasis
- **d.** Gonorrhea
- **e.** Trypanosomiasis

4 Organize these numerically from most specific (1) to least specific (8).

_____ Domain	_____ Phylum
_____ Class	_____ Genus
_____ Family	_____ Species
_____ Kingdom	_____ Order

5 Fungal infections are called _____.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ In their mature form, apicomplexans move by pseudopods.

_____ Protists are eukaryotic cells and include animal-like, plant-like, and fungi-like organisms.

_____ *Rhizopus* is an example of a unicellular fungus.

_____ Protists are grouped based on their form of sexual reproduction.

_____ Diatoms are an example of a plant-like protist.

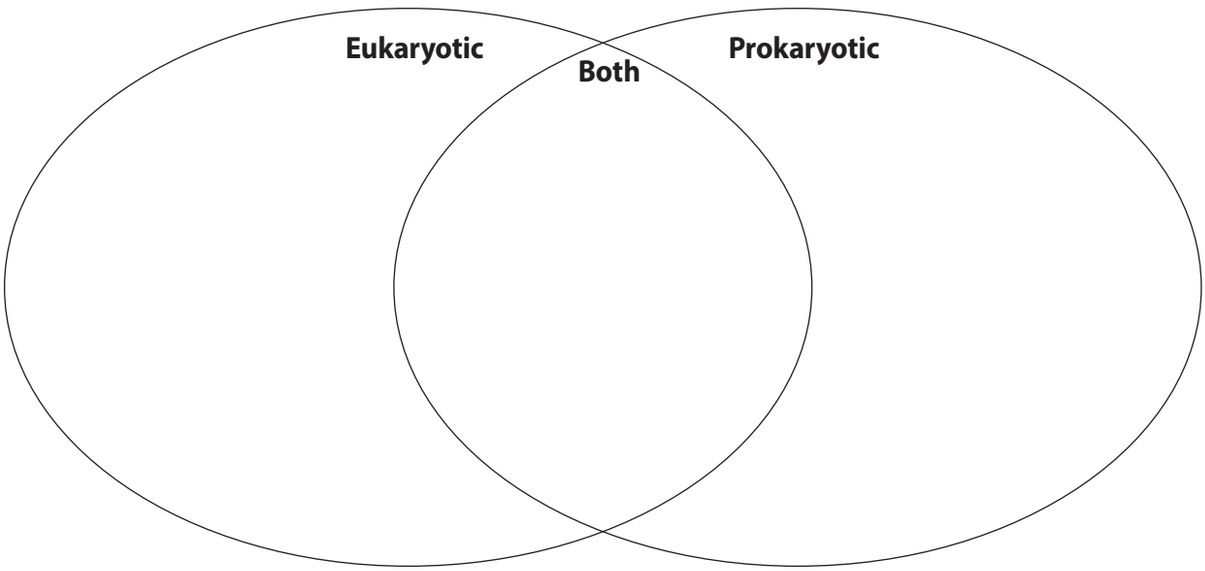
_____ Yeast are the only unicellular fungus.

_____ Prokaryotic cells are typically smaller than eukaryotic cells.

7 Select all that apply to an opportunistic pathogen:

a. Include fungi.
b. Can be unicellular.
c. Are often photosynthetic.
d. An example is *Candida albicans*.
e. Tend to affect a healthy host.

8 Fill in the Venn diagram with the nine listed statements as they relate to prokaryotic and/or eukaryotic cells.



Can be pathogenic	Nucleus absent	Domain Archaea
Domain Bacteria	Fungi	Can be unicellular
Can be multicellular	Nucleated	Ribosomes present

Critical Thinking

1 As compared to the availability of drugs to treat prokaryotic infections, the pharmacopeia of drugs that can safely and effectively treat eukaryotic infections is fairly limited. Why is it especially challenging to develop safe anti-protozoan drugs for human and/or animal use?

Name _____ Date _____

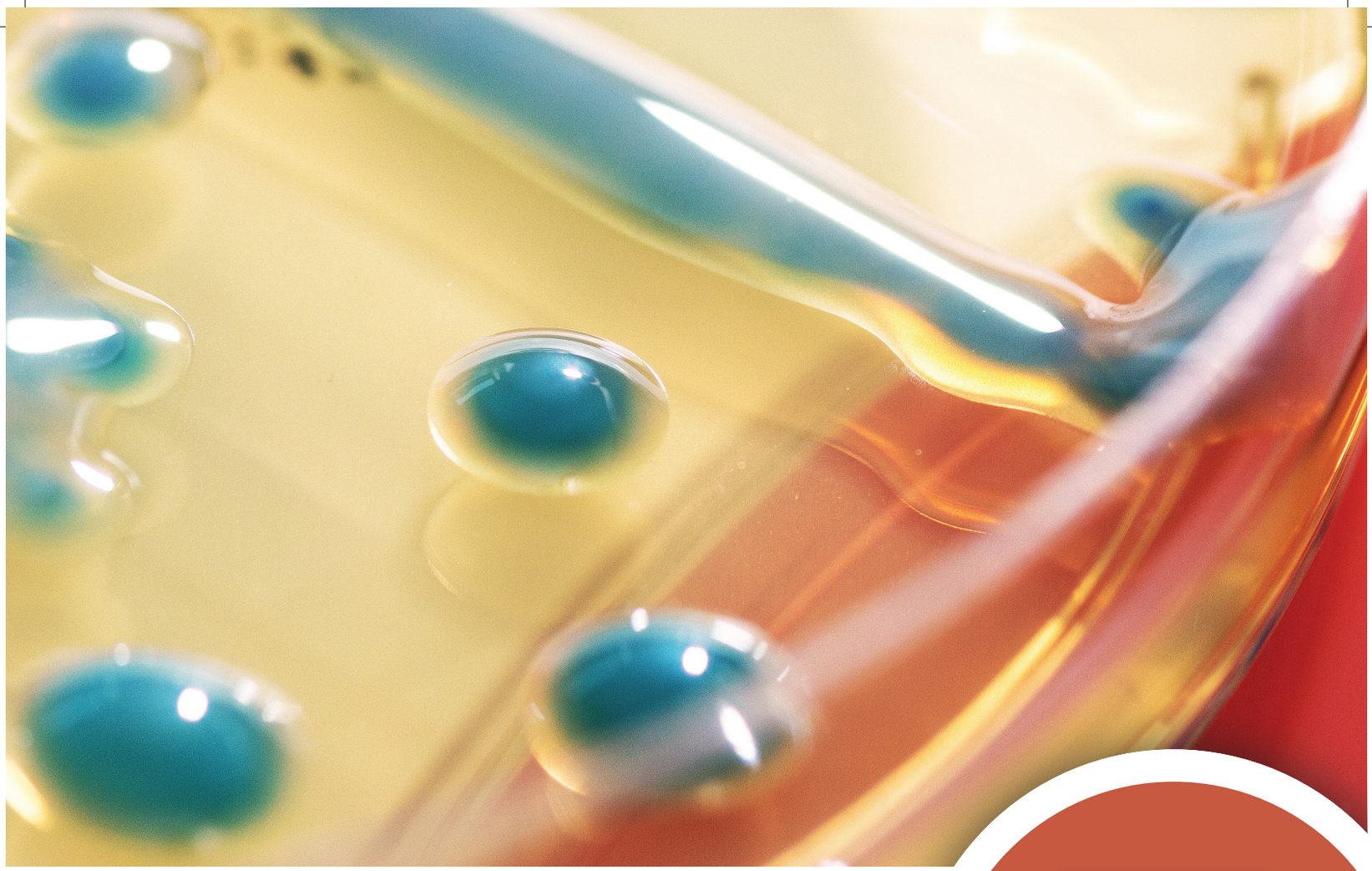
Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 2
DATA SHEET
(continued)

2 The endosymbiotic theory describes how eukaryotic cells evolved when an ancient ancestor engulfed (endocytosed) prokaryotic cells. The engulfed cells and the host cell had a symbiotic relationship—a mutually beneficial relationship. Over time, the cells in this symbiotic relationship increasingly relied on one another to the point that they became inseparable. Eventually those engulfed cells that were photosynthetic became chloroplasts and those that were non-photosynthetic became mitochondria. Based on the figures and descriptions in this laboratory, which cells do you think were engulfed first, photosynthetic cells or non-photosynthetic cells? **Provide support for your answer**.

— Lab Notes —

1



SECTION

2

Aseptic Technique and Bacterial Growth and Enumeration

In This Section

EXERCISE 3 Common Aseptic Transfer and Inoculation Methods 49

EXERCISE 4 Streak Plate Isolation Methods 69

EXERCISE 5 Spread Plate Isolation Method 81

EXERCISE 6 Colony Morphology 87

EXERCISE 7 Growth Patterns on Slants 101

EXERCISE 8 Growth Patterns in Broth 107

EXERCISE 9 Ubiquity of Microorganisms 111

EXERCISE 10 Standard Plate Count 119

— Lab Notes —

EXERCISE 3

Common Aseptic Transfer and Inoculation Methods

After doing this exercise, you should be able to:

1. Identify the various formats of growth media and properly inoculate them.
2. State the goals of aseptic methods.
3. Correctly apply aseptic methods while handling bacterial cultures and media.
4. Describe the necessary aspects of aseptic culture transfer.
5. Compare complex media to defined media and provide examples of each.
6. Define the terms *mixed culture* and *pure culture*.
7. Describe ways to minimize risks when working with BSL-2 agents.
8. Properly label microbial samples.

Background

Growing bacteria is a basic procedure in any microbiology lab. We start by **inoculating** (transferring) some bacteria into a **growth media**, a liquid, solid, or semisolid substance that provides necessary nutrients for specimen growth. Growth media (or simply, media) come in hundreds of formulations. The recipe used depends on the goal, from generalized growth to bacterial species differentiation and/or isolation. Here we'll review some basics about growth media and the aseptic methods for inoculating such media. In later laboratory exercises, we'll discuss more sophisticated inoculation methods used for species isolation (e.g., the streak plate isolation) and the collection of media used for differentiating bacterial species from one another.

In general, growth media have either a defined or complex formulation. **Defined media** (sometimes called synthetic media) get their name from the fact that each organic and inorganic ingredient is precisely known and quantified, which means these media are accurately reproducible. Glucose minimal salts media is an example. In contrast, **complex media** (also called enriched media) contain ingredients such as milk proteins, blood, or yeast extracts, less precisely defined organic nutrients. Most pathogens are fastidious (require complex nutrient sources) and will only grow on enriched media. In this lab exercise, we'll use a complex media called **nutrient broth**, which contains peptone (a solution of partially broken-down proteins) and beef extract.

Two Important Media Formats: Liquid and Solid

As their name suggests, **broth media** are liquids. Liquid media are great for quickly growing large quantities of bacteria. This is because high-volume batches can be readily prepared and easily inoculated. However, sometimes a solid media format is required.

To make solid media, we simply add **agar** to liquid media. Agar is purified from seaweed. It has the same solidifying effect as gelatin, but it's better for most bacterial growth applications because it remains solid at standard incubation temperatures and most pathogens can't digest it. The thickened media can be poured into sterile petri plates to make **agar plates**, upright test tubes to make **agar deeps**, or tilted test tubes to make **agar slants** (Fig. 3.1).

Plated media are used for isolating species, differential testing, and quantifying bacterial levels. We won't use plated media in this lab exercise, but we'll use them later. Agar slants are used for a variety of biochemical tests and, because they take up less space than plated media, they're useful for maintaining stock cultures. Most deeps are used to determine specific bacterial metabolic properties. No matter what media we use, each requires aseptic inoculation.

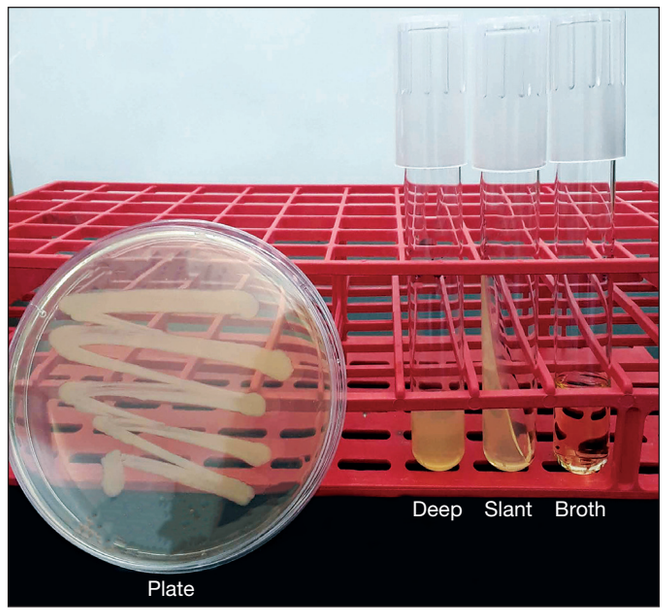


FIGURE 3.1 Media Formats

Aseptic Inoculation Methods

When growing bacteria in the lab, we start with a **culture**, a sample containing bacteria. If the sample contains only one species, we call it a **pure culture**. In contrast, a **mixed culture** contains more than one species. No matter what type of culture we start with, when transferring it to new media we must avoid contaminating it with additional microbes from the environment and we must avoid exposing ourselves and our surroundings to the microbes in our sample. To achieve this, we use **aseptic methods** (*a* = without; *septic* = contamination), which involve precise sample handling methods combined with using sterile media and sterile inoculating tools.

Sterile Media

One goal of aseptic methods is to avoid contaminating our culture with unwanted microbes. Thus, the growth media must be sterile before it's inoculated. There are a few ways to sterilize media, but the most common approach is to use an **autoclave**, a chamber that steam heats a sample under pressure. In addition to sterilizing the media, anything that will directly contact the sample must also be sterile. This includes the interior of the plastic or glass containers that hold the media. There are a couple of ways to do this: the media can be mixed and poured into the tubes and then immediately autoclaved so the media and glassware are simultaneously sterilized, or the sterilized media can be poured into presterilized petri plates and other plastic or glassware.

Sterile Inoculating Tools

All inoculating tools used to transfer the sample to the sterile media must also be sterile. There are many inoculating tools available, but in this lab exercise, we'll use a **wire inoculating needle** and a **wire inoculating loop** to pick up small amounts of a culture and transfer it to our media (Fig. 3.2). An inoculating loop has a handle that holds a thin tungsten, platinum, or nichrome wire twisted into a small loop at the end. An inoculating needle is basically the same as a loop, except the wire is straight. To sterilize the loop or needle, the tool's wire filament is inserted into an incinerator or Bunsen burner flame until the filament glows red-hot (Fig. 3.3). If presterilized disposable inoculating loops or needles are used, then the incineration/flame sterilization process is skipped. Sometimes other inoculating tools like pipettes are used; these are covered in Appendices C and D.

Protecting Yourself and Others

When properly performed, aseptic methods help you avoid sample contamination, but more importantly these techniques protect you and others from exposure to potential pathogens. You must do two things to accomplish this. First, limit how much you expose your sample to the environment. Second, limit sample agitation to avoid the formation of airborne particles (e.g., aerosols and droplets) when handling the culture. We'll delve into each process as we walk through the inoculation procedures. For now, here is a list of things to keep in mind:

- **Pay attention to your environment.** Your workspace should be clutter free. Never perform transfers over your books and papers because you may unknowingly contaminate them.
- **Thoroughly label all media.** Arrange all media in advance and clearly label them with your name, the date, the type of medium (e.g., nutrient broth, or NB for short), and the bacterium's name (or the sample name, such as Unknown #3) (Fig. 3.4). Never label lids. Instead, label the body of your tubes with tape, paper held on with a rubber band, or by writing directly on the glass. For plastic plates, write directly on the base (not the lid). Your instructor will tell you their labeling preferences. Whatever labeling method used, be sure you position labels so they don't obscure your view of the media.
- **Keep test tubes in a test-tube rack.** Never lay tubes on the table; they may leak or roll off (Fig. 3.5).
- **Take your time.** You are handling potentially dangerous microbes. Working at a frenzied pace leads to accidents.

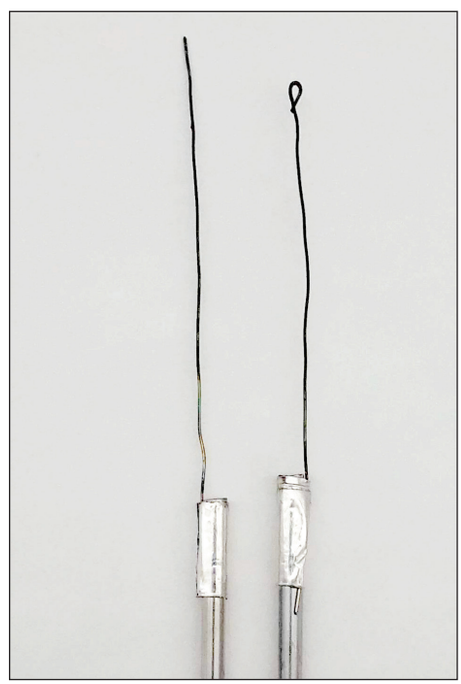


FIGURE **3.2** **Wire Inoculating Needle and Loop**

- **Never hold a tube by its cap**. Caps are designed to come off and are therefore a bad way to hold a sample. Plus, during incubation, caps are generally kept loose to allow for sample aeration. This is true for push caps and screw caps.
- **Use your dominant hand to hold inoculating tools.** With a relaxed grasp, hold your inoculating tool like a pencil (Fig. 3.5).

A Special Note on BSL-2 Agents

The primary concern with BSL-2 organisms is the formation of aerosols, which can lead to infection if inhaled and/or environmental contamination. Aerosols are problematic because we are generally unaware of their production, and they remain suspended in the air long after the procedure is complete. Because of the risks associated with BSL-2 pathogens, most college teaching laboratories have reduced, or even eliminated, the use of BSL-2 organisms. We've followed suit in this lab manual while trying to honor the fact that many people taking an introductory microbiology course want to work in health-care settings, places where BSL-2 organisms are unavoidable. As such, we often include "optional" BSL-2 bacteria in the exercises and simply leave the decision up to your instructor as to when (or if) you will use these agents.

Even if you do not use BSL-2 agents in the lab, most microbiology instructors will train you to protect yourself and others against these microbes. Unless your instructor

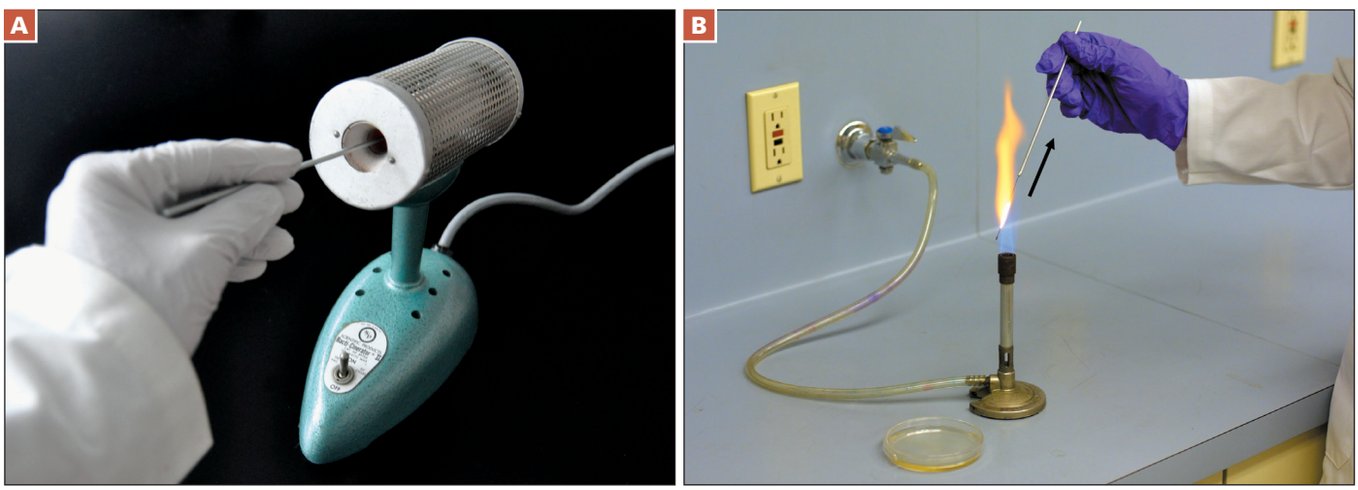


FIGURE **3.3** **Using an Incinerator or Bunsen Burner to Sterilize Inoculating Tools** • (A) An incinerator or (B) a Bunsen burner can be used to sterilize inoculating loops and needles. In either case, the full length of the inoculating tool's wire filament must be heated until red-hot, which takes about 3–7 seconds.

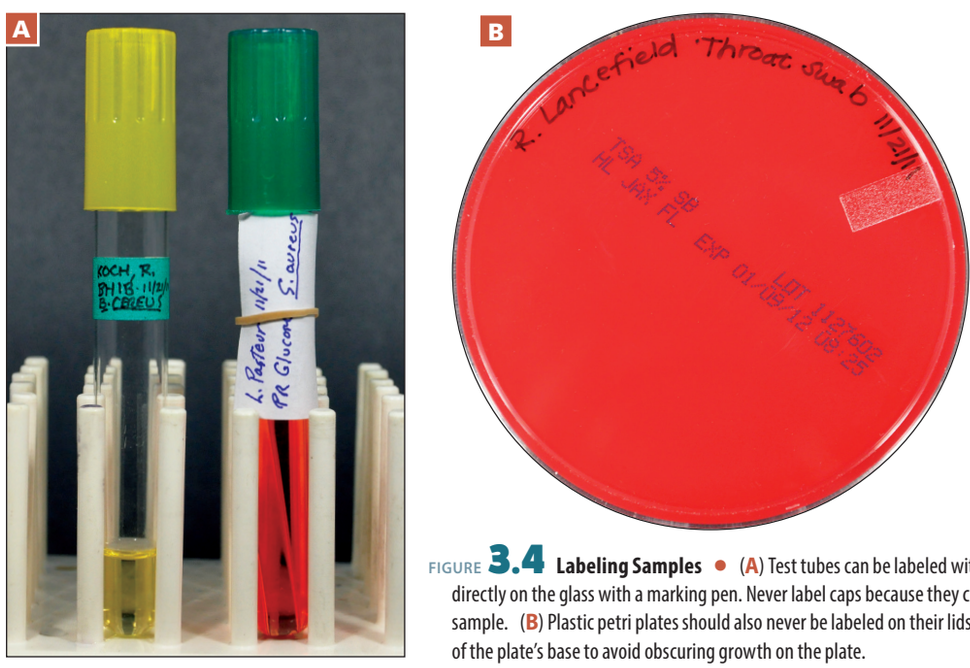


FIGURE **3.4** **Labeling Samples** • (A) Test tubes can be labeled with tape, paper labels, or directly on the glass with a marking pen. Never label caps because they can be separated from the sample. (B) Plastic petri plates should also never be labeled on their lids. Instead, label at the edge of the plate's base to avoid obscuring growth on the plate.

says otherwise, you should assume you are to use BSL-2 precautions for lab exercises that involve live bacterial specimens.

Throughout this manual, we emphasize techniques that minimize aerosol production, but they are even more essential when handling BSL-2 organisms. Your instructor will have specific guidelines on how to handle BSL-2 organisms. Precautions may include any (or all) of the following depending on the exercise and the equipment available. Whatever the dictated guidelines are, for your own health (and the health of others), take every precaution seriously.

- Performing tests in a biosafety cabinet is often recommended, but not all teaching laboratories have these installed.

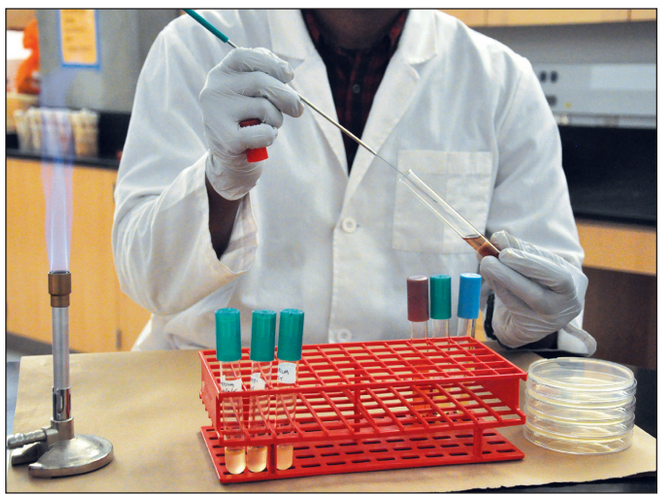
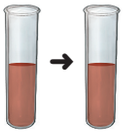


FIGURE **3.5** **A Microbiologist at Work** • Materials are neatly positioned and the work space is not cluttered. The Bunsen burner is accessible, but not so close as to be a major fire hazard. Test tubes are never laid on a table, instead they should be stored upright in a test-tube rack. Notice how the microbiologist holds the loop like a pencil and their little finger is holding the tube's cap—the cap is not on the table.

- An electric incinerator can reduce aerosol exposure because any aerosols that may develop while flaming inoculating tools do not escape the incinerator's chamber.
- Using sterile disposable loops/needles or sterile wooden sticks that don't require flaming after use can also help reduce aerosol formation. However, this approach increases the burden of biological waste management.
- Always wear lab safety gear (lab coat, gloves, and eye protection) and lab appropriate attire as dictated by your instructor (e.g., closed-toe shoes, long pants/skirt, shirt that covers your shoulders and the entirety of your torso).
- Clearly identify BSL-2 organisms. Red caps on tube cultures or BSL-2 labels on plate cultures can be helpful.

Inoculating Various Non-plated Media

In this lab, you'll use broth, slant, and plated stock cultures to inoculate various forms of non-plated media. Refer to the steps and procedural figures in the following Lab Techniques to help you understand each method. Always put on your safety gear before performing any procedure involving live microbes.



Transferring Culture from a Broth to Another Broth *(continues)*

Lab Technique

Figure 3.6 on pages 54–55 provides a photographic review of each step in the following process.

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Mix the Sample:** Mix the broth culture with a vortex mixer or by drumming your fingers along the tube. Do not mix so vigorously that broth enters the cap. Once it's mixed, loosen the cap and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with a Loop:** Holding the tube at an angle, dip the inoculating loop into the broth and then carefully withdraw the loop. Avoid touching any part of the tube with your loop. At this point, there should be a visible film of broth in your loop.
7. **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Broth:** With your free hand, pick up the sterile broth tube and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.
9. **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.
10. **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent, if you flame the loop with the film present you'll produce aerosols.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening (don't let broth slosh out of the tube), replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

Transferring Culture from a Broth to Another Broth *(continues)*

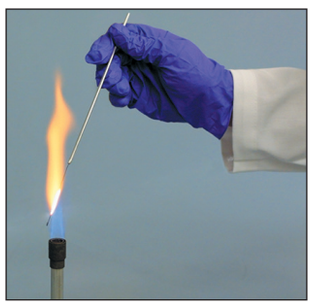
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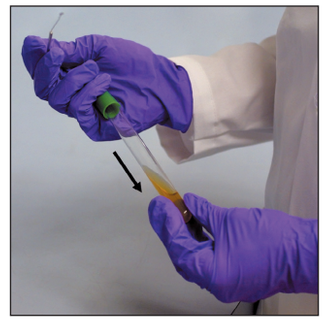
1 **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.



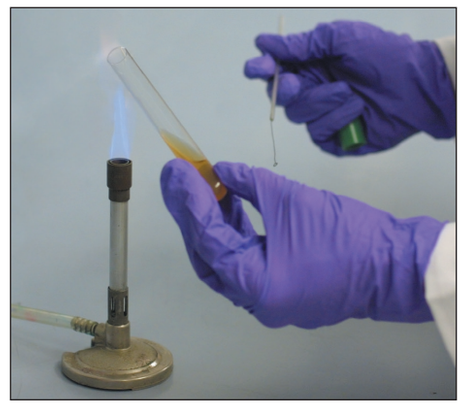
2 **Mix the Sample:** Mix the broth culture with a vortex mixer or by drumming your fingers along the tube. Do not mix so vigorously that broth enters the cap. Once it's mixed, loosen the cap and return the tube to the rack.



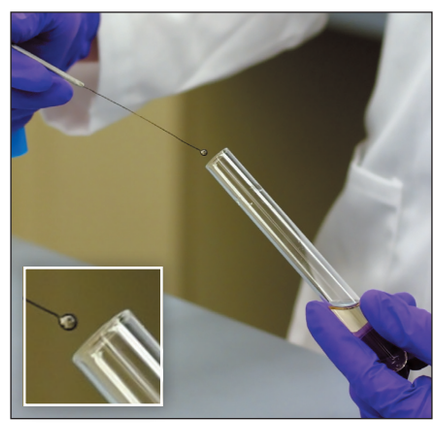
3 **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.



4 **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.



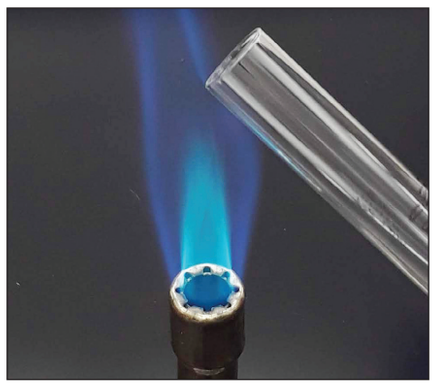
5 **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.



6 **Collect the Sample with a Loop:** Holding the tube at an angle, dip the inoculating loop into the broth and then carefully withdraw the loop. Avoid touching any part of the tube with your loop. At this point, there should be a visible film of broth in your loop.

FIGURE **3.6** **Transferring Culture from a Broth to Another Broth** • *(continues)*

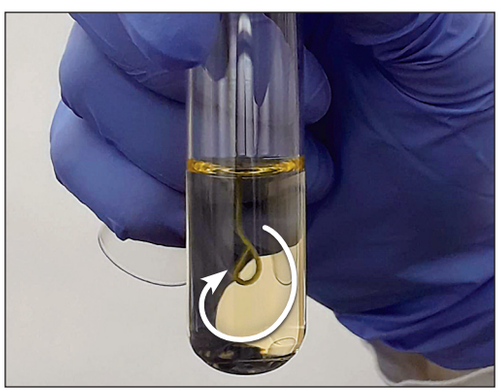
Transferring Culture from a Broth to Another Broth *(continued)*



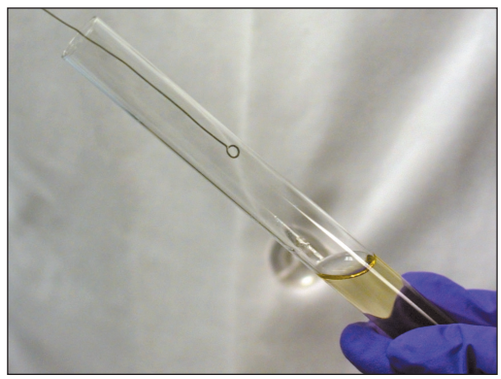
7 **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.



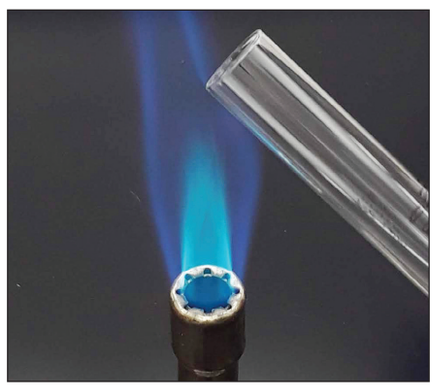
8 **Open the Sterile Broth:** With your free hand, pick up the sterile broth tube and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.



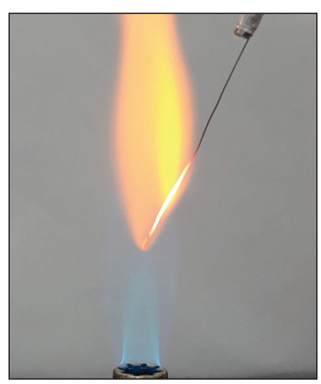
9 **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.



10 **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent, if you flame the loop with the film present you'll produce aerosols.

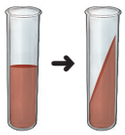


11 **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening (don't let broth slosh out of the tube), replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.



12 **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

FIGURE **3.6** *(continued)* **Transferring Culture from a Broth to Another Broth**



Transferring Culture from a Broth to an Agar Slant

The steps in this process are very similar to what you use to inoculate from a broth to another broth (Fig. 3.6). The main difference is Step 9, where instead of dipping the **inoculum** (collected sample) into a broth, you'll perform a fishtail inoculation (Fig. 3.7).

2

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end. If it's not, pinch it closed.
2. **Mix the Sample:** Mix the broth culture with a vortex mixer or by drumming your fingers along the tube. Do not mix so vigorously that broth enters the cap. Once it's mixed, loosen the cap and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with a Loop:** Holding the tube at an angle, dip the inoculating loop into the broth and then carefully withdraw the loop. Avoid touching any part of the tube with your loop. At this point, there should be a visible film of broth in your loop.
7. **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Slant:** With your free hand, pick up the sterile slant tube and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.
9. **Fishtail Inoculation of the Sterile Slant:** Starting at the bottom of the slant, gently drag the inoculating loop across the slant surface in a zigzag pattern until you reach the top of the slant (Fig 3.7). ***Note:*** If you use too much pressure, you'll gouge the agar.
10. **Remove the Loop:** Carefully remove the loop. Avoid catching it on the lip of the tube.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

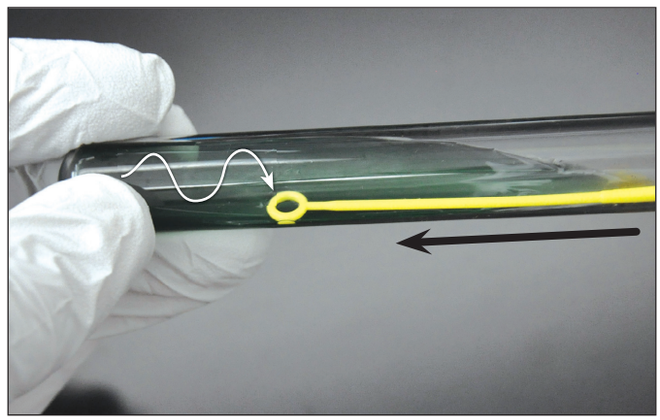
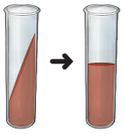


FIGURE **3.7** **A Fishtail Inoculation** • A zigzag (or fishtail) inoculation is used to inoculate an agar slant. (A disposable loop was used in this photo.)



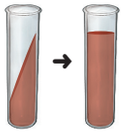
Transferring Culture from a Slant to a Broth

The primary difference for this procedure versus the others is you'll collect some sample off a slant culture and introduce it to a broth.

Lab Technique

2

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Loosen the Cap on the Slant Sample:** Loosen, but don't remove, the cap on the slant culture and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with a Loop:** Holding the tube at an angle, use the inoculating loop to collect a pin-head amount of the culture growing on the slant's surface. Be very gentle so that you do not gouge the agar. Then, withdraw the loop. Avoid touching any part of the tube with it.
7. **Re-flame the Stock Tube's Opening:** While holding your loop hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Broth:** With your free hand, pick up a tube of sterile broth and repeat the process of removing the cap with your loop hand's little finger and flaming the lip of the open tube.
9. **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.
10. **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent, if you flame the loop with the film present you'll produce aerosols.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening (don't let the broth slosh out of the tube), replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.



Transferring Culture from a Slant to a Deep

The steps in this process are very similar to the prior inoculations; the main difference is you will use an inoculating needle to introduce the sample into an agar deep (Fig. 3.8).

2

1. **Check the Needle:** Ensure your inoculating needle is reasonably straight, if not, straighten the wire. ***Caution!*** Be gentle! The wire easily pulls out of the handle.
2. **Loosen the Cap on the Slant Sample:** Loosen, but don't remove, the cap on the slant culture and return the tube to the rack.
3. **Sterilize the Inoculating Needle:** Hold the inoculating needle in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface.
4. **Open the Stock Sample:** Pick up the culture tube with your free hand. Then, using the little finger of the hand holding your needle, remove the tube's cap. Hold the cap with your pinky; do not put it down.
5. **Flame the Stock Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
6. **Collect the Sample with an Inoculating Needle:** Holding the tube at an angle, gently collect a small amount of culture onto the needle and carefully withdraw it. Avoid touching any part of the tube with your needle.
7. **Re-flame the Stock Tube's Opening:** While holding your needle hand still, flame the mouth of the open tube, replace the cap, and return the tube to the rack.
8. **Open the Sterile Agar Deep:** With your free hand, pick up the sterile agar deep tube and repeat the process of removing the cap with your needle hand's little finger and flaming the lip of the open tube.
9. **Stab Inoculate the Sterile Agar Deep:** In as straight a line as possible, slowly stab the middle of the agar deep until the needle is as close to the bottom of the agar as possible without having the handle touch the agar (Fig. 3.8).
10. **Remove the Needle:** Carefully remove the needle. Avoid catching it on the lip of the tube.
11. **Flame the Newly Inoculated Tube's Opening:** Keeping your needle hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
12. **Re-sterilize the Needle:** Before putting the needle down, re-flame the filament to sterilize it.

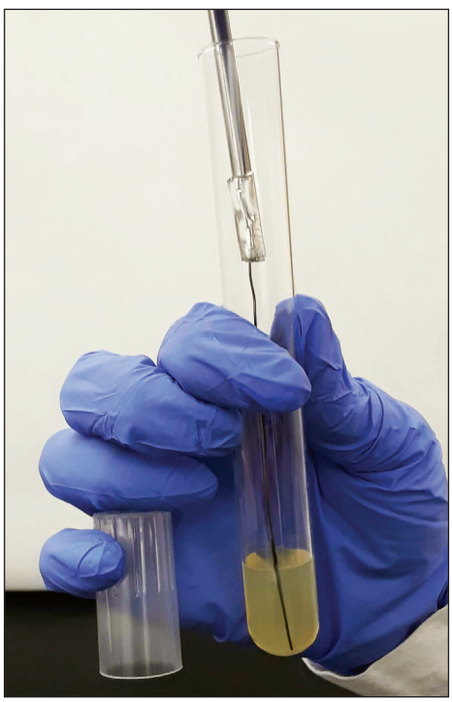
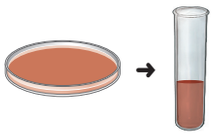


FIGURE **3.8** **Stab Inoculation of an Agar Deep** • Agar deeps are stab inoculated with a needle. Use as straight a motion as possible to inoculate the middle of the agar.



Lab Technique

Transferring Culture from a Plate to a Broth

In this process, you will use an inoculating loop to pick a colony from an agar plate culture and introduce it into agar broth. A **colony** is a visible collection of cells that are genetically alike. Therefore, if you inoculate a single colony into your media and do not introduce any contaminants into the sample, your resulting cultures should be pure. Whenever you open the plate, be sure you hold the lid like a clam shell. You can either set the plate on the table or hold the plate in your hand to do this (Fig. 3.9).

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Loosen the Cap on the Sterile Broth Tube You'll Inoculate:** Loosen, but don't remove, the cap on the tube of broth you'll inoculate and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface. Allow the loop to cool for about 20–30 seconds before going to the next step.
4. **Pick a Colony from a Stock Plate:** Open the plate containing your bacterial stock and gently pick up a colony with the loop. Do not gouge the agar while selecting a colony. To prevent contaminants from falling into the plate, keep the lid over the sample like a "clam shell." Withdraw the loop once you have a colony on it, put the plate lid on the culture, and set the plate down on the table.
5. **Open the Sterile Broth Tube:** With your non-loop hand, pick up the sterile tube of broth that you'll inoculate. Then, using the little finger of the hand holding your loop, remove the tube's cap and hold the cap with your pinky; do not put the cap down.
6. **Flame the Broth Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times (don't let broth slosh out of the tube). If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
7. **Inoculate the Sterile Broth:** Dip the loop into the sterile broth and gently swirl the loop.
8. **Remove the Loop:** Before removing the loop, tap the loop's face on the inside of the tube to remove the film within the loop. Be persistent. If you flame the loop with the film present, you'll produce aerosols.
9. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
10. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.

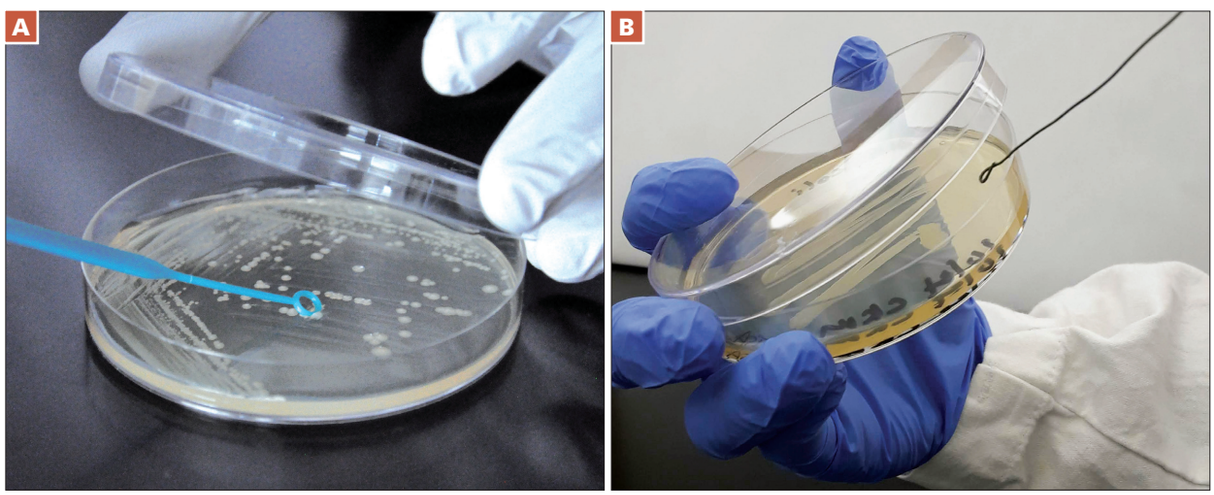
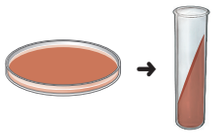


FIGURE **3.9** **"Picking" a Colony from an Agar Plate** • When opening an agar plate, using a "clam shell" angle can prevent contaminants from falling into the sample. (A) You can place the plate on the table to do this; or (B) hold the plate in your hand.

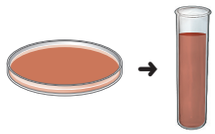


Transferring Culture from a Plate to a Slant

Here you'll pick a colony from an agar plate culture and introduce it to an agar slant. To prevent contamination, remember to hold the plate's lid in a clam shell position over the agar (Fig. 3.9).

2

1. **Check the Loop:** Ensure your inoculating loop has a fully closed circle at the end, if not, pinch it closed.
2. **Loosen the Cap on the Sterile Slant You'll Inoculate:** Loosen, but don't remove, the cap on the slant you'll inoculate and return the tube to the rack.
3. **Sterilize the Loop:** Hold the inoculating loop in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface. Allow the loop to cool for about 20–30 seconds before going to the next step.
4. **Pick a Colony from a Stock Plate:** Open the plate containing your bacterial stock and gently pick up a colony with the loop. To prevent contaminants from falling into the plate, keep the lid over the sample like a "clam shell." Withdraw the loop once you have a colony on it, put the plate lid on the culture, and set the plate down on the table.
5. **Open the Sterile Slant Tube:** With your non-loop hand, pick up the sterile slant that you'll inoculate. Then, using the little finger of the hand holding your loop, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
6. **Flame the Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
7. **Fishtail Inoculation of the Sterile Slant:** Starting at the bottom of the slant, gently drag the inoculating loop across the slant surface in a zigzag pattern until you reach the top of the slant (Fig 3.7). ***Note:*** If you use too much pressure, you'll gouge the agar.
8. **Remove the Loop:** Carefully remove the loop. Avoid catching it on the lip of the tube.
9. **Flame the Newly Inoculated Tube's Opening:** Keeping your loop hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
10. **Re-sterilize the Loop:** Before putting the loop down, re-flame the filament to sterilize it.



Transferring Culture from a Plate to a Deep

This time you'll use a needle instead of a loop to pick a colony from an agar plate culture and introduce the inoculum into an agar deep.

Lab Technique

2

1. **Check the Needle:** Ensure your inoculating needle is reasonably straight, if it's not, straighten the wire. ***Caution!*** Be gentle! The wire easily pulls out of the handle.
2. **Loosen the Cap on the Sterile Deep You'll Inoculate:** Loosen, but don't remove, the cap on the agar deep you'll inoculate and return the tube to the rack.
3. **Sterilize the Needle:** Hold the inoculating needle in your dominant hand like a pencil and heat the length of the wire filament until each section glows orange. Once the filament is sterilized, do not put it down and don't touch it to any surface. Allow the loop to cool for about 20–30 seconds before going to the next step.
4. **Pick a Colony from a Stock Plate:** Open the plate containing your bacterial stock and gently pick up a colony with the loop. To prevent contaminants from falling into the plate, keep the lid over the sample like a "clam shell." Withdraw the needle once you have a colony on it, put the plate lid on the culture, and set the plate down on the table.
5. **Open the Agar Deep Tube:** With your non-needle hand, pick up the sterile deep that you'll inoculate. Then, using the little finger of the hand holding your needle, remove the tube's cap. Hold the cap with your pinky; do not put the cap down.
6. **Flame the Tube's Opening:** Holding the open tube at a slight angle, quickly pass the mouth of the tube through the flame a few times. If you're using an electric incinerator, hold the mouth of the tube near the entrance of the incinerator.
7. **Stab Inoculate the Sterile Agar Deep:** In as straight a line as possible, slowly stab the middle of the agar deep until the needle is as close to the bottom of the agar as possible without having the handle touch the agar (refer to Fig 3.8).
8. **Remove the Needle:** Carefully remove the needle. Avoid catching it on the lip of the tube.
9. **Flame the Newly Inoculated Tube's Opening:** Keeping your needle hand still, flame the tube's opening, replace the cap, and return the tube to the rack. Sample caps should be kept loose for incubation, but not so loose that they will easily fall off.
10. **Re-sterilize the Needle:** Before putting the needle down, re-flame the filament to sterilize it.

Clinical Applications

Health care-acquired infections (HAIs), also known as **nosocomial infections**, are infections that develop as a medical intervention complication. The U.S. Centers for Disease Control and Prevention estimates that in American hospitals alone, there are about 1.7 million HAIs and roughly 99,000 HAI associated deaths each year. One way to prevent these infections is to practice proper aseptic methods. Anytime a health-care provider collects a patient sample (e.g., tissue biopsy, blood, etc.), administers an injected drug, dresses a surgical wound, delivers a baby, inserts a catheter or IV, or performs any sort of invasive procedure, they apply aseptic methods to keep patients safe. Furthermore, since patient samples often contain BSL-2 agents, health-care providers must know how to safely handle such pathogens.

2

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Bunsen burner
- ❑ Inoculating loop
- ❑ Inoculating needle
- ❑ Materials for labeling samples (e.g., marking pen, tape, etc.)
- ❑ Vortex mixer (optional)
- ❑ Nutrient broth culture of *Staphylococcus epidermidis* (or another BSL-1 bacterium) (Part B)
- ❑ Nutrient agar slant culture of *Staphylococcus epidermidis* (or another BSL-1 bacterium) (Part C)
- ❑ Nutrient agar streak plate of *Staphylococcus epidermidis* (or another BSL-1 bacterium) (Part D)
- ❑ Sterile nutrient broth (NB) tubes, 4 per lab group (1 broth tube is used in each exercise)
- ❑ Sterile nutrient agar (NA) slants, 3 per lab group (1 slant tube is used in Parts A, B, and D—a slant is not used in Part C)

PROCEDURE

Your instructor may or may not have you perform all parts (A–D) of the exercise (Table 3.1). Pay attention to their instructions and proceed in the order they direct you.

TABLE **3.1** Inoculations for Parts A–D

Procedure Part	Source	Destination	Inoculating Tool
A (simulation)	Sterile broth sample	Sterile broth	Loop
		Sterile slant	Loop
B	Broth sample	Sterile broth	Loop
		Sterile slant	Loop
C	Slant sample	Sterile broth	Loop
		Sterile deep	Needle
D	Plate sample	Sterile broth	Loop
		Sterile slant	Loop
		Sterile deep	Needle

PART A: Practicing Aseptic Methods with a Sterile "Stock" Sample

For this part of the exercise, you'll simulate inoculating a broth and a slant. During the next lab session—after potential contaminants have had a chance to grow—you'll check your samples to see if you managed to keep them sterile.

1. Clear your workspace as much as possible.
2. Put on your safety gear (lab coat, safety glasses, and gloves).
3. If using a Bunsen burner, light it and adjust the flame so a small blue cone is at the center (Fig. 3.10). You do not want a large orange flame. If using an incinerator, turn it on.
4. Obtain two tubes of sterile broth and one sterile slant.
5. Label each of your three tubes with your name, the date, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short). Normally you'd also add the bacterium's name to each label, but we won't be using any bacteria for this practice. Instead, you're going to see if you can keep your samples sterile. As such, label one of the sterile broth tubes "simulated stock" and label each of your other two tubes "simulated inoculation."

6 Use the "simulated stock" to inoculate your broth and slant tubes. To become comfortable with your aseptic methods, inoculate the sample broth three separate times. Do the same for the slant.

7 When you're done, put all three of your labeled tubes wherever your instructor directs you to put them for incubation. Samples are typically incubated overnight at 35–37°C.

→ Next Lab Session

1 Remove your "sterile" practice tubes from the incubators and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your tubes as your instructor directs.



FIGURE **3.10** **Bunsen Burner Flame** • When properly adjusted, a Bunsen burner produces a two-cone flame. The inner cone (red arrow) is the hottest and is where the inoculating tool wire is inserted for sterilization. The outer cone (white arrow) is used to heat the mouths of open glassware items.

PART B: Transferring Bacteria from a Broth Culture to Non-plated Media

For this part of the exercise, you'll transfer bacteria from a stock bacterial broth into fresh sterile broth and onto a sterile agar slant. You'll check your results the next lab session, after the bacteria have had a chance to grow.

1 Clear your workspace as much as possible.

2 Put on your lab coat, eye protection, and gloves.

3 Obtain a stock broth bacterial culture. Your instructor may use *Staphylococcus epidermidis* or some other bacterium.

4 Obtain a tube of sterile broth and a sterile agar slant. Label each tube with your name, the date, the name of the bacterium you will grow, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short).

5 To inoculate the broth media, follow the directions for transferring a culture from a broth to another broth (p. 53–55).

6 To inoculate the agar slant, follow the directions for transferring culture from a broth to an agar slant (p. 56).

7 After you've completed both transfers, put your newly inoculated samples wherever your instructor directs you to put them for incubation. Samples are usually incubated at 35–37°C overnight. Save or dispose of the original culture as your instructor directs.

→ Next Lab Session

1 Remove your cultures from the incubators and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your samples as your instructor directs.

PART C: Transferring Bacteria from a Slant Culture to Non-plated Media

In this part of the exercise, you'll transfer bacteria from a stock bacterial slant into sterile broth and onto a sterile agar deep. You'll check your results the next lab session, after the bacteria have had a chance to grow.

1 Clear your workspace as much as possible.

2 Put on your lab coat, eye protection, and gloves.

3. Obtain a stock slant bacterial culture. Your instructor may use *Staphylococcus epidermidis* or some other bacterium.
4. Obtain a tube of sterile broth media and a sterile agar deep. Label each tube with your name, the date, the name of the bacterium you will grow, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short).
5. To inoculate the broth media, follow the directions for transferring a culture from a slant to a broth (p. 57).
6. To inoculate the agar deep, follow the directions for transferring culture from a slant to an agar deep (p. 58).
7. After you've completed both transfers, put your newly inoculated samples wherever your instructor directs you to put them for incubation. Samples are usually incubated at 35–37°C overnight. Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. Remove your cultures from the incubator and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.
2. Dispose of your samples as your instructor directs.

PART D: Transferring Bacteria from a Plated Culture to Non-plated Media

In this part of the exercise, you'll transfer bacteria from a stock bacterial plate into fresh sterile broth, to a sterile agar slant, and into a sterile agar deep. You'll check your results the next lab session, after the bacteria have had a chance to grow.

1. Put on your lab coat, eye protection, and gloves.
2. Obtain a stock plate of bacteria. Your instructor may use *Staphylococcus epidermidis* or some other bacterium.
3. Obtain a tube of sterile broth, a sterile agar slant, and a sterile agar deep. Label each tube with your name, the date, the name of the bacterium you will grow, and the name of the medium (e.g., nutrient broth or nutrient agar, NB and NA for short).
4. To inoculate the broth media, follow the directions for transferring a culture from a plate to a broth (p. 59).
5. To inoculate the agar slant, follow the directions for transferring culture from a plate to a slant (p. 60).
6. To inoculate the agar deep, follow the directions for transferring culture from a plate to a deep (p. 61).
7. After you've completed all three transfers, put your samples wherever your instructor directs you to put them for incubation. Samples are usually incubated at 35–37°C overnight. Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. Remove your cultures from the incubator and examine them for growth. Record your observations and answer the data sheet questions at the end of this lab.
2. Dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 3

DATA SHEET

Common Aseptic Transfer and Inoculation Methods

During the Lab

1 Describe the appearance of growth on/in each **solid** medium for the lab exercises you performed.

Part A	Part B	Part C	Part D
Slant:	Slant:	Deep:	Slant: Deep:

The degree of cloudiness (turbidity) is often used to qualitatively describe a bacterium's growth in a liquid medium. Using the following scoring scale, note the turbidity of your **broth** samples for each exercise you performed:

+ + + for "very turbid"

+ + for "somewhat turbid"

+ for "barely turbid"

0 for "not turbid"

Part A	Part B	Part C	Part D

2 Name at least three ways contaminants could enter a sample in Part A.

_____

_____

3 Considering the cultures used to inoculate each medium in this exercise, how many different microbial types should you expect to see on/in each medium? Explain your answer.

Lab Related Questions

1 Which of the following is (are) required for proper aseptic transfer of a bacterial specimen? (Select all that apply.)

a. A sterile work surface.
b. Sterile media.
c. Limited exposure of the sample to the environment.
d. A sterile inoculating tool.
e. Knowledge of the specimen's biosafety level.

2 Which of the following is (are) described as complex media? (Select all that apply.)

a. Blood agar
b. Glucose minimal salts media
c. Nutrient broth
d. Nutrient agar
e. Skim milk agar

3 Some of the following steps should not be used if you want to aseptically transfer a culture **from a plate to a slant**. First, draw a line through the steps you would not use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Flame the inoculating needle.

_____ Loosen the slant tube's cap and return the tube to the rack.

_____ Open the plate using a "clam shell" position for the lid.

_____ Flame the mouth of the slant tube and secure the cap.

_____ Perform a fishtail inoculation of the slant surface.

_____ Take the cap off the slant and set the cap on the table.

_____ Stab the agar slant with the inoculating tool.

_____ Pick up a colony with the inoculating tool.

4 Name the four things you should list on every specimen label:

-
-
-
-

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 3
DATA SHEET
(continued)

5 Which of the following is (are) mismatched? (Select all that apply.)

a. Broth media: inoculated with a needle
b. Slant media: inoculated with a loop
c. Deep media: inoculated with a loop
d. Colony: consists of a single bacterial species
e. Plate: solid media

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ For defined media, each ingredient is precisely known and quantifiable.

_____ Agar is a solidifying agent that most pathogens will digest as a nutrient source.

_____ Clinical samples are usually handled as though they may contain BSL-1 infectious agents.

_____ Slant cultures are ideal for growing large volumes of sample.

_____ A mixed culture contains at least two different media.

7 The best media format for bacterial species isolation is:

a. Broth media.
b. Slant media.
c. Semisolid media.
d. Plate media.
e. Agar deeps.

8 A major concern in working with BSL-2 pathogens is the formation of _____, which can lead to infection through inhalation.

Critical Thinking

1 Some students might notice a difference in density (turbidity) of growth in nutrient broth tubes inoculated from nutrient broth versus inoculated from a nutrient agar slant or plate. Suggest some reasons why a difference may occur.

2 Suppose you performed a transfer from a nutrient broth stock to a glucose minimal salts slant and upon checking your sample the next day, you do not see any growth. Assuming you did everything correctly during the transfer, why might the organism have failed to grow?

EXERCISE 4

Streak Plate Isolation Methods

After doing this exercise, you should be able to:

1. Use three streak plate isolation patterns to inoculate plated media.
2. Correctly apply aseptic techniques while handling bacterial cultures and media.
3. State the risks associated with environmental sampling.
4. Describe how bacterial isolation methods are clinically useful.
5. Define the following terms: *colony*, *colony-forming unit*, *mixed culture*, *pure culture*, and *etiological agent*.

Background

A microbial culture containing two or more species is called a **mixed culture**. In contrast, a **pure culture** contains only a single species. Generally, the first step in identifying an organism is to isolate it from a mixed sample. A common **isolation technique** is the **streak plate**.

In the streak plate isolation method, a bacterial sample is streaked onto an agar plate. The streaking process spreads out the sample so individual cells are scattered across the agar surface. If the medium being used supports the microbe's growth, then sufficiently isolated (separated) cells will grow into individual **colonies** that are visible to the naked eye. A colony is a collection of genetically identical cells that originated from a **colony-forming unit (CFU)**. In some cases, a CFU may be an individual cell that was seeded onto the plate, but a pair or even a small chain or cluster of cells can also give rise to a colony. Cells from an isolated colony (one that is not touching another colony) can be transferred to a sterile medium to start a pure culture.

A variety of streak plate inoculation patterns exist; the one used depends on the inoculum's source and the microbiologist's preference. Although streak patterns range in complexity, all are designed to separate deposited cells (CFUs) on the agar surface to generate isolated colonies. The quadrant streak pattern and T-streak pattern are useful when dealing with concentrated samples, whereas a simple zigzag (continuous streak) pattern may be useful for low concentration samples.

The Quadrant Streak Plate Isolation Pattern

As the name suggests, a quadrant streak pattern involves dividing the plate you'll inoculate into quadrants (four sections). You can think of it as a process that dilutes the sample on a solid surface.

Using a sterile loop, the source sample is inoculated into the first section. From there, the sample is spread to the other three sections; we do not add any more sample. To sequentially dilute the sample, the inoculating loop is flame sterilized between each section. The hope is that in at least one section (usually the last section, where the sample is most diluted), the cells will be spread far enough apart that they will grow into isolated colonies (Fig. 4.1). Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Collect the Sample onto a Sterile Loop:** Your stock sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). No matter what the source sample's nature, you'll need to get some of it on a sterile loop. Refer to Exercise 3, page 59, for specific directions on obtaining a sample from a broth sample or a sample growing on agar medium.

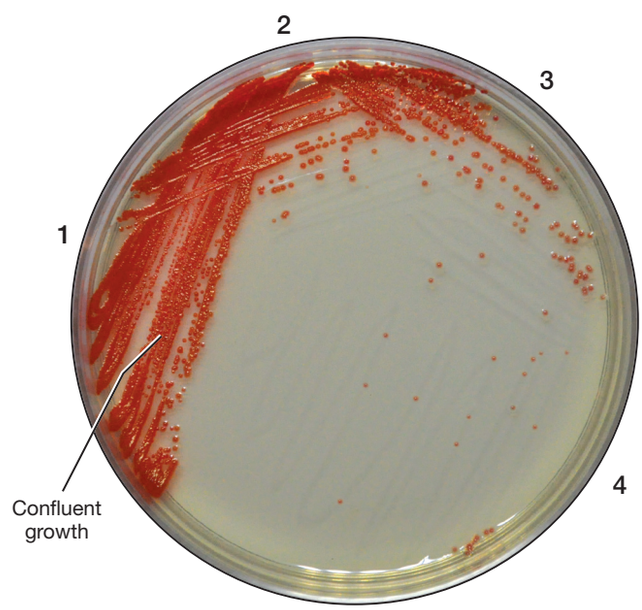


FIGURE **4.1** **Quadrant Streak Plate of *Serratia marcescens*** • Note how the growth density decreases as we progress clockwise around the plate from Quadrant 1 to 4. On this plate, some isolation is achieved in the second quadrant, but the microbiologist would not know that at the time of streaking, so all four quadrants are streaked in the hope that isolation will occur in at least one of them.

2. **Positioning the Plate for Inoculation:** Hold your sample-loaded loop in your dominant hand like a pencil. With your free hand, you'll position the plate for inoculation. You have two options for this:
   - **Option 1:** Leave the sterile agar plate on the table and using your free hand, slightly lift the lid into a "clam shell" configuration. In this position, the lid shields the agar surface from airborne contamination (Fig 4.2A).
   - **Option 2:** Hold the plate in your free hand and slightly open it so the lid and plate have a "clam shell" orientation and the lid is in place to serve as a shield against airborne contaminants (Fig. 4.2B).

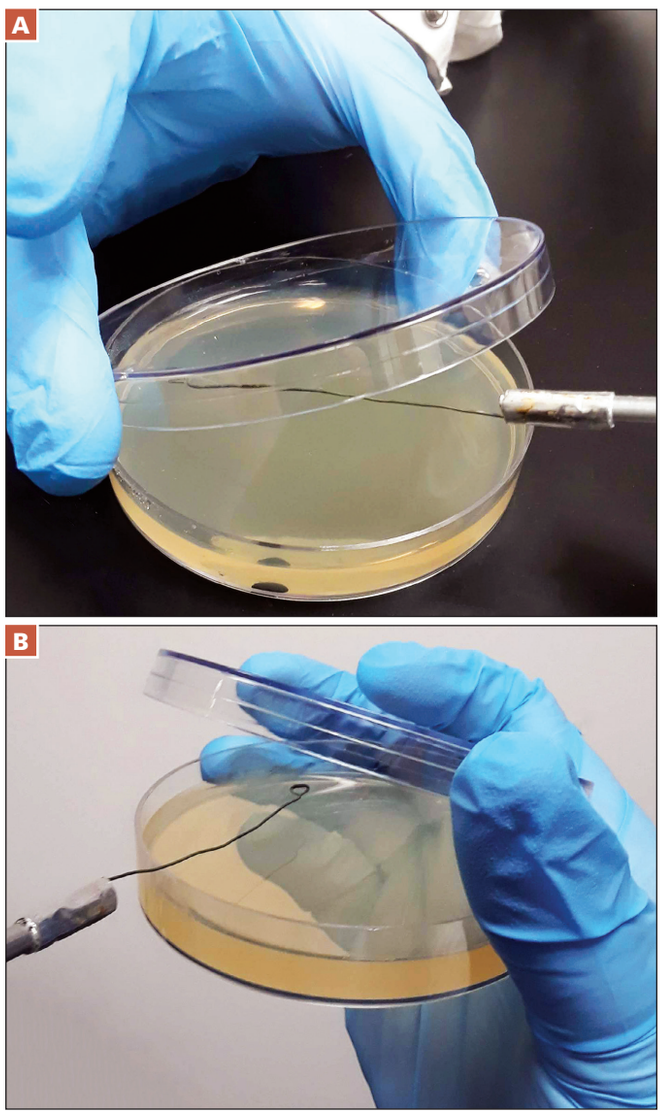


FIGURE **4.2** **Positioning a Plate for Inoculation** • (A) You can place the plate on the table and hold the lid in a "clam shell" configuration to shelter the agar from airborne contamination. (B) If you prefer having the culture closer to eye level, you can use your non-loop hand to hold the plate in a "clam shell" configuration.

3. **Inoculate the First Quadrant:** Starting at the edge of the plate, lightly drag the loop back and forth across the agar surface's first quadrant (Fig. 4.3). Be careful not to cut the agar surface. The loop should contact the agar with slight pressure at an angle like that shown in Figure 4.4.

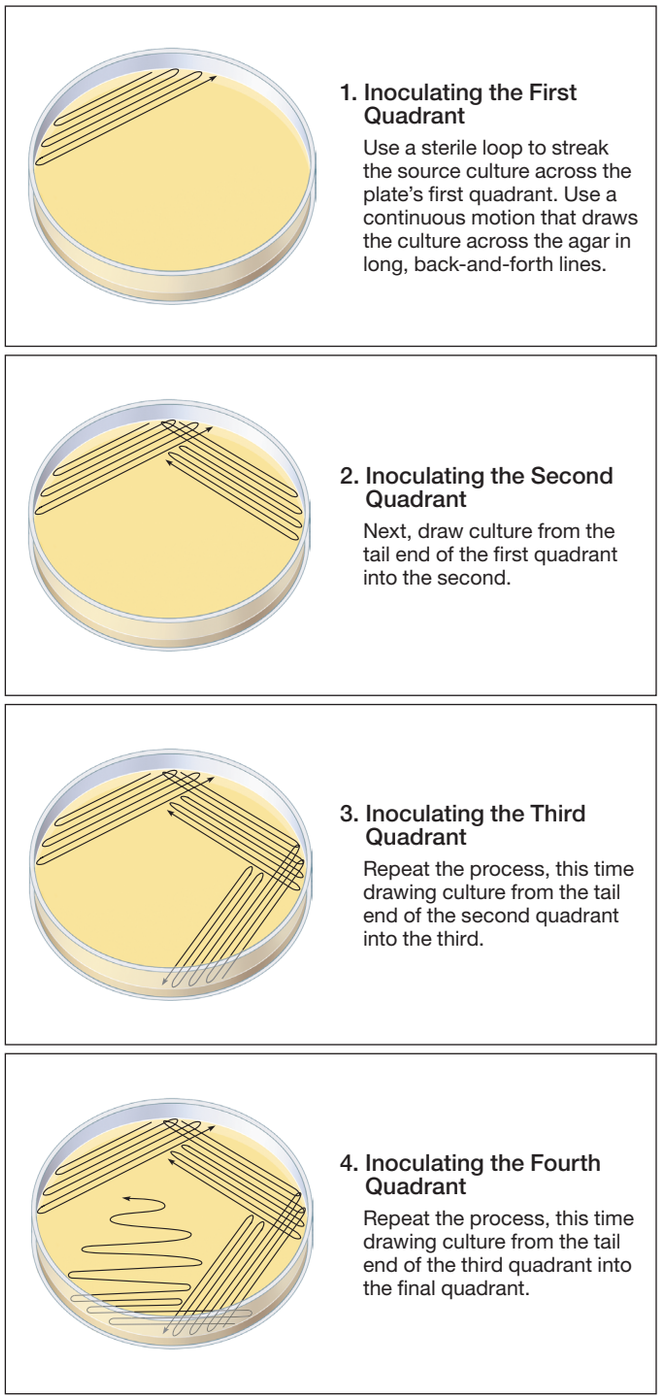


FIGURE **4.3** **Performing a Quadrant Streak Pattern** •

4. **Sterilize the Inoculating Loop:** Remove the loop from the plate and replace the lid. Flame sterilize the loop's wire filament using a Bunsen burner's flame or an incinerating machine. Ensure that each section of the wire is heated to a glowing state. Allow the loop to thoroughly cool before proceeding (about 30 seconds). In some cases, your instructor may encourage you to cool the loop by gently touching it to a part of the agar surface that has not been inoculated. You should follow their preferences for this.
5. **Inoculate the Second Quadrant:** The culture for this quadrant will come from the first quadrant. You do not go back into the source culture. Gently drag your sterile, cooled loop across the tail end of the first streak pattern and into the second quadrant as shown in Figure 4.3.
6. **Sterilize the Inoculating Loop:** Sterilize the loop and let it cool.
7. **Inoculate the Third Quadrant:** Repeat the streaking process, this time drag the loop across the tail end of the second quadrant and into the third section (Fig. 4.3).
8. **Sterilize the Inoculating Loop:** Sterilize the loop and let it cool.
9. **Inoculate the Fourth Quadrant:** Repeat the streaking process, but this time, drag the loop across the tail end of the third quadrant and into the fourth (Fig. 4.3).
10. **Sterilize the Loop:** Sterilize the loop and set it down.
11. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

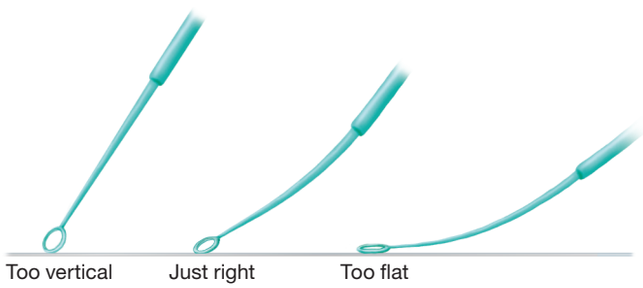


FIGURE **4.4** **Proper Loop Angle and Pressure**

The T-streak Plate Isolation Pattern

The T-streak method is a variation on the quadrant streak, but instead of dividing the plate into four sections, three sections are designated (Fig. 4.5). There is no advantage of one method over the other; it comes down to personal preference. Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Section the Plate:** Use a marking pen to draw a "T" on the base of the plate you'll inoculate (Fig. 4.5).
2. **Collect the Sample onto a Sterile Loop:** Your stock sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). No matter

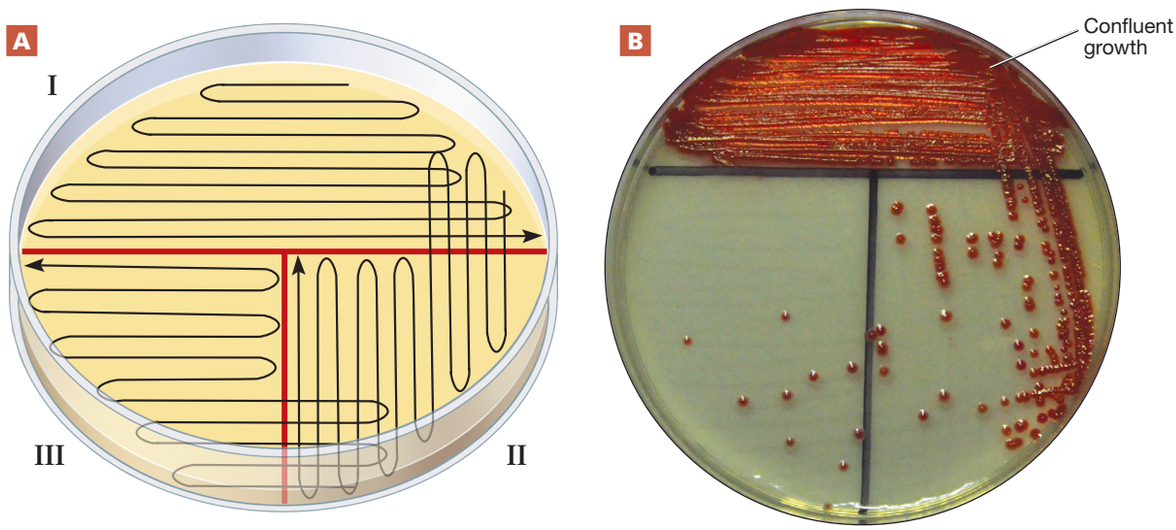


FIGURE **4.5** **T-streak of *Serratia marcescens*** (A) This method divides the plate into three sections. The regions are designated by drawing lines (shown in **red**) in the shape of a "T" on the plate's bottom. The first region (**I**) is the largest (about one-third of the agar's surface) and is streaked with the source sample. After sterilizing the loop, draw it across the tail end of the first section a few times followed by four or five streaks into the second region (**II**). The final section (**III**) is similarly inoculated. (B) A T-streak of *Serratia marcescens*.

what the source sample's nature, you'll need to get some of it on a sterile loop. Refer to Exercise 3 Lab Techniques, pages 53–61, for specific directions on obtaining a sample from a broth sample or a sample growing on agar medium.

3. **Positioning the Plate for Inoculation:** Hold your loop-laden sample in your dominant hand like a pencil. With your free hand, you'll position the plate for inoculation. You have two options for this:
   - **Option 1:** Leave the sterile agar plate on the table and using your free hand, slightly lift the lid into a "clam shell" configuration. In this position the lid shields the agar surface from airborne contamination (Fig. 4.2A).
   - **Option 2:** Hold the plate in your free hand and slightly open it so the lid and plate have a "clam shell" orientation and the lid is in place to serve as a shield against airborne contaminants (Fig. 4.2B).
4. **Inoculate the First Section:** Being careful that you don't cut the agar, use the loop to streak your sample across the larger section of the agar—the area above the "T" you drew on the plate.
5. **Sterilize the Inoculating Loop:** Remove the loop from the plate and replace the lid. Flame sterilize the loop's wire filament using a Bunsen burner's flame or an incinerating machine. Ensure that each section of the wire is heated to a glowing state. Allow the loop to thoroughly cool before proceeding (about 30 seconds). In some cases, your instructor may encourage you to cool the loop by gently touching it to a part of the agar surface that has not been inoculated. You should follow their preferences for this.
6. **Inoculate the Second Section:** The culture for this section will come from the first section. You do not go back into the source culture. Gently drag your sterile, cooled loop across the tail end of the first streak pattern and into the second section as shown in Figure 4.5.
7. **Sterilize the Inoculating Loop:** Sterilize the loop and let it cool.
8. **Inoculate the Third Section:** Repeat the streaking process, this time dragging the loop across the tail end of the second section and into the third section (Fig. 4.5).
9. **Sterilize the Loop:** Sterilize the loop and set it down.
10. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

Zigzag (Continuous) Plate Inoculation Pattern

This inoculation pattern is usually performed for low concentration samples (e.g., certain environmental samples) or when you know you are dealing with a pure culture and species isolation is not necessary. Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Obtain Your Sample:** You'll use a sterile swab to collect your sample and inoculate it onto an agar plate. Your sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). Alternatively, it may be directly from the environment (e.g., a swipe of a surface).
2. **Position the Plate for Inoculation:** Hold the swab comfortably in your dominant hand and lift the lid of the petri plate with the other. As described in the quadrant and T-streak procedures, use the lid as a shield to protect the agar from airborne contamination.
3. **Inoculate the Agar:** Lightly drag the cotton swab across the agar surface in a zigzag pattern, rolling it as you do so. Be careful not to cut the agar surface (Fig. 4.6).
4. **Replace the Lid and Dispose of the Swab:** Cover the plate, set it down, and then dispose of the swab in a biohazard container.
5. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

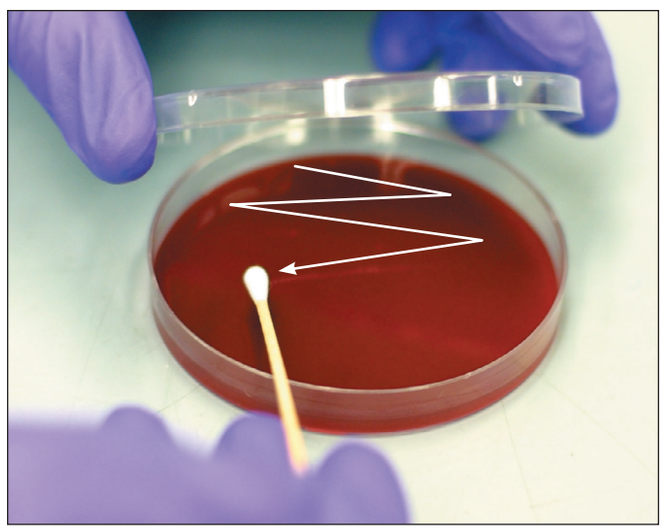


FIGURE **4.6** **Zigzag (Continuous) Inoculation with a Swab**

A Hybrid Plate Inoculation: Zigzag Inoculation Combined with Streak Plating

This pattern is a hybrid of the zigzag and quadrant plate inoculation patterns. It is usually performed as the initial streak for isolation of two or more bacterial species in a mixed culture with suspected high cell density. Like the quadrant method, the plate is inoculated across four sections, but unlike the quadrant method, the first quadrant is inoculated with a swab. Before you start working, always declutter your work space and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Obtain Your Sample:** You'll use a sterile swab to collect your sample and inoculate it onto an agar plate. Your sample might be a liquid or it may be growing on solid agar media (i.e., a plate or slant). Alternatively, it may be directly from the environment (e.g., a swipe of a surface).
2. **Position the Plate for Inoculation:** Hold the swab comfortably in your dominant hand and lift the lid of the petri plate with the other. As described in the quadrant and T-streak procedures, use the lid as a shield to protect the agar from airborne contamination.
3. **Inoculate the Agar:** Lightly drag the cotton swab across the first quadrant of an agar plate. This replaces the first streaking pattern used in the quadrant isolation pattern.
4. **Replace the Lid and Dispose of the Swab:** Cover the plate, set it down, and then dispose of the swab in a biohazard container.
5. **Complete the Streak Pattern with a Loop:** Further streaking is performed with an inoculating loop as described in Steps 5–10 of the quadrant isolation pattern (see p. 71).
6. **Incubate the Plate:** Plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

Clinical Applications

If you've ever had your throat swabbed to check for streptococcal pharyngitis (strep throat), then you've been a beneficiary of bacterial isolation methods. The techniques you'll perform in this lab are used every day in clinical labs to isolate and identify an **etiological agent** (causative agent) of a patient's bacterial or fungal infection. Viral pathogens are isolated with different methods. By isolating the causative agent, microbiologists can further characterize the pathogen for antimicrobial drug susceptibility. Drug susceptibility data helps clinicians prescribe a drug regimen that will optimally target the pathogen and improve patient outcomes. Being able to isolate bacteria is also central to food, drug, and water quality testing processes, measures that prevent millions of infections every year.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Bunsen burner or electrical incinerator
- ❑ Inoculating loop
- ❑ Sterile swab (Part C only)
- ❑ Materials for labeling samples (e.g., marking pen, tape, etc.)
- ❑ Vortex mixer (only if using a broth stock culture)
- ❑ Tryptic soy agar (TSA) plates (3; one plate for each part: A, B, and C—fewer plates if not performing all three parts)
- ❑ Tube (1) of sterile distilled water (Part C only)
- ❑ Broth or plated cultures of *Micrococcus luteus* and *Staphylococcus epidermidis* (or some other combination of BSL-1 bacteria) (Parts A and B)

PROCEDURE

In this procedure, you'll learn how to isolate individual organisms from a mixed culture, the first step in producing a pure culture. We'll explore three related streaking techniques, the choice of which one is used depends on the sample's concentration (suspected and/or known bacterial levels). Your instructor may have you do some or all of the following four parts. Pay attention.

PART A: Performing a Quadrant Streak

For this part, you'll use the quadrant streak plate isolation pattern to inoculate a plate of enriched media. During the next lab session—after your bacteria have had a chance to grow—you'll check your plate to see if you achieved colony isolation.

1. Clear your workspace as much as possible.
2. Put on your safety gear (lab coat, safety glasses, and gloves).
3. If using a Bunsen burner, light it and adjust the flame so a small blue cone is at the center. You do not want a large orange flame. If using an incinerator, turn it on.
4. Obtain a stock sample of *Micrococcus luteus* (or another bacterium as your instructor directs). The stock may be growing in a broth or on a plate, it depends on what your instructor wants you to use.

5 Label the plate you will inoculate with your name, the date, and the name of the medium (e.g., tryptic soy agar, TSA for short), and the bacterium's name.

6 Follow the quadrant inoculation procedure described on pages 69–71 to transfer a loopful of the bacterial stock to the agar plate.

7 When you're done, put your plate where your instructor directs you to put it for incubation. Samples are typically incubated overnight at 35–37°C. Remember, plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

2

→ Next Lab Session

1 Remove your plate from the incubators and examine it for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your plate as your instructor directs.

PART B: Performing a T-streak

Here you'll perform a T-streak pattern to inoculate an agar plate. You'll check your results the next lab session after the bacteria have had a chance to grow.

1 Clear your workspace as much as possible.

2 Put on your safety gear (lab coat, safety glasses, and gloves).

3 If using a Bunsen burner, light it and adjust the flame so a small blue cone is at the center. You do not want a large orange flame. If using an incinerator, turn it on.

4 Obtain a stock sample of *Staphylococcus epidermidis* (or another bacterium as your instructor directs). The stock may be growing in a broth or on a plate, it depends on what your instructor wants you to use.

5 Label the plate you will inoculate with your name, the date, and the name of the medium (e.g., tryptic soy agar, TSA for short), and the bacterium's name.

6 Follow the T-streak inoculation procedure described on pages 71–72 to transfer a loopful of the bacterial stock to the agar plate.

7 When you're done, put your plate where your instructor directs you to put it for incubation. Samples are typically incubated overnight at 35–37°C. Remember, plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

→ Next Lab Session

1 Remove your plate from the incubators and examine it for growth. Record your observations and answer the data sheet questions at the end of this lab.

2 Dispose of your plate as your instructor directs.

PART C: Zigzag Inoculation of an Environmental Sample

This part involves sampling the environment with a sterile swab and then performing a zigzag inoculation of the collected sample. ***Caution!*** Before proceeding, read the following section on environmental sampling.

Environmental Sampling

Environmental sampling is interesting, but it can be hazardous. Never perform environmental sampling unless your instructor expressly tells you to do so, and even then only sample environments your instructor has approved.

You must apply strict caution whenever you're sampling the environment. After all, microbes in our environment infect us all the time, and that's *before* we purposely grow them, a process that concentrates their numbers and increases the infection risk. Because of these risks, many instructors only perform such lab exercises with microbiology majors in upper level courses. Some institutions have entirely forbidden such lab exercises for nonmajors and severely restrict the environments that majors can sample (often body sites, bodily fluids, and facilities that contact such fluids are barred from sampling).

The bottom line is, if you sample the environment, there is a good chance that you'll isolate a dangerous pathogen and concentrate it to an infectious dose level. Moreover, that isolated agent is likely to be fully **virulent** (able to exert its full disease-causing potential) as

opposed to **attenuated** (weakened) like the strains we typically use in teaching lab exercises. As such, if you do perform such lab exercises, you must strictly follow *all* BSL-2 safety measures and only perform such exercises in a lab that is specifically designated as a BSL-2 facility (see the Introduction, pp. 2–4, for specifics on BSL-2 safety practices and facilities).

1. Clear your workspace as much as possible.
2. Put on your lab coat, eye protection, and gloves.
3. Label the plate you'll inoculate with your name, the date, and the name of the medium (e.g., tryptic soy agar, TSA for short), and the nature of the sample (e.g., swab of lab door handle).
4. Obtain a tube of sterile water and a sterile cotton swab. Dip the swab in sterile water. Before removing the swab, remove excess water by pressing the swab against the inside of the tube of sterile water. (For more details, refer to the environmental sampling section of Appendix B, p. 475).
5. Use the damp swab to swipe an environmental surface. Rotate the swab as you sample the surface.
6. Follow the zigzag inoculation procedure described on page 72 to transfer the environmental sample to an agar plate. Dispose of your swab in an approved biological hazard container.
7. When you're done, put your plate where your instructor directs you to put it for incubation. Samples are typically incubated overnight at 35–37°C. Remember, plates are incubated in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.

→ Next Lab Session

1. Remove your plate from the incubator and examine it for growth. Record your observations and answer the data sheet questions at the end of this lab.
2. Dispose of your plate as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 4

DATA SHEET

Streak Plate Isolation Methods

During the Lab

1 Answer the following questions for each part you completed.

Part A (Quadrant Streak)

Describe the growth on your plate. (If none, explain specifically why you may not have growth. You must be more specific than just writing "I did it wrong.")

Did you achieve colony isolation? If so, in what quadrant(s)?

What would you change for next time?

Part B (T-Streak)

Describe the growth on your plate. (If none, explain specifically why you may not have growth. You must be more specific than just writing "I did it wrong.")

Did you achieve colony isolation? If so, in what quadrant(s)?

What would you change for next time?

Part C
(Zigzag Environmental Streak)

Describe the growth on your plate. (If none, explain specifically why you may not have growth. You must be more specific than just writing "I did it wrong.")

Did you achieve colony isolation?

How many different types of colonies did you detect?

What would you change for next time?

2 Considering the stock cultures used for Parts A and B, how many different microbial types should you expect to see on the plates you inoculated?

Lab Related Questions

1 Which of the following applies to the quadrant streak isolation pattern? (Select all that apply.)

a. You should use an inoculating needle.
b. You will enter the stock samples 4 times, once for each quadrant inoculated.
c. You should use an inoculating loop.
d. The plated media is sterilized after inoculation.
e. A swab is used to start the inoculation, and then you use an inoculating loop.
f. The stock sample used must be a pure culture.
g. This method can be used to obtain a pure culture.

2 Which of the following applies to the zigzag isolation pattern? (Select all that apply.)

a. It can be used if you have a pure culture.
b. It is useful for samples with a low bacterial concentration.
c. It is performed with a sterile swab.
d. It can only be used if the stock sample is a broth culture.
e. It involves a single continuous motion for inoculation.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 4
DATA SHEET
(continued)

3 Assume a T-streak is performed and no colony isolation is observed. What could have led to this scenario? (Select all that apply.)

a. Too much bacterial culture was used to inoculate the plate.
b. The loop was not sterilized between plate sections.
c. The sample was contaminated.
d. The streaks did not overlap between sections.
e. The sample was incubated too long.

4 Describe two things that add to the hazard of environmental sampling.

_____

_____

_____

_____

5 In teaching labs we often use pathogens that are weakened and therefore less likely to cause disease. Such microbes are described as _____.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ A pure culture is derived from a single colony.

_____ One CFU will give rise to multiple colonies.

_____ A CFU is always a single cell.

_____ The quadrant streak plate is essentially a dilution of a sample on a plate.

_____ Viral pathogens can be isolated using the techniques described in this lab exercise.

7 A pathogen that can exert its full disease-causing effects is said to be _____.

8 The best definition of an etiological agent is:

a. the causative agent of an infection.
b. a drug resistant strain of bacteria.
c. a pure isolate of a pathogen.
d. a contaminant of a pure culture.
e. the way an infection is passed from one person to another.

Critical Thinking

1 How can colony sheen, shape, and coloration help you know if you have a pure culture or not?

2 Assume a T-streak is performed and colony isolation is observed in the first section of the plate, but no growth occurs in the other plate sections. What most likely led to this scenario?

EXERCISE 5

Spread Plate Isolation Method

After doing this exercise, you should be able to:

1. Properly perform a spread plate inoculation.
2. State the goals of the spread plate method.
3. Correctly apply aseptic techniques while handling bacterial cultures and media.
4. Describe when a spread plate method is appropriate to use.

Background

In the spread plate method, we add a diluted microbial sample to an agar plate and uniformly spread the sample across the media's surface with a sterile spreader, often a glass rod. Provided the sample is properly diluted, colony forming units (CFUs) will be deposited far enough apart on the agar surface to grow into individual colonies.

Not only is the spread plate method useful for colony isolation, it's also used for enumerating how many live bacteria are in a sample. For enumeration, only plates that grow between 25–250 colonies are considered valid samples. Plates that grow over 250 colonies are labeled as TNTC (too numerous to count) and plates that fall below the 25-colony threshold are designated as TFTC (too few to count). The 250-colony limit is the upward cap because good colony isolation is unlikely the closer we get to the 300 mark. The lower limit is set at 25 to limit the impact that potential contaminants have on colony counts. Because this method is great for bacterial enumeration, it's often used in food and water quality testing protocols.

Performing the Spread Plate Method

Generally, multiple plates are inoculated with successively diluted samples. Here we provided a description of how one plate would be inoculated, but the process would be repeated for each dilution being analyzed. As a reminder, before you begin any lab exercise involving live microbes, declutter your work space and put on your lab safety gear (lab coat, safety glasses, and gloves).

1. **Label Plate(s):** Label the base of each plate you'll inoculate with your name, the date, the organism name (or the sample source if the specific organism is unknown), and any other relevant information (e.g., dilution information).
2. **Arrange Your Work Space:** To minimize fire risk, arrange the alcohol jar, Bunsen burner, and agar plate as shown in Figure 5.1.
3. **Open the Plate You'll Inoculate:** Lift the plate's lid and use it as a shield to protect the agar surface from airborne contamination.
4. **Add Sample to the Plate:** Use a pipette to deposit the designated inoculum volume onto the agar surface (Fig. 5.2). (Refer to Appendices C and D for how to use pipettes.) When finished, dispose-able pipettes and pipette tips are discarded into a biohazardous waste container.

To prevent the inoculum from soaking into the agar, the remaining steps should be completed within about 15 seconds of adding the sample.

5. **Sterilize the Spreading Tool:** Dip the glass spreading rod into alcohol and then pass it through the flame to ignite the alcohol (Fig. 5.3). Remove the rod from the flame and allow the alcohol to completely burn off. Do not leave the rod in the flame; the combination of the alcohol and brief flaming are enough to sterilize it. ***Caution!*** **Do not drop flaming alcohol onto the work surface, and especially not back into the alcohol-filled jar. If the alcohol in the jar ignites, place the lid over it to smother the flame.**



FIGURE **5.1** **Spread Plate Set-up** • The spread plate method requires a Bunsen burner, a screw-cap jar with alcohol, a glass spreading rod, and the plate you'll inoculate. The shown arrangement reduces the chance of accidentally catching the alcohol on fire. Notice the cotton in the jar's bottom to reduce the chance of breaking the glass rod.

6. **Cool the Spreader:** After the alcohol has burned off the spreader, cool the spreader by touching it to the agar surface away from the inoculum. As always, shield the agar surface with the plate's lid to prevent contaminants from entering the plate.
7. **Spread the Inoculum:** Carefully rotate the plate so the rod spreads the sample over the full surface of the media, including the edges (Fig. 5.4A). Alternatively, place the plate on an inoculating turn table and spread the inoculum (Fig. 5.4B).
8. **Finishing the Inoculation:** Remove the rod from the plate, replace the plate's lid, and return the rod to the alcohol.

2

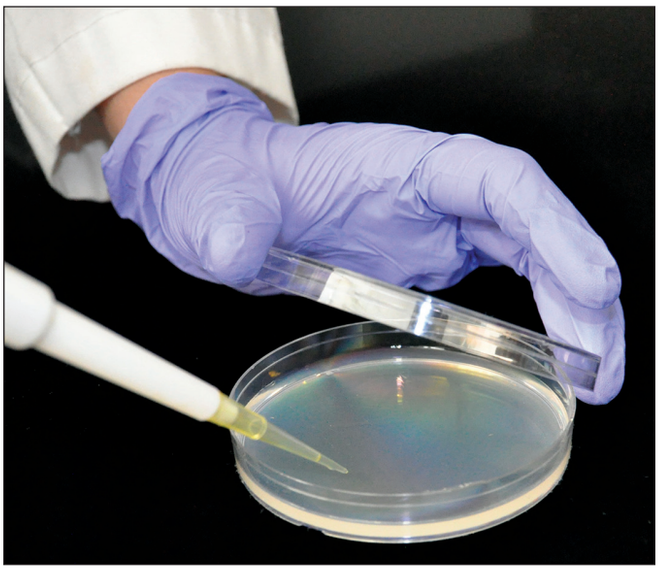


FIGURE **5.2** **Adding the Inoculum to the Plate** • Use a pipette to deposit the inoculum near one side of the agar surface.



FIGURE **5.3** **Flaming the Glass Rod** • Dip the glass spreader into some alcohol and then flame the spreader to burn off the alcohol.

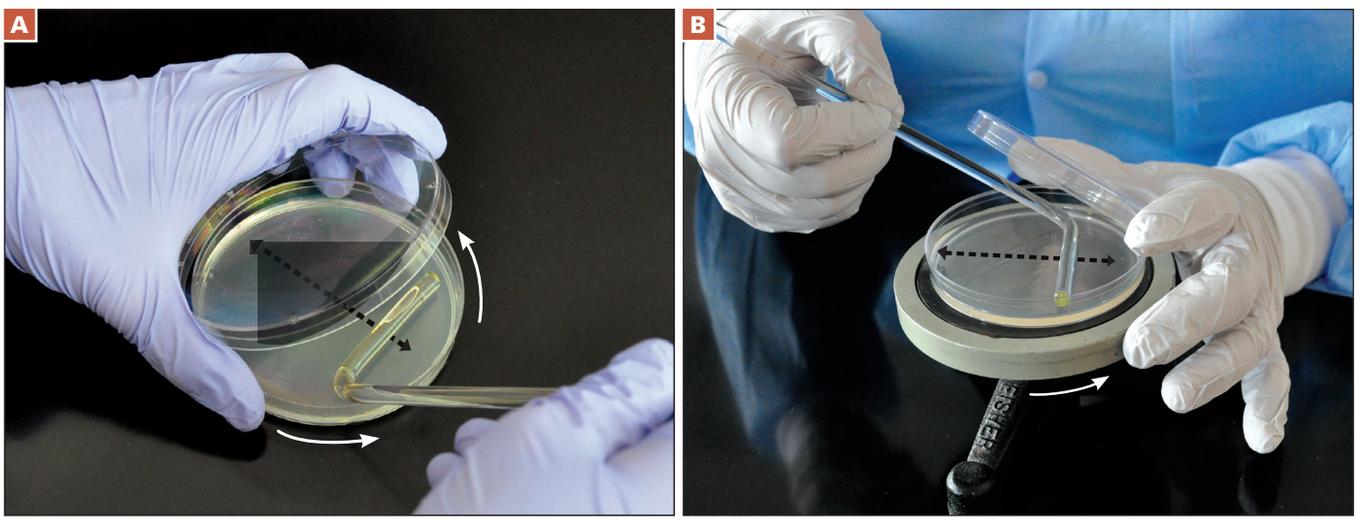


FIGURE **5.4** **Spreading the Inoculum** • (A) Carefully rotate the plate so the rod spreads the sample over the full agar surface, including the edges. (B) An inoculating turntable makes it easy to rotate the plate during the spread plate method.

Clinical Applications

USAID estimates that every day about 1,900 children die of diarrheal diseases linked to poor sanitation and water quality. Effective and widespread water sanitation is one of the hallmarks of an industrialized nation, and it's a process that heavily relies on microbiologists monitoring our water supply. One way this is done is with the spread plate method; a technique that helps microbiologists enumerate the number of live bacteria in a sample. If the level of pathogens exceeds a safe level, then boil water advisories are instated until water quality is restored. The spread plate method is also used to detect and enumerate potential biological contaminants in foods and medications.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Bunsen burner
- ❑ Inoculating loop
- ❑ Materials for labeling samples (e.g., marking pen, tape, etc.)
- ❑ Vortex mixer (optional)
- ❑ Sterile plastic transfer pipettes (6)
- ❑ Glass spreading rod (or 4 sterile disposable spreaders)
- ❑ If using a non-disposable glass spreader, you'll also need a glass, screw-cap jar with cotton at the bottom and enough isopropyl alcohol to only cover the spreading part of the glass rod.
- ❑ Capped microtubes each containing 1 mL of sterile water (4)
- ❑ Empty sterile microtube (1)
- ❑ Nutrient agar (NA) plates (4)
- ❑ Nutrient broth culture of *Staphylococcus marcescens* (or another BSL-1 bacterium)
- ❑ Nutrient broth culture of *Escherichia coli* (or another BSL-1 bacterium)

PROCEDURE

Here you'll perform a spread plate inoculation as an isolation procedure, but it's also commonly used to quantify the number of live bacteria in a liquid sample. Before you begin, clear your workspace as much as possible and put on your safety gear (lab coat, safety glasses, and gloves).

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so the alcohol-filled jar is not near the Bunsen burner.
2. **Use the Empty Sterile Microtube to Setup Your Culture Stock:** Label the empty microtube "non-dilute stock." Use a sterile pipette to add a few drops of *E. coli* culture to the sterile tube. Then use a second pipette (or a new sterile pipette tip) to add *S. marcescens* culture to the same microtube. Cap the tube and mix the sample with a vortex mixer or by gently drawing and dispensing the mixture in and out of the tube a couple of times with the pipette. Do not spray the mixture! Properly dispose of pipettes or pipette tips into the appropriate biohazardous waste container.
3. **Label the Four Microtubes of Water:** Label the four microtubes containing sterile water "A," "B," "C," and "D."
4. **Label Your Plates:** Label the four nutrient agar plates "A," "B," "C," and "D."
5. **Add Bacteria to Tube A:** Use a sterile inoculating loop to transfer a loopful of the "non-dilute stock" you prepared in Step 2 above into Tube A and mix the contents with the loop or a vortex mixer. If using a vortex mixer, cap the tube before mixing.
6. **Add Bacteria to Tube B:** Using a flame sterilized and cooled loop, transfer a loopful of the mixture in Tube A to Tube B and mix well with the loop or a vortex mixer.
7. **Add Bacteria to Tube C:** Using a flame sterilized and cooled loop, transfer a loopful of the mixture in Tube B to Tube C and mix well with the loop or a vortex mixer.
8. **Add Bacteria to Tube D:** Using a flame sterilized and cooled loop, transfer a loopful of the mixture in Tube C to Tube D and mix well with the loop or a vortex mixer.
9. **Add Bacteria to Plate A:** Use a sterile pipette to add a couple of drops of culture from Tube A to Plate A. Properly dispose of the pipette/pipette tip into a biohazardous waste container. Spread the inoculum with a glass rod. (Follow the steps for performing the spread plate method that were presented on pp. 81–82.)
10. **Inoculate the Remaining Plates:** Repeat Step 9 for Tubes B, C, and D and Plates B, C, and D, respectively. Do your best to transfer the same volume to each plate.
11. **Incubate Your Plates:** Plates are incubated overnight at 35–37°C in an inverted position so condensation won't accumulate on the lid and drip back onto the culture.
12. **Clean up:** Place Tubes A, B, C, D, and your "non-diluted stock" where your instructor directs (typically in a non-sharps biological waste container for autoclaving).

Next Lab Session

1. Retrieve your plates from the incubator and examine them for growth. *S. marcescens* produces reddish-orange colonies and *E. coli* produces buff-colored colonies.
2. Record your observations and answer the data sheet questions at the end of this lab. Dispose of your plates as your instructor directs.

2

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 5

DATA SHEET

Spread Plate Isolation Method

During the Lab

1 Record your observations in the table provided.

Organism	Which plates had isolated colonies (A, B, C, and/or D)?	Comments
E. coli		
S. marcescens		

2 When performing the lab, what would be the primary consequence of failing to evenly spread the inoculum over the agar surface?

_____

_____

Lab Related Questions

1 Which of the following is (are) used in the spread plate method? (Select all that apply.)

a. Inoculating needle
b. Nutrient broth
c. Nutrient agar
d. Agar deeps
e. Bunsen burner
f. Glass spreader
g. Alcohol

2 Some of the following steps should *not* be used if you want to aseptically transfer a culture to perform the spread plate method. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Select a colony with a sterile loop and inoculate it onto the agar plate.

_____ Add inoculum to a plate with a swab.

_____ Spread the liquid inoculum on the agar surface.

_____ Mix the stock inoculum.

_____ Use a sterile pipette to add the inoculum to an agar plate.

_____ Incubate the plate in an inverted position.

3 Assume you use the spread plate method to grow a series of bacterial samples. Following incubation, you make the following observations:

- Plate A (non-diluted stock A): 500 red colonies; 10 green colonies; 5 white colonies.
- Plate B (10-fold dilution of stock A): 50 red colonies; 1 green colony; 0 white colonies.

Based on this information, if you were to inoculate a third plate (Plate C), which was inoculated using a 10-fold dilution of the stock that was used to inoculate Plate B, what do you predict you'd see following incubation?

a. Plate C would look like Plate A.
b. Plate C would look like Plate B.
c. Plate C would have about 20 red colonies, 1–5 green colonies, and 0 white colonies.
d. Plate C would have 5–10 red colonies and no green or white colonies.

4 Why must solid media be used for colony isolation?

Critical Thinking

1 Suppose you have two organisms in a mixture and Organism A is 1,000 times more abundant than Organism B. Will you (without counting on good luck!) be able to isolate Organism B using the spread plate method? Explain your answer.

2 To get isolated colonies on a plate inoculated using the spread plate method, there can only be about 300 cells in the inoculum. What will happen if the inoculum's cell density significantly exceeds this number?

EXERCISE 6

Colony Morphology

After doing this exercise, you should be able to:

1. Describe bacterial colony morphological features.
2. Name things that can affect colony morphological features.
3. Measure and describe bacterial colony sizes.
4. Discuss why noting bacterial colony morphology is useful in microbiology.
5. Safely handle bacterial cultures and properly dispose of such cultures.

Background

Previously we reviewed inoculation techniques for growing isolated bacterial colonies on solid media. Here we'll review the features we note when observing isolated bacterial colonies. Carefully documenting bacterial colony appearance can help you determine what species are in a sample. Bacterial colony diversity is an important indicator of whether you're dealing with a pure or mixed culture. If the sample is pure (contains only one bacterial species), then every colony on the plate should have the same general **colony morphology**, observable physical characteristics such as color, shape, texture, colony margins, and other features we'll review herein (Fig. 6.1). In contrast, a mixed culture will yield diverse colonies (Fig. 6.2).

Examples of Colony Morphological Features

Round, Shiny, Convex Colonies

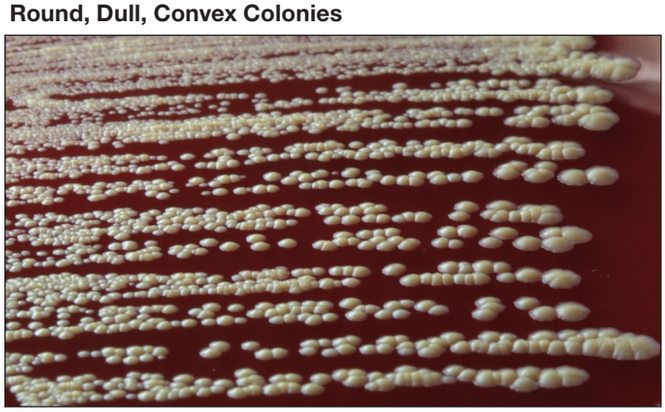


These buff-colored colonies are a 48 hour culture of *Providencia stuartii* on nutrient agar. *P. stuartii* is a common isolate from urine samples of catheterized patients. It's highly resistant to antibiotics.

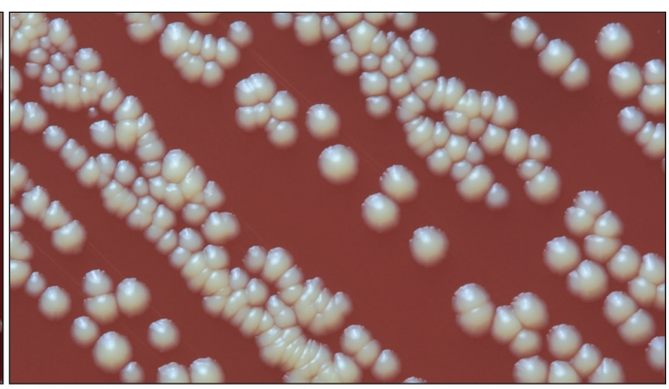


The colonies of the soil and water bacterium *Chromobacterium violaceum* grown on sheep blood agar are purple (hence "violaceum"). **Figure 6.4** shows how nutrient availability affects *C. violaceum's* pigment production.

Round, Dull, Convex Colonies



Dry, buff-colored *Corynebacterium xerosis* colonies on sheep blood agar photographed from the side. *C. xerosis* is an opportunistic pathogen.



This is a close-up of the same *C. xerosis* colonies pictured to the left, but here they have been photographed from above.

FIGURE **6.1** **Bacteria Exhibit Diverse Colony Morphology**

An organism's genetic makeup combined with environmental factors (e.g., nutrient availability, temperature, and incubation time) influence colony morphology (Figs. 6.3 and 6.4). For example, *Serratia marcescens* makes a red pigment called **prodigiosin**. However, the enzyme that helps make this pigment tends to work best below body temperature. As such, we are unlikely to see the red pigmentation unless the bacteria are grown below

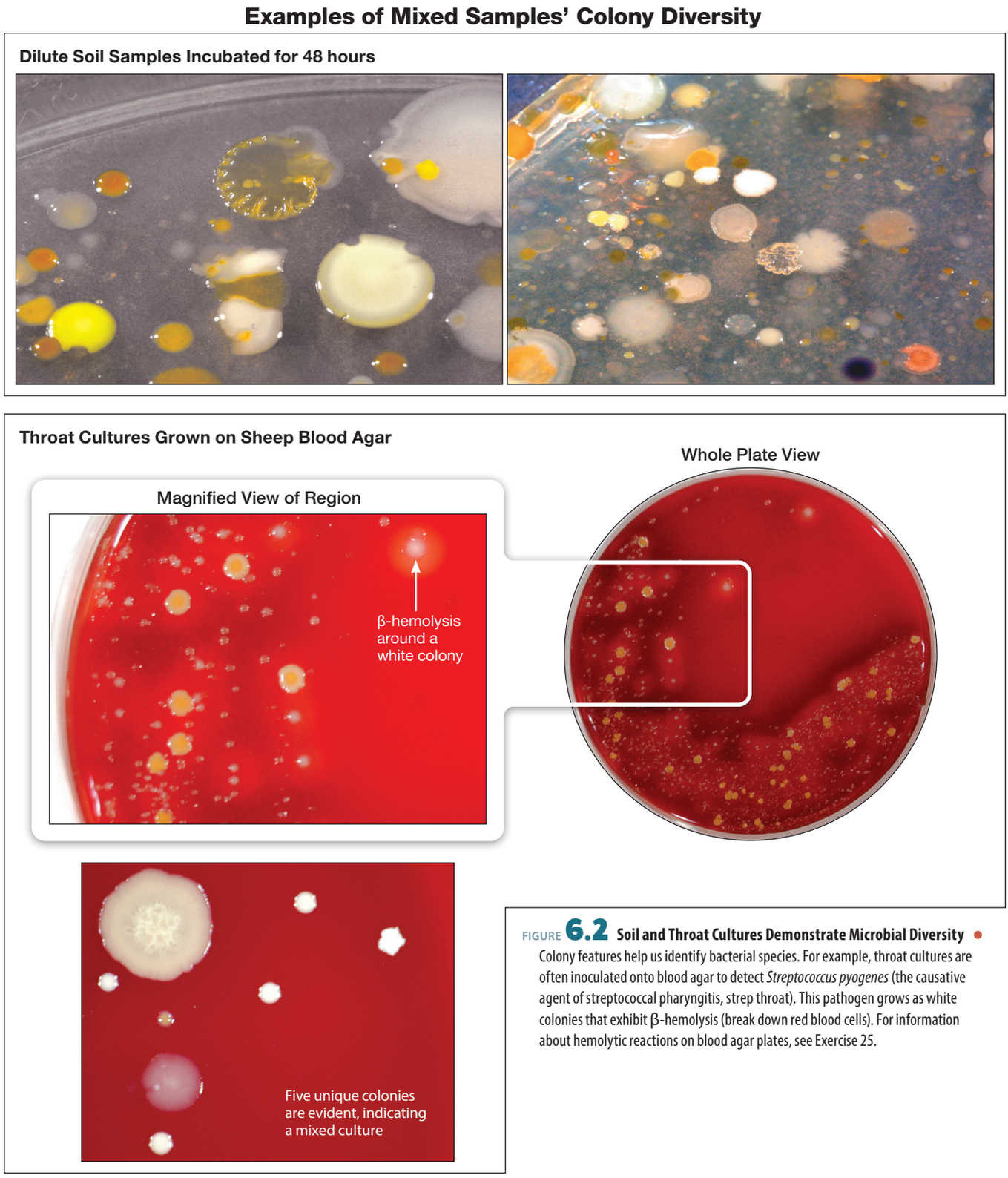


FIGURE **6.2** **Soil and Throat Cultures Demonstrate Microbial Diversity** ● Colony features help us identify bacterial species. For example, throat cultures are often inoculated onto blood agar to detect *Streptococcus pyogenes* (the causative agent of streptococcal pharyngitis, strep throat). This pathogen grows as white colonies that exhibit β-hemolysis (break down red blood cells). For information about hemolytic reactions on blood agar plates, see Exercise 25.

37°C. Colony morphological characteristics can be observed with the naked eye, a hand lens, a stereo (dissecting) microscope, and/or a colony counter (Fig. 6.5). Morphological features worth documenting include: colony size, shape (form), margin (edge), elevation, consistency, surface texture, color, and optical properties (Fig. 6.6). Even members of the same genus can manifest diverse morphological features (Fig. 6.7).

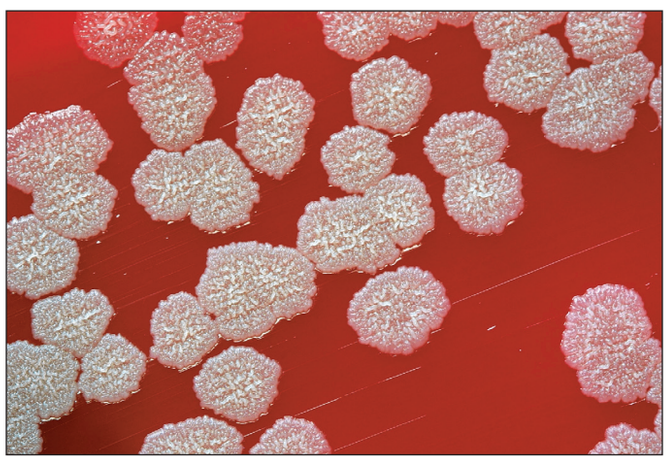
- **Size:** We note the diameter of round colonies. For noncircular colonies, we note the colony length and width. In general, colonies are described as **punctate/punctiform** (pinpoint size colonies that are generally smaller than 1 mm), small (1–2 mm), medium (3–4 mm), or large (greater than 5 mm) (Fig. 6.8).
- **Shape** (form): Colonies may be described as **round** (circular), **irregular**, **rhizoid** (root-like), **filamentous**, or **spindle** shaped (wide in the middle and tapered at the ends) (Figs. 6.9 and 6.10).
- **Margin** (edge): The edges or margins of a colony may be **entire** (smooth, with no irregularities), **undulate** (wavy), **lobate** (lobed), **filamentous** (unbranched strands), or **rhizoid** (root-like branching) (Fig. 6.11).
- **Elevation:** Colonies can appear **flat**, **raised**, **convex**, **pulvinate** (very convex), and **umbonate** (raised in the center) (Fig. 6.12).
- **Optical Properties** (opacity, translucency, color): Opaque colonies limit light transmission (they are not transparent), while translucent colonies allow light to pass through them and therefore appear "clear" or "see-through" (Fig. 6.13).
- **Consistency:** The growth may be **moist**, **mucoid** (sticky like mucus), **butyrous** (buttery), or **dry** (Fig. 6.14).
- **Surface Texture:** Colonies can appear **smooth**, **rough**, **rugose** (wrinkled), **granular**, **shiny**, or **dull** (Fig. 6.14).

Features such as colony shape, margin, surface, texture (shiny or dull), and color are best viewed by observing from above. If your instructor says it is safe to do so, you can observe the colonies with the lid off the culture to get a better idea of how light reflects off the colonies. If your instructor allows it, you may also check texture by touching the growth with an inoculating loop or wooden stick. When finished, flame the loop to sterilize it or dispose of the wooden stick in the appropriate biohazard container.

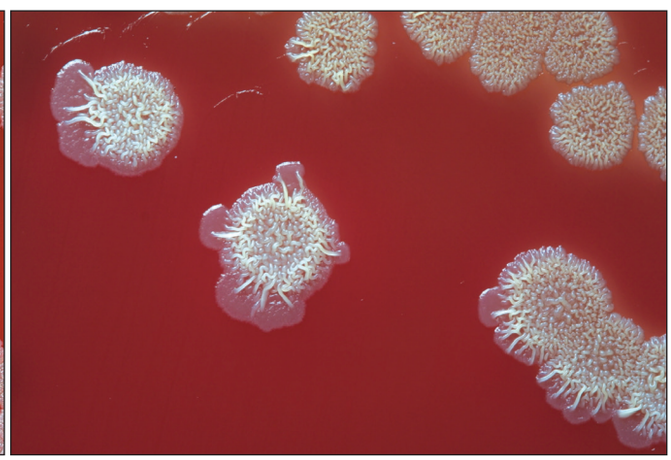
Colony elevation is best viewed with the plate tilted slightly at eye level. Opacity and translucence are best viewed by placing the plate on a colony counter or holding it (lid on) so it is illuminated from behind (transmitted light). Colony dimensions are best measured from the plate's base rather than through the lid.

When reporting colony morphology, it is important to include the medium and the incubation time and temperature, all of which can affect a colony's appearance.

Factors that Affect Colony Features: Incubation Time



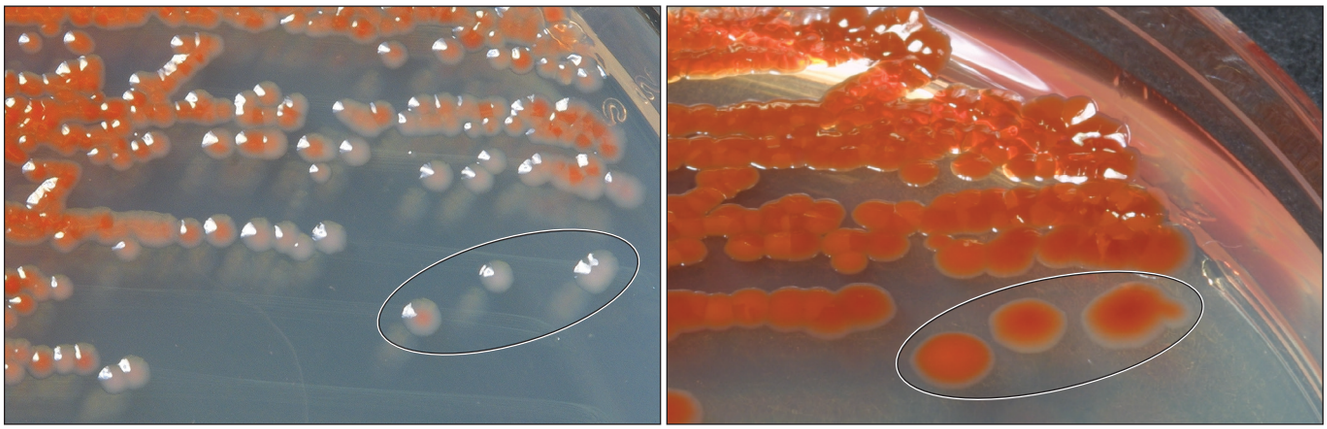
Close-up of *Bacillus subtilis* on sheep blood agar after 24 hours of incubation.



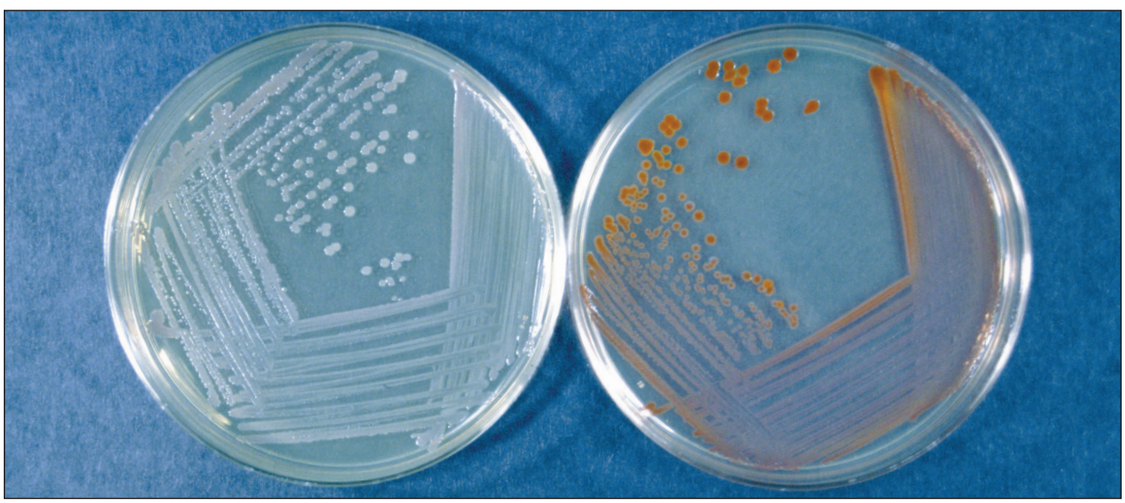
Close-up of *Bacillus subtilis* culture after 48 hours of growth. Note the wormlike extensions.

FIGURE **6.3** **Effect of Incubation Time on Colony Morphology**

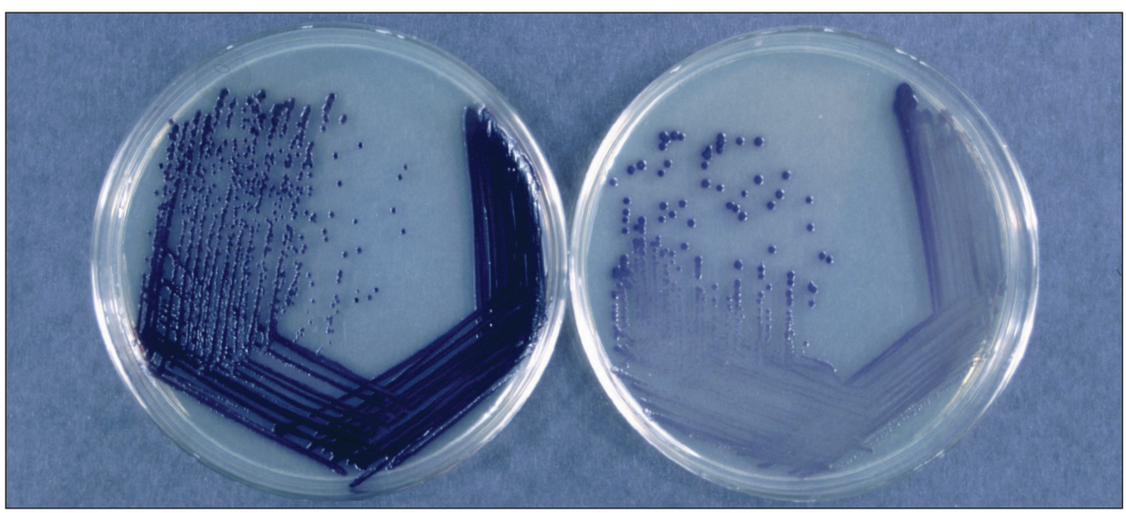
Factors that Affect Colony Features: Incubation Time, Temperature, and Nutrients



Effect of Time: *Serratia marcescens* grown on nutrient agar for 24 hours (left) and after 48 hours (right). The colonies grew darker over time.



Effect of Temperature: *Serratia marcescens* produces less orange pigment when grown at 37°C (left) than when grown at 25°C (right).



Effect of Nutrients: *Chromobacterium violaceum* produces a much more intense purple pigment when grown on tryptic soy agar (left) than when grown on nutrient agar (right), a less nutritious medium.

FIGURE **6.4** **Environmental Factors Can Alter Colony Pigmentation**

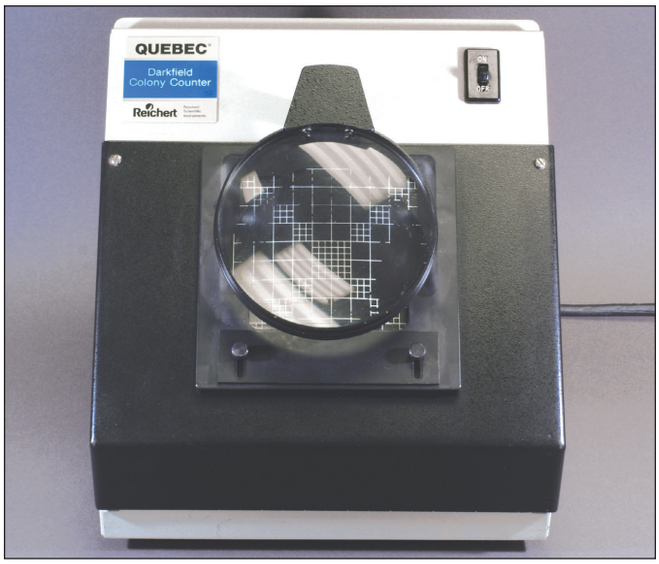


FIGURE **6.5** **Colony Counter** • A colony counter provides magnification and transmitted light to help us see subtle differences in colony shape and size. However, colony color and many other features are best determined with reflected light. The grid in the background is a counting aid; each big square is 1 square centimeter.

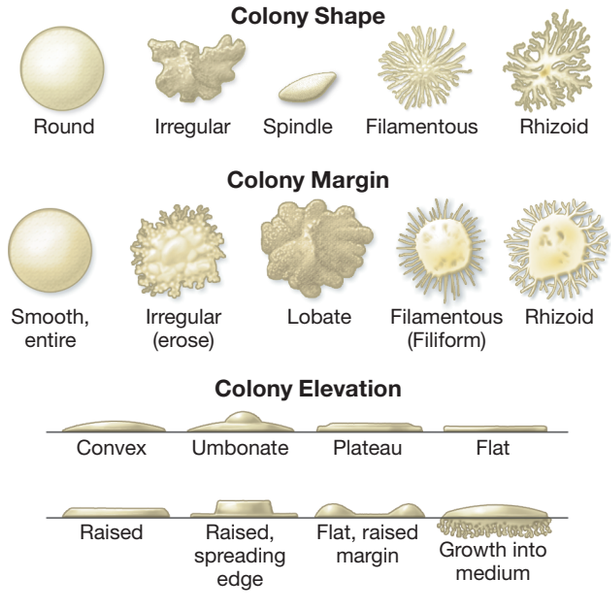
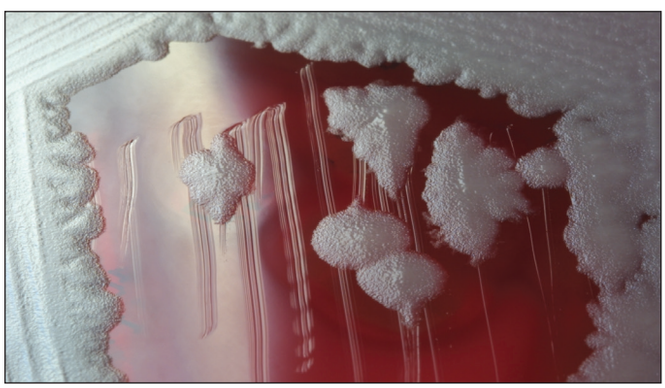


FIGURE **6.6** **Bacterial Colony Morphology Examples** • These terms describe colony morphology. Other descriptions include surface texture, consistency, elevation, color, and optical properties.

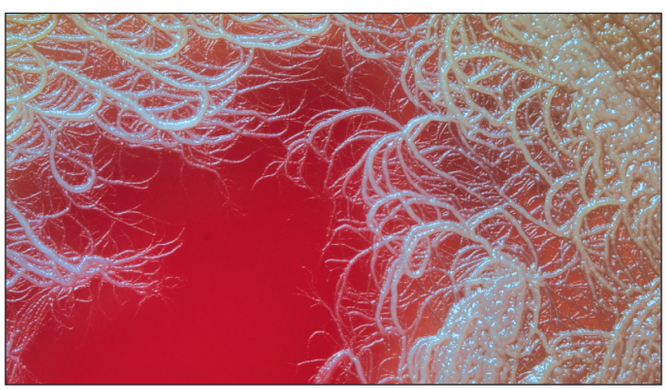
Morphological Diversity Among Members of the Same Genus



Bacillus cereus grown on sheep blood agar (SBA) produces distinctively large (up to 7 mm), gray, granular, irregular colonies. They often produce a "mousy" smell. Also note the distinctive extensions of growth along the streak line.



Bacillus anthracis colonies on SBA resemble *B. cereus*, but are usually smaller and adhere to the medium more tenaciously. It must be handled at *least* in a BSL-2 laboratory, and sometimes BSL-3 if the cell density is high enough, so it is unlikely you will be testing its tenacity!



Bacillus mycoides produces distinctive, rapidly spreading, rhizoid (note the branching) colonies. It is a common isolate from soil and is shown here on SBA.



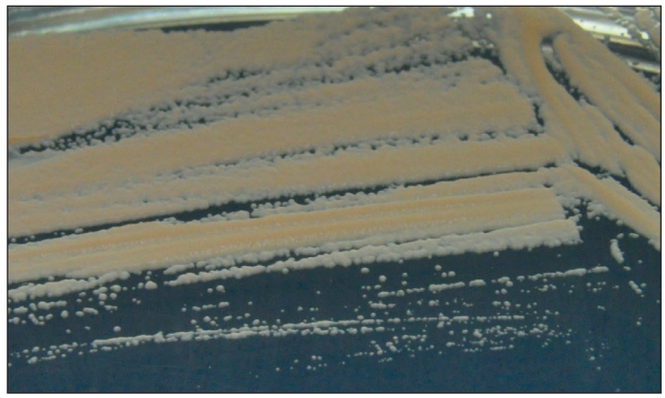
This unknown *Bacillus* isolated as a laboratory contaminant produced a wrinkled, irregular colony with an irregular (wavy) margin on tryptic soy agar.

FIGURE **6.7** **Comparison of Four *Bacillus* Species Colonies** •

Colony Size



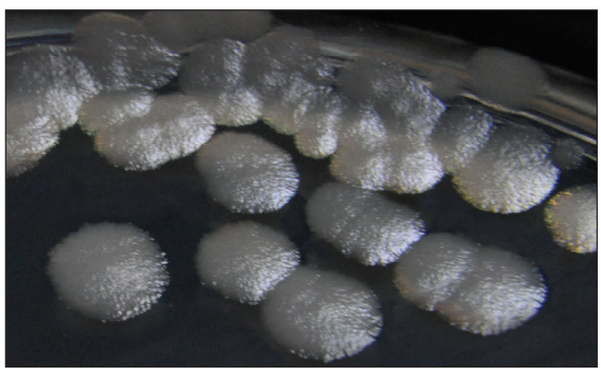
Punctiform: These are *Mycobacterium smegmatis* colonies growing on sheep blood agar. This slow-growing relative of *M. tuberculosis* forms punctate colonies that are less than 1 mm in diameter.



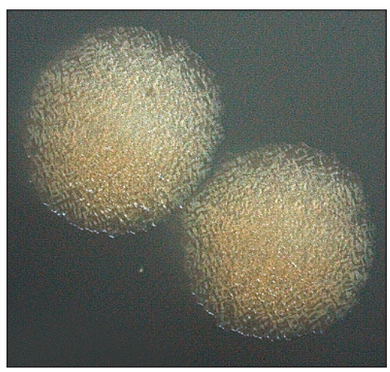
Punctiform: *Kocuria rosea* can be isolated from water, dust, and salty foods. Even after growing for 96 hours, colonies are smaller than 0.5 mm in diamter.

FIGURE **6.8** **Examples of Punctate Colonies**

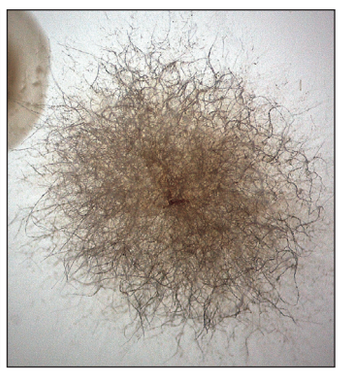
Colony Shape: Filamentous



These are *Rhizobium leguminosarum* colonies on brain-heart infusion agar. The colonies are about 5 mm in diamter, filamentous, convex, circular, and mucoid with translucent edges. This bacterium aids in nitrogen fixation by forming root nodules in many legumes (*rhiz* = "root").



These are *R. leguminosarum* colonies as viewed through a stereo microscope. The bacteria are growing on brain-heart infusion agar.



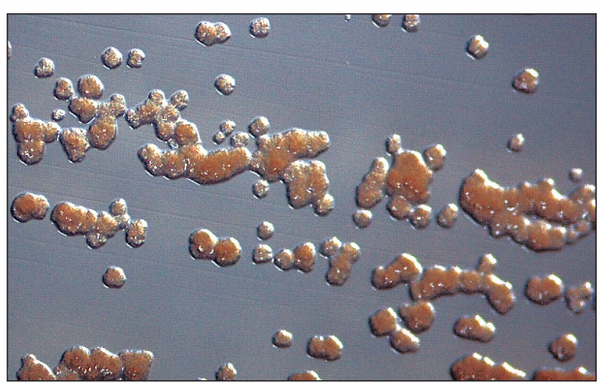
Some molds grow as filamentous colonies. This is an unidentified mold mycelium viewed with a stereo microscope.

FIGURE **6.9** **Examples of Filamentous Shaped Colonies**

Colony Shape: Irregular



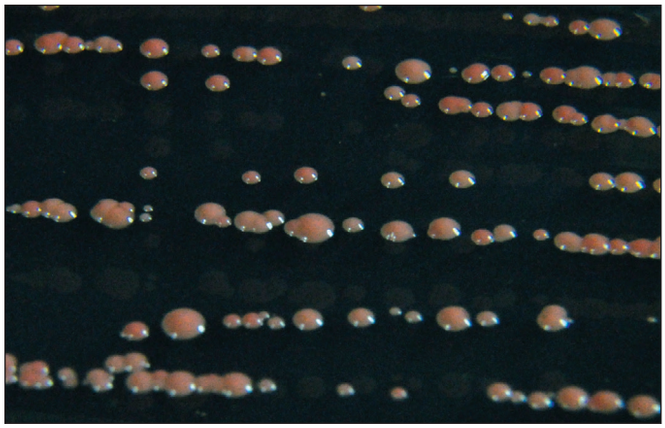
This unidentified contaminant grew on tryptic soy agar and was isolated from a laboratory tabletop. In addition to its irregular shape, it has a lobed margin and wrinkled (rugose) surface. Its widest dimension was approximately 5 mm. This photo was taken through a stereo microscope.



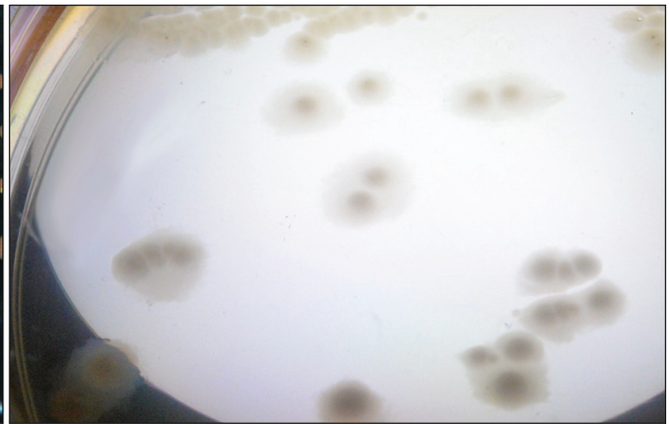
Viewing *Kocuria rosea* colonies with a stereo microscope reveals irregular shaped colonies with a wrinkled surface.

FIGURE **6.10** **Examples of Irregularly Shaped Colonies**

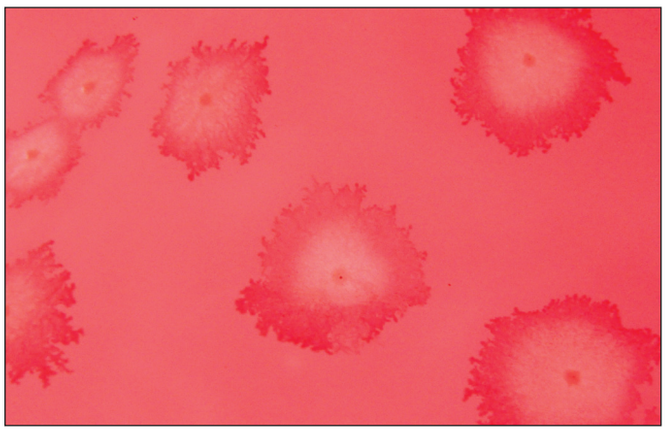
Common Colony Margins



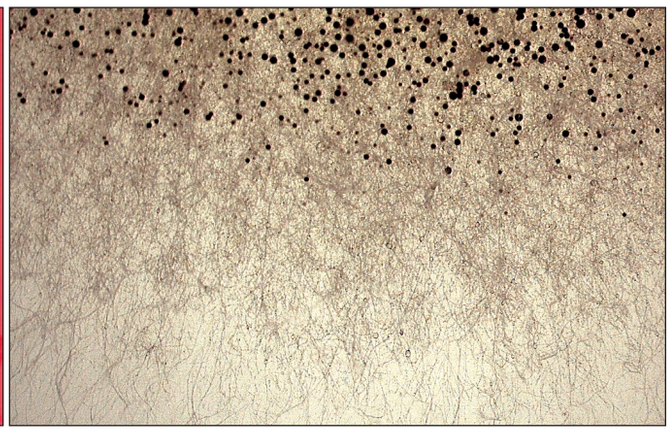
Smooth Entire Margin: These circular and convex *Rhodococcus rhodochrous* colonies were grown on brain-heart infusion agar for 48 hours. They are about 1 mm in diameter with a smooth margin and a shiny, pink surface (hence "*rhodo*"). *Rhodococcus* species are soil organisms.



Spreading Margin: *Erwinia amylovora* colonies have a raised center with an irregular, spreading margin. These colonies were grown on nutrient agar 48 hours at 25°C and ranged in size from 4 to 6 mm in diamter. *E. amylovora* is a plant pathogen; its name literally means "Erwin (Smith's) starch devourer."



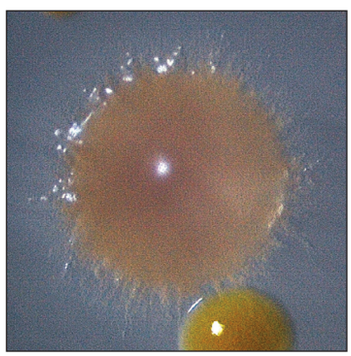
Rhizoid Margin: *Clostridium sporogenesis*, a common soil bacterium, was grown anaerobically on sheep blood agar and viewed via a stereo microscope. Note the irregular, flat, colonies have a raised center and a spreading margin of branched, tangled filaments (like the mythological creature Medusa, who had snakes for hair!). Colonies were 2 to 6 mm.



Rhizoid Margin: Fungi (other than yeast) are naturally filamentous and sometimes the filaments are branched, as in this lab contaminant (probably *Aspergillus niger*). It takes magnification to see that the margin is rhizoid and not simply filamentous. The black spheres are asexual spores called conidia.



Lobed Margin: Note the lobed margin and rugose surface of this lab contminant. After 48 hours of incubation at 35°C, this circular colony was about 1 cm in diameter.



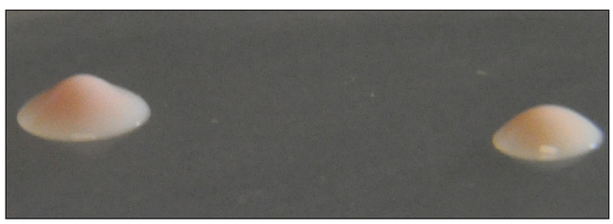
Filamentous Margin: This unidentified lab contaminant's colony margin is filamentous. The colony is also circular, convex, shiny, and about 2 mm in diameter.

FIGURE **6.11** **Examples of Colony Margins**

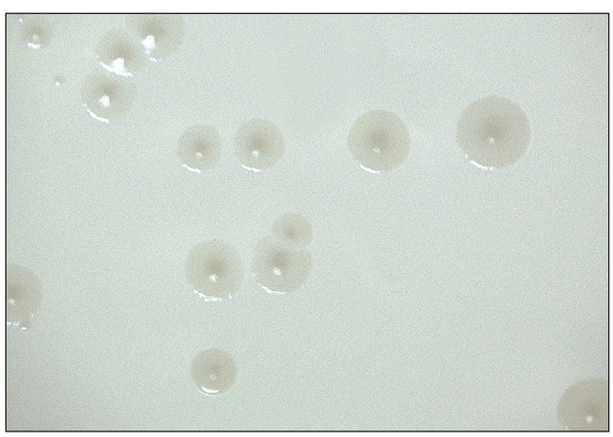
Colony Elevation



Flat: This unknown soil isolate is flat only around the edge. The center is slightly concave like a bowl and at the very center is another small, raised ring surrounding another concavity. It's almost like an automobile tire around the wheel and hub. Very unusual! Also note the dull surface.



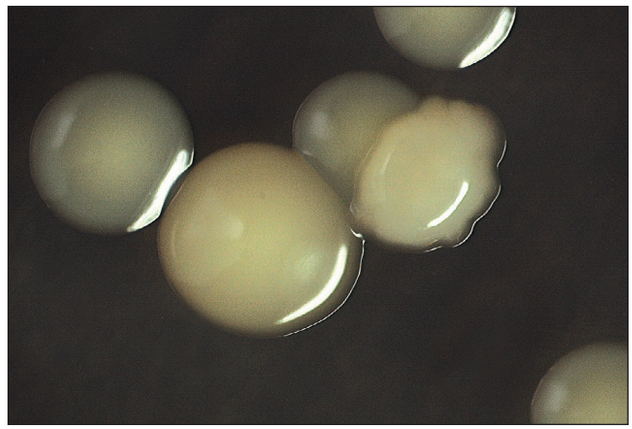
Umbonate: The colony of this anaerobic lab contaminant on the left is truly umbonate. The one on the right is getting there. Their diameters are about 3 mm.



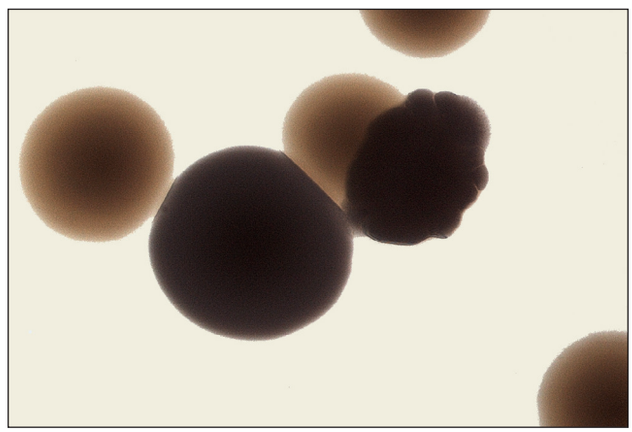
Umbonate: These are *Enterococcus faecium* colonies grown on tryptic soy agar for 48 hours and photographed via a stereo microscope. The colonies are 1–2 mm in diameter, white, and circular with an entire margin. They are also umbonate—flat except for the central bump. *E. faecium* is found in human and animal feces.

FIGURE **6.12** **Examples of Colony Elevations**

Optical Properties



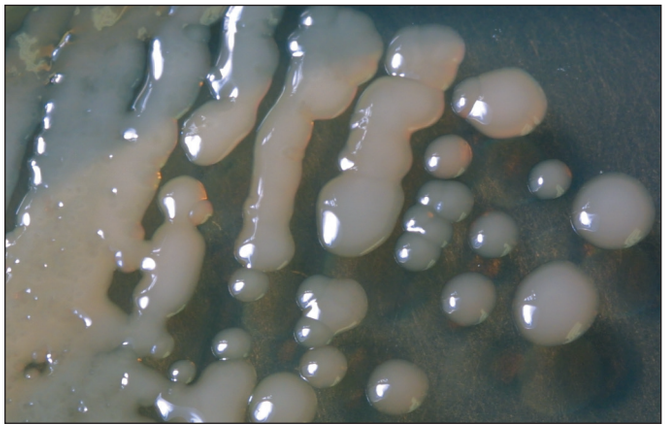
If we only observe reflected light, optical properties can be hard to determine. These colonies were photographed with a stereo microscope using refected light. There are slight differences in size and color, and one is irregular in shape, whereas the others are circular.



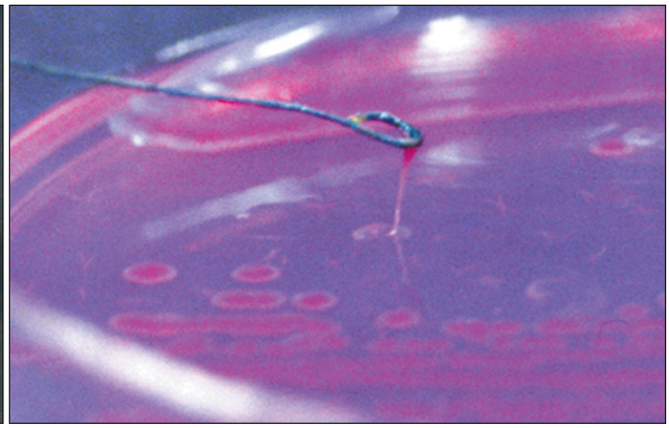
When the same colonies from the photograph above are illuminated with transmitted light, it becomes obvious that the medium-sized round colonies are translucent, whereas the others are opaque.

FIGURE **6.13** **Viewing Optical Properties with Transmitted Light**

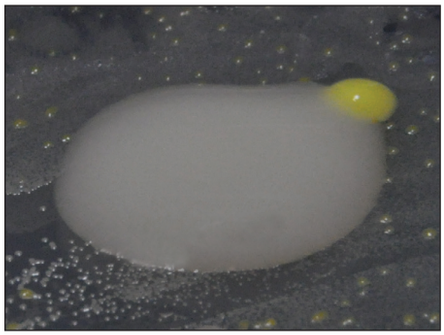
Colony Consistency and Texture



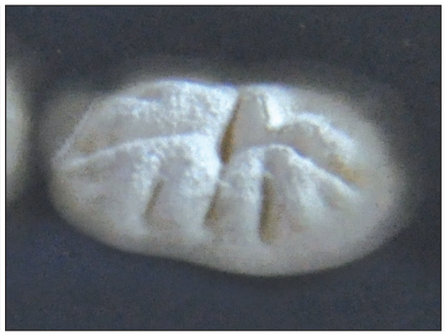
Mucoid Consistency/Shiny Texture: These *Klebsiella pneumoniae* colonies grown on nutrient agar are mucoid, raised, and shiny. While it's a normal inhabitant of the human intestinal tract, it is associated with community-acquired pneumonia and nosocomial urinary tract infections.



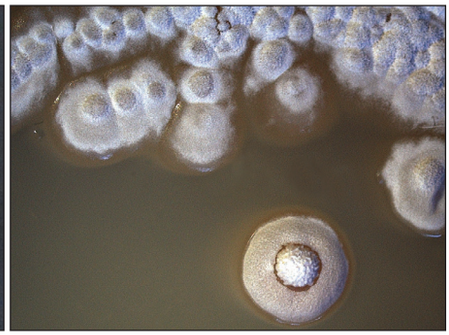
Mucoid Consistency/Shiny Texture: *Pseudomonas aeruginosa* is found in soil and water and it easily infects burn wounds. Clinical samples tend to grow as either mucoid colonies with a shiny texture (as shown here), or as "fried-egg" colonies—large, smooth colonies with flat edges and an elevated center.



Butyrous Consistency/Dull Texture: This unidentified 12 mm colony was found on a glycerol yeast extract plate inoculated with a diluted soil sample. Butyrous (butyrum means "buttery") colonies have the consistency of melted butter. This colony is dull in texture as compared to the shiny yellow colony to its right.



Dry Consistency/Rugose Texture: This is *Streptomyces griseus* grown on brain-heart infusion agar. The circular colony has entire margins. At a later stage of development, this bacterium produces yellow reproductive spores. These bacteria have an "earthy" smell. The plate this colony was on made the whole incubator smell like soil!



Dry Consistency/Granular Texture: This is *Streptomyces violaceus* viewed with the stereo microscope after 3 weeks of incubation at 25°C. The colonies are umbonate, dry, and granular. Members of this genus share a growth pattern similar to common fungi—but, they are Gram-positive bacteria and the similarities are only coincidental.

FIGURE **6.14** **Diversity of Colony Consistency and Texture**

Clinical Applications

Recognizing different bacterial growth morphologies on agar plates is a useful step in species identification; it's often the first indication that one organism is different from another. Once a pure culture has been confirmed by an appropriate staining procedure (this is not always done), cells can be transferred to a sterile medium, grown, and maintained as a pure culture, which then acts as a source of that microbe for identification or other purposes.

In some cases, bacterial colonies have such a distinct appearance on certain media, that visual properties alone can indicate the presence of a pathogen. For example, *Pseudomonas aeruginosa*, a bacterium that commonly causes wound infections and **otitis externa** (outer ear infections such as swimmer's ear), makes a green-blue pigment called **pyocyanin**. This distinct coloration makes it easy to detect *P. aeruginosa* colonies on certain solid media (Fig. 6.15). Some bacteria exhibit unique growth patterns, such as the swarming growth of *Proteus* species. This swarming ability is thought to help *Proteus* species such as *P. mirabilis* and *P. vulgaris* cause urinary tract infections in catheterized patients (Fig. 6.16).

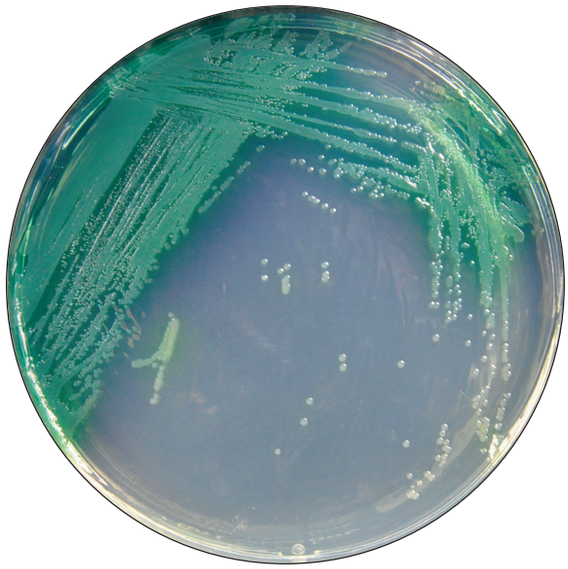


FIGURE **6.15** **Pyocyanin Pigment** • Here *Pseudomonas aeruginosa* is growing on tryptic soy agar. The characteristic blue-green pigment pyocyanin is evident.

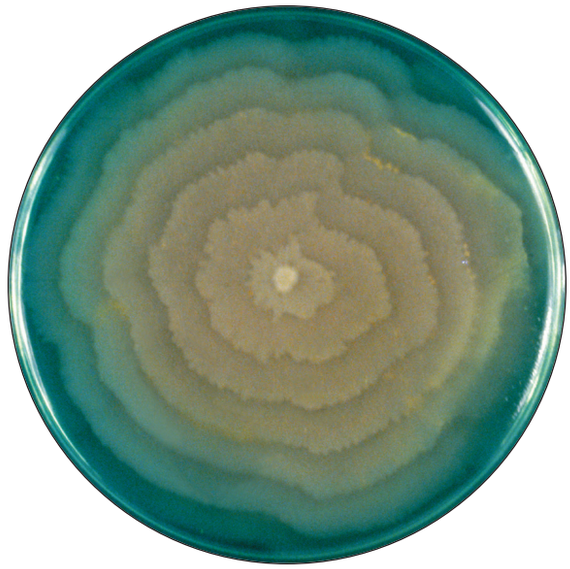


FIGURE **6.16** **Swarming Growth** • Members of the genus *Proteus* will swarm at certain intervals and produce a pattern of concentric rings because of their motility. This photograph demonstrates the swarming behavior of *P. vulgaris* on DNase agar.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Metric ruler
- ❑ Plates inoculated with bacterial samples (e.g., plates from Exercises 4 and/or 5 would work)
- ❑ Colony counter, stereo (dissecting) microscope, and/or hand lens (magnifying glass) (optional)
- ❑ Tryptic soy agar (TSA) or brain-heart infusion agar (BHIA) streak plate cultures of any of the following microbes (optional):
  - ☐ *Bacillus subtilis*
  - ☐ *Corynebacterium xerosis*
  - ☐ *Kocuria rosea*
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Micrococcus luteus*
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available, i.e., ATCC 27203)
  - ☐ *Pseudomonas aeruginosa* (Strain ATCC 39327 is a BSL-1 strain, other strains may be BSL-2)
  - ☐ *Proteus mirabilis* (BSL-2)

PROCEDURE

Today you will view and describe the growth on plates saved from prior exercises (e.g., Exercises 4 and/or 5) and/or prepared streak plates your instructor provides. Before you begin, put on your safety gear (lab coat, safety glasses, and gloves). ***Safety Note:*** Many microorganisms are opportunistic pathogens, so handle every plate carefully. Do not open plates cultured from the environment, plates with known BSL-2 organisms on them, or plates containing "fuzzy" growth. The presence of a fuzzy colony suggests fungal growth containing spores that can spread easily and contaminate the laboratory and other cultures. If in doubt about what you can and cannot open, check with your instructor.

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it is not cluttered.
2. **Describe Observed Colonies:** Use the terms in Figure 6.6 and in the exercise's background information to describe some representative colonies on the plates you're observing (e.g., plates from Exercises 4, 5, and/or plates provided by your instructor). You may also find Figures 6.7 through 6.14 useful. If you see a distinctive feature that has not been given a name, make up one! Just make it descriptive and easily understood by others. That's what the early microbiologists did to compile the list you have been given.
3. **Measure Observed Colonies:** Use a metric ruler to measure colony sizes (mm scale). Try to find punctate, small, medium, and large colonies. Include colony measurements with your colony descriptions.
4. **Record Your Findings:** Record colony features and measurements in the data sheet at the end of this lab.
5. **Clean up:** Unless you are told to save the samples, discard plates into an appropriate biohazard container.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 6

DATA SHEET

Colony Morphology

During the Lab

1 Use the terms provided in the laboratory exercise background and figures to describe representative colonies on the plates you examined. Draw separate sketches for the colonies as seen from above and in elevation (from the side). Use a colony counter for magnification if necessary. Measure colony diameters, and include them with your descriptions along with incubation times and temperatures, and culture sources.

Organism/Plate	Colony Description	Colony Sketch

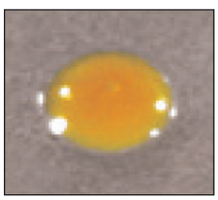
2 Based on your observations, which plates contained pure cultures and which (if any) contained mixed cultures?

Lab Related Questions

1 Which of the following does not describe colony elevation?

a. Rugose
b. Raised
c. Flat
d. Pulvinate
e. Concave

2 Provide terms to describe the pictured bacterial colony.



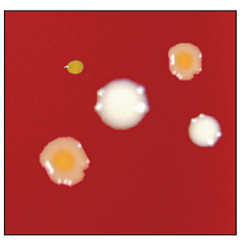
Shape: _____

Margin: _____

Elevation: _____

Texture: _____

3 Based on the following pictured bacteria, how many different species are most likely present? Explain your reasoning.



_____

_____

_____

_____

4 Upon growing bacteria isolated from a burn wound, you observe a greenish-blue bacterial colony on the agar plate. Which of the following is the most likely candidate for the bacterium's identity?

a. *Serratia marcescens*
b. *Pseudomonas aeruginosa*
c. *Streptococcus pyogenes*
d. *Streptomyces griseus*
e. *Kocuria rosea*

5 What feature(s) help *Streptococcus pyogenes* stand out from other bacteria when grown on sheep's blood agar?

_____

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Colony morphology may be affected by incubation temperature.

_____ Bacteria of the same genus may have different colony features.

_____ Colony optical properties are best determined by illuminating a sample with reflected light.

_____ A mixed culture will contain uniform appearing colonies.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 6
DATA SHEET
(continued)

7 *Serratia marcescens* makes a pigment called _____,

which has a _____ coloration.

8 The presence of a fuzzy colony on an agar plate most likely suggests the presence of:

a. A true pathogen.
b. An opportunistic pathogen.
c. *Streptococcus pyogenes*.
d. A bacterial contaminant.
e. A fungus.

Critical Thinking

1 Suppose you observe a bacterial plate that contains numerous colonies and aside from colony size, the bacteria have identical morphological features. Can you safely assume you have a mixed culture? Why or why not?

2 Assume you grew three bacterial samples on brain-heart infusion agar and you obtained the following results:

Plate 1 incubated 37°C for 48 hours: All bright-yellow pigmented colonies.

Plate 2 incubated at 35°C for 24 hours and 37°C for the last 24 hours: All light-yellow pigmented colonies.

Plate 3 incubated at 35°C for 48 hours: All white colonies.

If we assume the cultures were identical and consisted of the same pure culture, then what is the *most likely* explanation for these findings? How could you most easily test your hypothesis?

— Lab Notes —

EXERCISE 7

Growth Patterns on Slants

After doing this exercise, you should be able to:

1. Describe morphological features of bacterial cultures cultivated on agar slants.
2. Discuss the advantages of using agar slants over agar plates.
3. Safely handle bacterial cultures and properly dispose of such cultures.

Background

Agar slants are readily sealed and easily stored in test-tube racks, which means they take up less space than agar plates. These features make slant media a great alternative for cultivating and storing pure stock cultures. That said, slants are not useful for isolating pure cultures, that is best done using plated agar.

Organisms cultivated on slants may display diverse growth characteristics. Many of the organisms you will see in this laboratory exercise have **filiform** growth (dense and opaque with a smooth edge), but you will see differences in other features. Growth on slants can vary in texture (e.g., moist or **friable** [dry, crusty]), optical properties (e.g., **opaque**, **translucent**), and margin (e.g., **smooth**, **lobed**, **spreading/effuse**, **filamentous**, **rhizoid** [branched], or **echinulate** [spiny]). Be advised, margins can be difficult to evaluate—and possibly unobservable—if the slant surface was not correctly streaked with an inoculating loop in a defined zigzag (fishtail inoculation) and/or if the organism covers the entire slant with its edge butting up against the glass.

Figure 7.1 illustrates some of these features. Color is also a variable feature of growth on slants. All the organisms in Figure 7.2 are naturally **pigmented** (an obvious difference) and all but one is filiform (the second from the left is effuse). As with growth on plated media, environmental factors such as incubation time and temperature can influence microbial growth patterns. Figure 7.3 illustrates how temperature affects *Serratia marcescens*' pigmentation.

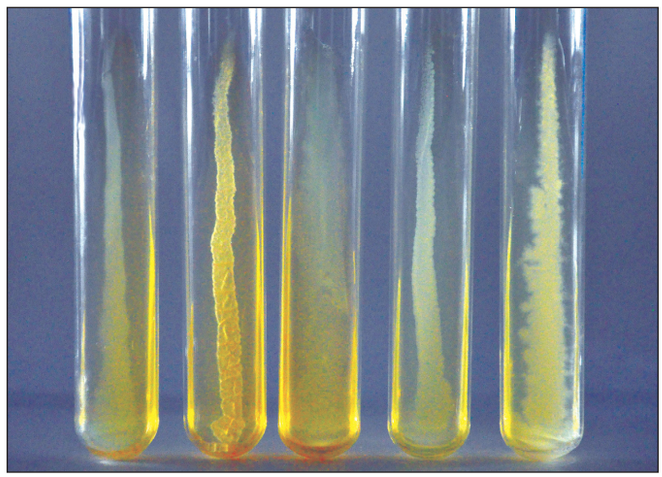


FIGURE **7.1** **Growth Patterns on Slants** • From left to right: filiform (smooth, even growth) and translucent; friable and opaque; effuse and translucent; echinulate and opaque; and rhizoid and opaque.



FIGURE **7.2** **Pigment Production on Slants** • These organisms all naturally produce a pigment. From left to right: *Staphylococcus epidermidis* (white), *Pseudomonas aeruginosa* (green), *Chromobacterium violaceum* (violet), *Serratia marcescens* (red/orange), *Kocuria rosea* (rose), *Micrococcus luteus* (yellow). All but *Pseudomonas*, which is effuse, have an entire (smooth) edge. The waviness you see is due to the inoculation pattern. Bacteria grow where you put them!

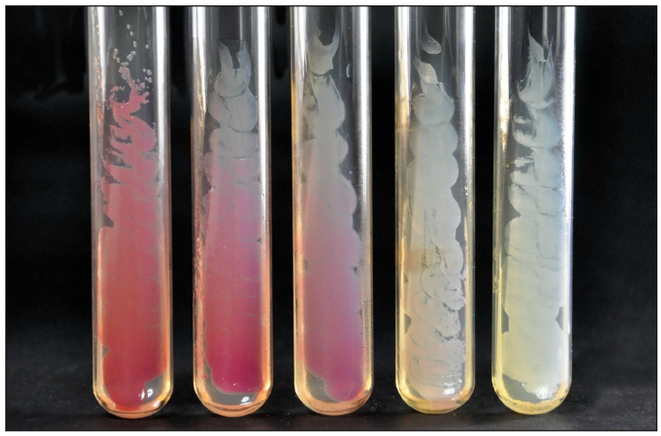


FIGURE **7.3** **Influence of Temperature on Pigment Production in *Serratia marcescens*** ● *Serratia marcescens* was grown for 48 hours on tryptic soy agar slants at five different temperatures. From left to right: 25°C, 30°C, 33°C, 35°C, and 37°C. A difference of 2°C makes the difference between being pigmented or not!

Clinical Applications

Slants are usually inoculated with pure cultures, so a streak plate has already been done and colony morphology has been described ahead of culturing samples on a slant. Still, growth characteristics on slants can provide useful information when attempting to identify an organism. As you'll see in later lab exercises, many biochemical tests that are central to bacterial identification are slant media.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Slant cultures of the following recommended bacteria:
  - ☐ *Bacillus subtilis*
  - ☐ *Corynebacterium xerosis*
  - ☐ *Kocuria rosea*
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Micrococcus luteus*
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available—i.e., ATCC 27203)
  - ☐ *Proteus mirabilis* (BSL-2)
- ❑ Uninoculated tube of the slant medium used for the recommended cultures

PROCEDURE

In Exercise 6, you examined colony growth on agar plates. Today you will be looking at the growth patterns of up to seven organisms on prepared agar slants. If your instructor had you save your plates from Exercise 6, you will compare the growth patterns on the two different types of media. Before you begin, put on your safety gear (lab coat, safety glasses, and gloves).

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it is not cluttered.
2. **Describe All Observed Growth:** Working with your group, examine the slants and describe the different growth patterns on the data sheet at the end of this lab exercise. Include a sketch of a representative portion of each sample. Use the uninoculated tube for comparison if growth is difficult to see on any of the slants.
3. **(Optional) Compare Growth to Prior Plates:** Compare the slant growth to the colonies on the corresponding agar plates from Exercise 6.
4. **Clean up:** Unless you are told to save the samples, discard the slants as directed by your instructor.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 7

DATA SHEET

Growth Patterns on Slants

During the Lab

1 Describe the growth on your slants, including margin, texture, and color. Draw a picture of a representative segment of each observed slant culture and include relevant information about the incubation conditions (e.g., time, temperature, and the medium used).

Organism	Description and Sketch
Control Tube	

2 Based on your observations, discuss similarities between the slant cultures observed in this exercise and the plated cultures you observed in Exercise 6. If you did not perform Exercise 6, look back at the materials and procedure for that lab exercise and hypothesize what similarities you may have observed.

Lab Related Questions

1 Which of the following is (are) false regarding slant cultures? (Select all that apply.)

a. They can be used to maintain a pure culture.
b. They can be used to observe bacterial pigmentation.
c. They can be used to isolate a pure culture.
d. They cannot be used to observe bacterial texture.
e. They are commonly used in biochemical tests.

2 Match the following:

_____ Filiform	a. Produces colored growth
_____ Spreading edge	b. Smooth texture with solid edge
_____ Transparent	c. Solid growth seeming to radiate outward
_____ Friable	d. Easily transmits light
_____ Pigmented	e. Rough texture with crusty appearance

3 The surface of a slant culture is usually inoculated using a:

a. streak isolation pattern.
b. stab inoculation with an inoculating needle.
c. fishtail inoculation with an inoculating loop.
d. drop of broth culture from a pipette.
e. sterilized glass rod.

Critical Thinking

1 Why is a bacterial slant impractical for isolating a pure culture?

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 7
DATA SHEET
(continued)

2 Why are agar slants an ideal way to maintain pure stock cultures?

— Lab Notes —

EXERCISE 8

Growth Patterns in Broth

After doing this exercise, you should be able to:

1. Describe morphological features of bacterial cultures cultivated in broth media.
2. Safely handle bacterial cultures and properly dispose of such cultures.

Background

Microorganisms cultivated in broth display a variety of growth characteristics. Some organisms float on top of the medium and produce a type of surface membrane called a **pellicle**. Others sink to the bottom as **sediment**. Some bacteria produce **uniform fine turbidity,** and others appear to clump in what is called **flocculent** growth. Others show pigmentation or form a ring at the surface (Figs. 8.1 and 8.2). Recall that broth media are typically inoculated with a pure culture and the bacteria are introduced to the media using an inoculating loop.



FIGURE **8.1** **Growth Patterns in Trypticase Soy Broth (TSB)** • **From left to right:** Uniform fine turbidity; Flocculent growth, notice the flakes within the turbidity; Pellicle, a film floating on surface. This pellicle's consistency is illustrated by the portions hanging down into the broth, an artifact of handling during the photo shoot, as is the sediment at the bottom, which fell from the pellicle at the surface; Uninoculated TSB for comparison; Ring at surface, with fine turbidity and a small sediment. Also note the purple coloration of the organism; Sediment.

Clinical Applications

Bacterial genera, and frequently individual species within a genus, demonstrate characteristic growth patterns in broth that provide useful information when attempting to identify an organism. Furthermore, many biochemical tests that are used to definitively identify bacteria are broth culture formats.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile uninoculated brain–heart infusion broth (BHIB) control (1)
- ❑ Broth cultures of the following recommended bacteria:
  - ☐ *Bacillus subtilis*
  - ☐ *Corynebacterium xerosis*
  - ☐ *Kocuria rosea*
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Micrococcus luteus*
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available—i.e., ATCC 27203)
  - ☐ *Proteus mirabilis* (BSL-2)



FIGURE **8.2** **Pigmentation in Broth** • *Rhodospirillum rubrum* has a red color due to carotenoid pigments. It grows as a photoheterotroph in the presence of light and the absence of oxygen.

PROCEDURE

Today you will be examining the growth characteristics of up to seven different bacteria in broth. Before you begin, put on your safety gear (lab coat, safety glasses, and gloves).

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it is not cluttered.
2. **Describe All Observed Growth:** Working with your group, examine the broth samples and describe the different growth patterns on the data sheet at the end of this lab exercise. Include a sketch of a representative portion of each sample. Use the uninoculated tube for comparison.
3. **Clean up:** Unless you are told to save the samples, discard the broth cultures as directed by your instructor.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 8

DATA SHEET

Growth Patterns in Broth

During the Lab

1 Describe the growth of your broth cultures. Draw a picture of a representative segment of each observed culture and include relevant information about the incubation conditions (e.g., time, temperature, and the medium used).

Organism	Description and Sketch
Control Tube	

Lab Related Questions

1 Which of the following is (are) false regarding broth cultures? (Select all that apply.)

a. They can be used to isolate a pure culture.
b. They can be used to observe bacterial pigmentation.
c. They will not allow for colony formation.
d. They cannot be used to observe bacterial texture.
e. They are commonly used in biochemical tests.

2 Match the following:

_____ Flocculent	a. Evenly cloudy throughout
_____ Sediment	b. Growth at top around the edge
_____ Ring	c. Suspended chunks or pieces
_____ Pellicle	d. Growth on the bottom
_____ Uniform fine turbidity	e. Membrane at the top

3 A broth culture is usually inoculated using:

a. A streak isolation pattern.
b. A stab inoculation with an inoculating needle.
c. A fishtail inoculation with an inoculating loop.
d. A sterilized glass rod.
e. An inoculating loop.

Critical Thinking

1 What factors besides physical growth characteristics of the organism are important when recording data about an organism? Why?

2 You have two 5 mL broth cultures that were inoculated at the same time with the same bacterium. Both were incubated for 24 hours at room temperature. One culture is slightly turbid and, at least to the naked eye, the second tube of media appears perfectly clear. You have only 48 hours to determine if any bacteria at all grew in the second tube. Describe two ways that you could determine if bacteria are present using only bacteriological culture tools. (***Note:*** Assume *both* experiments must be completed in their entirety within 48 hours, you do not get 48 hours per experiment.)

EXERCISE 9

Ubiquity of Microorganisms

After doing this exercise, you should be able to:

1. List and describe the types of symbiotic relationships microbes have with humans.
2. Define the terms *source* and *reservoir* and differentiate between the two.
3. Describe colony morphological features for bacteria isolated from an environmental sampling.
4. Sample environmental sources for microbiological life.
5. Safely handle and dispose of samples collected from an environmental source.

CAUTION!

The U.S. Centers for Disease Control and Prevention cautions, "Either nonpathogenic or attenuated bacterial strains should be used when possible, especially in teaching laboratories." Before assigning this exercise, faculty should check institutional policies regarding cultivating unknown bacteria from environmental sources and from human subjects. Anytime such sampling is performed, BSL-2 agents that are not attenuated could be isolated. Faculty are strongly cautioned to review their institution's policies on students handling such samples. This lab exercise should only be performed in laboratories approved to handle BSL-2 agents.

Background

Microorganisms have a long, rich history on Earth and have successfully adapted to a wide range of habitats. Microbes of all sorts can be isolated from soil (even desert sands), water (over a large range of salinities), plants, and animals (including humans). Microbes are even found in seemingly uninhabitable sites such as hot acid pools (Fig. 9.1). Microbes are often described as "ubiquitous in nature," meaning they are everywhere.

Many microorganisms are **free-living**, they do not require a specific **host** to survive. And although bacteria are often discussed in the context of disease, which makes it easy for us to assume the majority are **pathogens**, the truth is most are **nonpathogenic** (do not cause disease). Instead, most bacteria are **saprophytes** (live off dead/decaying matter) and therefore perform the important ecosystem role of breaking down organic matter. Many perform important conversions of inorganic molecules and participate in **biogeochemical cycles**, such as the sulfur or nitrogen cycles.

Other microorganisms reside on or in another species; they have a **symbiotic relationship** with their host(s). The microbes that normally live in and on us are a part of our **normal microbiota**, or **microbiome**. Microbes and humans have evolved a variety of symbiotic relationships, including those that hurt the host (**parasitism**), help the microbe and leave the host unaffected (**commensalism**), or are mutually beneficial to the host and the microbe (**mutualism**). That said, many of the commensal and mutualistic strains inhabiting our bodies may cause disease if introduced to body sites where they do not belong. For example, *Escherichia coli* is a natural inhabitant of the colon; we have a mutualistic relationship with these bacteria. We provide them with a food source and shelter, and they in turn help us digest certain foods and make vitamin K for us. However, when *E. coli* are displaced into an area where they do not belong, they can cause an infection (e.g., *E. coli* entering the urinary tract can cause a urinary tract infection). When an infectious agent that normally lives in or on your body is responsible for an infection, we say the infection was



FIGURE **9.1** ***Sulfolobus* in the Sulfur Caldron, Yellowstone National Park** ● The pictured geologic feature of Yellowstone National Park makes abundant H_2S gas. *Sulfolobus* (domain Archaea) uses the H_2S as an energy source. These microbes oxidize the H_2S to sulfuric acid (H_2SO_4), which lowers the local environmental pH to 2 or less (about the equivalent of battery acid!). This extreme acidity breaks down rock and soil to produce the muddy conditions seen in the photo. The steam you see rising from the caldron is due to its high temperature. *Sulfolobus* species grow between 65°C and 85°C, that's roughly 150°F to 185°F! *Sulfolobus* usually gets its carbon from CO_2, just like plants.

derived from an **endogenous source**. Of course, other people, animals, and our environment are also potential pathogen sources, these are referred to as **exogenous sources.**

Environmental Sampling: A Cautionary Word

Environmental sampling is interesting and clinically useful, but it can be hazardous. Never perform environmental sampling unless your instructor expressly tells you to do so, and even then, only sample environments your instructor has approved.

2

You must apply strict caution whenever you're sampling the environment. After all, microbes in our environment infect us all the time, and that's *before* we purposely grow them, a process that concentrates their numbers and increases the infection risk (facilitates the pathogen reaching an **infectious dose** level). Because of these risks, many instructors only perform such exercises with microbiology majors in upper level courses. Some institutions have entirely forbidden such exercises for nonmajors and severely restrict the environments majors can sample (often body sites, bodily fluids, and facilities that contact such fluids are barred from sampling).

The bottom line is, if you sample the environment, there is a reasonable chance that you'll isolate a BSL-2 pathogen and concentrate it to an infectious dose level. Moreover, that isolated agent is likely to be fully **virulent** (able to exert its full disease-causing potential) as opposed to **attenuated** (weakened) like the strains we typically use in teaching lab exercises. As such, if you do perform such exercises, you must strictly follow *all* BSL-2 safety measures and only perform such exercises in a lab that is specifically designated as a BSL-2 facility (see the Introduction, pp. 2–4, for specifics on BSL-2 safety practices and facilities).

Clinical Applications

In this exercise you'll discover the ubiquitous nature of microorganisms. Even surfaces that appear clean are often covered in microbes. Our environment is an important source of infectious agents. A **source** is anything that spreads an infectious agent from its reservoir to a new host. A **reservoir** is any animate or inanimate habitat where a given pathogen is naturally found.

It's important to note that the terms *source* and *reservoir* are not synonyms. In some cases, the infectious agent's reservoir and the source of the infection are the same, but this is not a safe assumption. For example, humans are both the source and the sole reservoir for the smallpox virus. This fact made smallpox a terrific candidate for eradication. In contrast, *Clostridium botulinum*, the bacterium that cause botulism, naturally exists in soil. However, when people develop botulism, it is because they ingested a contaminated food (the source of the infection) versus directly ingesting soil (the natural reservoir). Discovering a pathogen's source and reservoir often requires environmental sampling.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Nutrient agar (NA) plates (8)
- ❑ Sterile cotton swab (1)
- ❑ Tube of sterile nutrient broth (NB) (1)

PROCEDURE

Today you'll sample your environment to observe what microbes are present and you'll qualitatively assess their relative abundance. Wear a lab coat, gloves, and eye protection when performing this procedure. If you are working in a lab group, then students in the group should share responsibility for inoculating the plates in this exercise.

1. **Situate Your Workspace:** Obtain your required materials and situate your workspace so it's not cluttered. Number the agar plates 1 through 8 (remember to label the base of the plates, not the lids).
2. **Plate 1:** Open Plate 1 and expose it to the air for 30 minutes or longer. Set it aside and out of the way of the other plates.
3. **Plates 2 and 3:** Dip your sterile cotton swab into the tube of sterile nutrient broth. Press the swab against the tube's inner wall to squeeze out excess media from the cotton tip. Then, use the damp swab to sample your desk/bench top; roll the swab on the

tabletop as you sample it. Once you have sampled the surface, streak Plates 2 and 3 in the pattern shown in Figure 9.2. As you streak the agar, gently roll the swab so you can ensure that the contaminated part of the swab contacts the agar. Also, use only light pressure so you don't cut into the agar. Dispose of the swab as your instructor directs.

4. **Plate 4:** With the lid off, hold Plate 4 directly in front of your mouth and cough several times onto the agar surface. Be sure you aren't facing anyone when you cough, instead, cough downward onto the plate to minimize spreading germs to others.
5. **Plate 5:** Rub your hands together, and then touch the agar surface of Plate 5 lightly with your fingertips. A light touch will do; touching too firmly will crack the agar. Your fingers will feel damp afterward, but the agar is sterile and safe to touch.

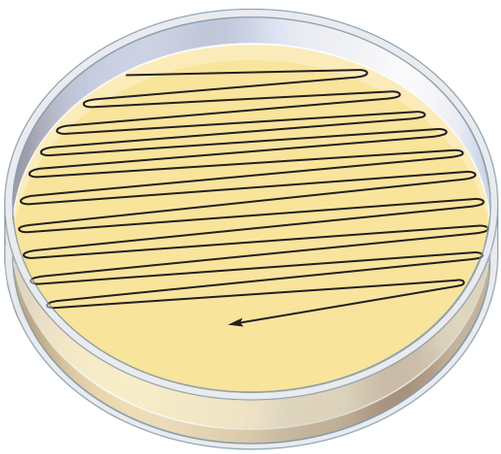


FIGURE **9.2** **Simple Streak Pattern on an Agar Plate** • Roll the swab as you inoculate the plate in a zigzag pattern that covers the entire plate surface. Do not press so hard that you cut the agar.

6. **Plate 6:** Remove the lid of Plate 6 and vigorously scratch your head above it. It is best to bend your head forward so your head/hair is above the plate. When inoculating, do so away from Plate 1 to avoid cross-contamination.
7. **Plates 7 and 8:** Leave Plates 7 and 8 covered; do not open them. These will be controls.
8. **Final steps:** Label the base of each plate with the date, the type of exposure it received, the incubation temperature (see Step 9), and your name.
9. **Incubate the plates:** Invert all plates and incubate them for 24 to 48 hours as follows:

   Plates 1, 2, and 8: incubate at 25°C

   Plates 3, 4, 5, 6, and 7: incubate at 35 ± 2°C

→ Next Lab Session

1. Remove your plates from the incubators and examine them for growth.
2. Record your observations and answer the data sheet questions at the end of this lab.

CAUTION!

Do not remove any of your plates' lids; you have cultivated unknown microbes and they should remain contained. Record your observations on the data sheet at the end of this lab exercise.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 9

DATA SHEET

Ubiquity of Microorganisms

During the Lab

1 Describe the growth of your samples. For each plate, choose two different colonies and draw each as seen from above and from the side. Simple line drawings are acceptable. Record the incubation time, temperature, and source of inoculum for each plate. Also include other useful colony information, such as color and relative abundance.

Organism	Colony Descriptions and Sketches
Control plates (7 and 8)	
Plate 1	
Plate 2	
Plate 3	
Plate 4	
Plate 5	
Plate 6	

Lab Related Questions

1 The best definition of a reservoir is:

a. The place where a pathogen naturally exists.
b. The agent that spreads the pathogen from one host to another.
c. The place where a host becomes infected with a given pathogen.
d. The infectious agent that causes a particular disease.
e. The place a given pathogen reproduces.

2 Assume you discover a prokaryotic pathogen that naturally resides on human skin. This agent is discovered to cause urinary tract infections, but only in immune compromised people. What term(s) appropriately describe(s) this newly identified agent and/or the infection it causes? (Select all that apply.)

a. This agent could be described as a fungal pathogen.
b. This agent would be described as an opportunistic pathogen.
c. This agent would be described as a true pathogen.
d. The described infection can be derived from an endogenous source.
e. The described infection can be derived from an exogenous source.

3 Match the following:

_____ Exogenous source	a. Microbes that naturally reside in and on us.
_____ Endogenous source	b. Microbes that cause disease in a compromised host.
_____ Pathogen	c. An infectious agent contracted from the host's body
_____ Opportunistic pathogen	d. A microbe that causes disease in an otherwise healthy host.
_____ Microbiome	e. A microbe contracted from outside the host's body.

4 A pathogen would be best described as having a _____ relationship with its host.

a. mutualistic
b. commensal
c. parasitic
d. exogenous source
e. opportunistic

5 Assume you isolate a bacterium from a lake water sample. This bacterium is found to live outside a host, but it can be swallowed by ducks as they drink lake water. The bacterium readily thrives in the duck gastrointestinal tract and seems to benefit from the association. Ducks who carry the bacterium are usually protected from a rare fungal disease that affects water fowl. Based strictly on this information, select the applicable statements from below.

a. This bacterium exhibits a mutualistic relationship with ducks.
b. This bacterium exhibits a commensal relationship with ducks.
c. This bacterium is free-living.
d. This bacterium is important in biogeochemical cycles.
e. This bacterium is a pathogen.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 9
DATA SHEET
(continued)

6 Consider the fungus *Candida albicans*. This fungus naturally resides in and on the human body and is known to cause disease (candidiasis) when the host's normal microbiota is disrupted. Referring to the background section of this lab, provide three terms and/or phrases that appropriately describe this agent.

Critical Thinking

1 How would growth on the uninoculated plates affect your interpretation of the other plates?

2 The plates you are using for this lab will be autoclaved to completely sterilize them. The measures taken to disinfect the tabletops (the source of the organisms on Plates 2 and 3) are not as extreme. Why?

— Lab Notes —

EXERCISE 10

Standard Plate Count

After doing this exercise, you should be able to:

1. Perform a standard plate count to determine the cell density of a stock bacterial sample.
2. Discuss the uses and limitations of the standard plate count method.
3. Define the terms *countable*, *TNTC*, and *TFTC* as they apply to the standard plate count method.
4. Aseptically perform serial dilutions.
5. Calculate dilutions and original cell density.
6. Convert from µL to mL and vice versa.

2

Background

Microbiology is the science of extraordinarily small organisms in extraordinarily large numbers, which makes counting them a challenge on two fronts! Fortunately, we don't need to count all the cells in a sample to learn how many of them we most likely have. Instead, we only need to count the cells in a known fraction of the sample and then use some basic calculations to determine how many cells are most likely in the original sample.

The **standard plate count method** (also called the viable plate count method) is a common and inexpensive procedure for determining how many living cells are in a liquid sample. The fact that we are only counting living cells is important because it sets this **direct count** enumeration procedure apart from counting methods that detect the number of cells in a sample, but does not differentiate living from nonliving cells. While this is certainly an advantage of the standard plate count method, there are some drawbacks to the method.

The major disadvantage is in dealing with mixed cultures. There are not universally optimal conditions that equally promote the growth of all species in a mixed sample, so growth conditions are bound to favor one species over another. Consequently, this method won't accurately enumerate all the microorganisms in a mixed culture; some species may not even grow under the prescribed growth conditions. Instead, we select the species we want to enumerate (or group of species with the same growth requirements) and optimize the procedure to enumerate those microbes. Notably, this disadvantage becomes an advantage when we want to enumerate a specific bacterial population, such as bacterial groups associated with fecal contamination of food or water (i.e., enterococci).

Recall in the streak plate isolation method (Ex. 4) we discussed the concept of **colony-forming units**, or **CFUs**. Here we again rely on the concept that a single cell, when sufficiently separated from neighboring cells on an agar plate, will generate one colony. Of course, we can't guarantee that every colony came from exactly one cell, some colonies could develop from two cells stuck together or groups of cells adhering to one another based on the organism's typical cellular arrangement. Therefore, rather than saying a single cell will produce a colony, it's more precise to say that one CFU will produce one colony. That said, microbiologists often use CFU/mL and cells/mL interchangeably but, they do so with a full understanding of the reviewed limitations.

In the standard plate count method, we plate diluted portions of a sample onto agar plates and then count the number of colonies that grow. The number of resulting colonies is then used to calculate a sample's **original cell density** (OCD), which is expressed as CFU/mL.

Performing and Calculating Serial Dilutions

As shown in Figure 10.1, a serial dilution is a sequence of controlled transfers down a line of dilution blanks, tubes containing a known volume of sterile diluent (e.g., water, saline, or buffer). The dilution series begins with a sample of unknown cell concentration (density) and ends with a very dilute mixture containing only a few—or no—cells. Each dilution blank in the series receives a known volume from the previous tube and delivers a known volume to the next, typically reducing (diluting) the cell density by 10- or 100-fold at each step.

As an example, let's assume the original broth culture in Figure 10.1 contains 1,000,000 CFU/mL. Following the first transfer, which is a 100-fold dilution (10 µL of sample added to 990 µL of diluent), Tube 1 would contain 10,000 CFU/mL (calculation: 1,000,000/100 = 10,000). Upon performing another 100-fold dilution into Tube 2, we would have 100 CFU/mL (calculation: 10,000/100 = 100). Therefore, if we were to inoculate 1 mL of the sample in Tube 2, we'd expect about 100 colonies to grow. Because the original sample's cell density is unknown, only the dilutions are recorded on the tubes. By convention, dilutions are expressed in scientific notation. Therefore,

a 10-fold dilution (or a 1/10 dilution) is written as 10^{-1} and a 100-fold dilution (also noted as 1/100 dilution) is written as 10^{-2}.

In Figure 10.1, you can see that the first transfer in the series is a simple dilution while successive transfers are compound dilutions. Both types of dilutions can be calculated using the following formula:

$$V_1D_1 = V_2D_2$$

2 In this formula, V_1 and D_1 are the starting volume and dilution, respectively. In contrast, V_2 and D_2 are the new (or resulting) volume and dilution.

Let's work through how to calculate the dilution in Tube 1 of Figure 10.1. In such a case, D_2 is the dilution we want to calculate (the new dilution), so that is the variable we need to isolate. Here's what the formula looks like when we isolate D_2 on one side of the equation:

$$D_2 = \frac{V_1D_1}{V_2}$$

Next, we plug in our known values. In this case we know V_1, D_1, and V_2:

- V_1 is 10.0 µL (the volume of the original non-diluted sample that we transferred into Tube 1).
- D_1 is the starting dilution (we are starting with a non-diluted sample, so $D_1 = 1.0$).
- V_2 is our final volume (10 µL of sample were to 990 µL of diluent, so Tube 1 contains a final volume of 1,000 µL).

$$\frac{10.0\ \mu L \times 1.0}{1{,}000\ \mu L} = \frac{10}{1{,}000} = 0.01 = 10^{-2}$$

That's nice, but what does 10^{-2} (or 0.01) mean? How much did we ultimately dilute the original sample? Well, if we started with a non-diluted sample at full concentration (1.0) and we now have a dilution of 0.01, then we made a 100-fold dilution. How do we know that?

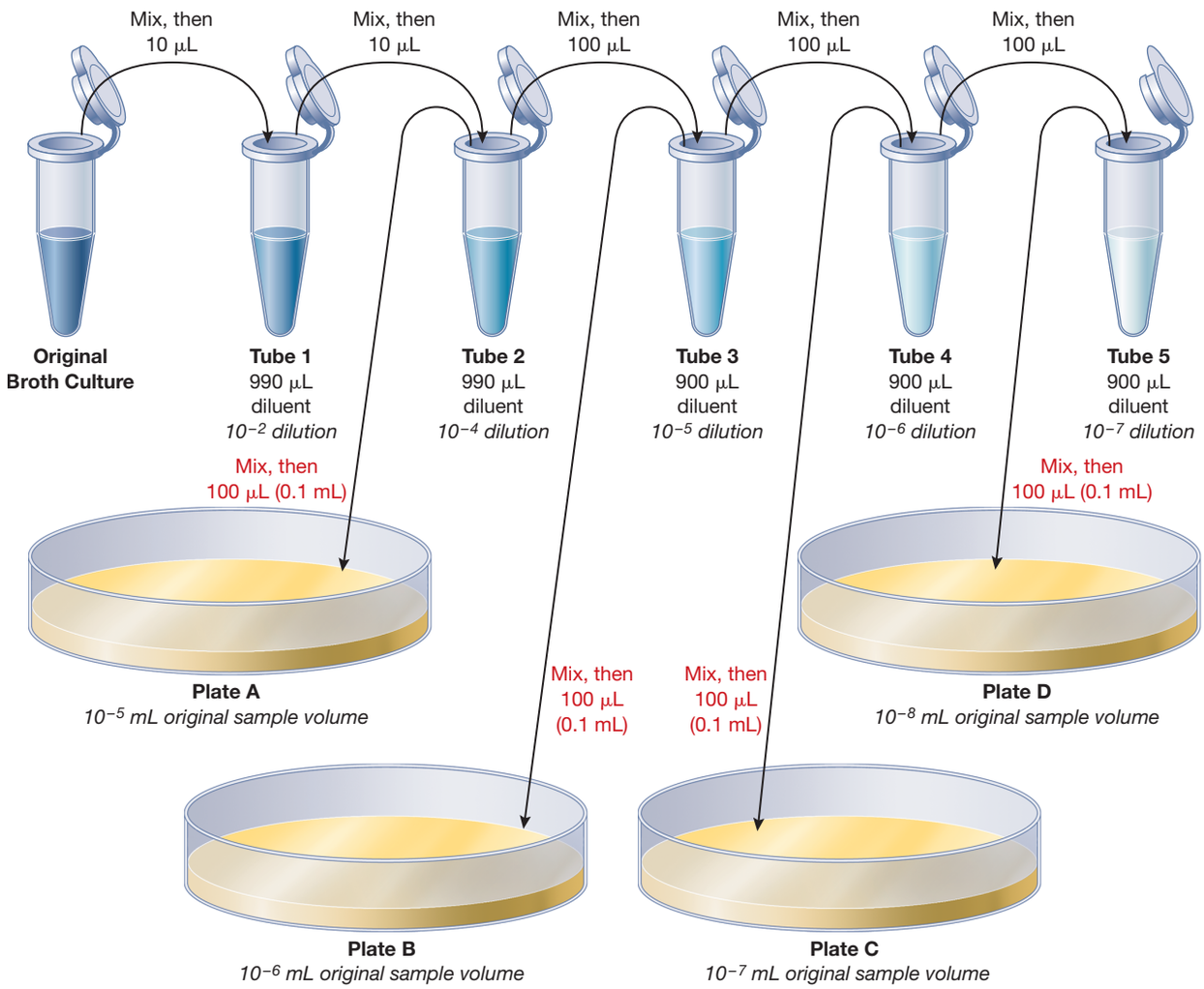


FIGURE **10.1** **Serial Dilution Procedural Diagram** ● This diagram shows the dilution scheme you will follow for the lab procedure. The dilution assigned to each tube (written below the tube) represents the proportion of original sample inside that tube. For example, if the dilution is 10^{-2}, the proportion of original sample inside the tube is 1/100th of the total volume.

$$\text{Fold dilution} = \frac{\text{Starting dilution}}{\text{Final dilution}} = \frac{1.0}{0.01} = 100$$

We can also calculate compound dilutions, such as the dilutions for the samples in Tubes 2 through 5 in Figure 10.1. In the first example we worked through, D_1 had a value of 1.0 to reflect it was not diluted (it was the original sample at full concentration). However, in this new scenario we are starting with a diluted sample, so D_1 is less than 1.0. Earlier we determined that Tube 1 contained a 0.01 or 10^{-2} dilution, this will therefore be our starting dilution, D_1. As an example, let's calculate the dilution in Tube 2 using the volumes reflected in Figure 10.1.

$$D_2 = \frac{V_1 D_1}{V_2} = \frac{10.0\ \mu L \times 0.01}{1{,}000\ \mu L} = 0.0001 = 10^{-4}$$

Counting Plates and Calculating Cell Density

Once all dilutions are prepared, the next step is to inoculate agar plates with a portion of the diluted samples. Not every dilution will be inoculated; the dilutions selected for inoculation and the volume inoculated depends on the original sample's *estimated* cell density. Ultimately, the goal is to generate at least one **countable plate**, a 100 mm agar plate that contains between 30 and 300 colonies (Fig. 10.2). A plate with fewer than 30 colonies is considered statistically unreliable and described as **TFTC** (Too Few to Count). A plate with more than 300 colonies is too dense to reliably obtain separated colonies and is categorized as **TNTC** (Too Numerous to Count).

There are several methods available for counting colonies on agar plates. A cheap yet effective approach is to use a handheld tally counter and a felt-tip marker. As colonies are counted, you simply mark each colony on the plastic base and simultaneously click the counter. This is the method most teaching labs will use, but more sophisticated counting methods do exist. For example, there are electronic pens that, when touched to the plastic petri dish below a colony, will record a tally. There are also software systems that capture an image of the plate and then allow a computer to count the colonies. Once all the plates containing between 30–300 colonies are counted, you can perform some simple calculations to determine the sample's original cell density (OCD), which is expressed as CFU/mL. The basic formula for this calculation is:

$$\text{OCD} = \frac{\text{CFU}}{\text{D} \times \text{V}}$$

In the bottom line of this equation (the denominator), we multiply D × V. This is mathematically equal to the volume of the *original* sample represented on the plate that was counted. So, another way to write this equation is as follows:

$$\text{OCD} = \frac{\text{CFU}}{\text{Original sample volume (mL)}}$$

Next, we plug in our known values. For this example, assume we are using inoculum from Tube 3 in Figure 10.1:

- CFU is the number of colonies on the plate. For this example, let's assume it is 37.
- If we don't know the original sample volume that was inoculated onto the plate, we can calculate it by multiplying D × V.
  - Since Tube 3 is a 10^{-5} dilution, we will plug in 0.00001 for D.
  - V is volume (expressed in mL) of the inoculum added to the agar plate. In Figure 10.1, we used 0.1 mL of sample from Tube 3 to inoculate the agar plate. ***Calculation note:*** In previous calculations where we were determining the dilution, we did not need to convert the volume from µL to mL. However, because OCD is expressed as cells per mL (cells/mL), we must convert all volume measurements to mL. This is done by moving the decimal to the left three place values. Mathematically, this is the same as dividing the µL amount by 1,000. When we do this, the 100 µL inoculated from Tube 3 becomes 0.1 mL.

$$\text{OCD} = \frac{37}{0.00001 \times 0.1\ \text{mL}} = \frac{37}{0.000001\ \text{mL}} = 37{,}000{,}000 = 3.7 \times 10^7\ \frac{\text{CFU}}{\text{mL}}$$

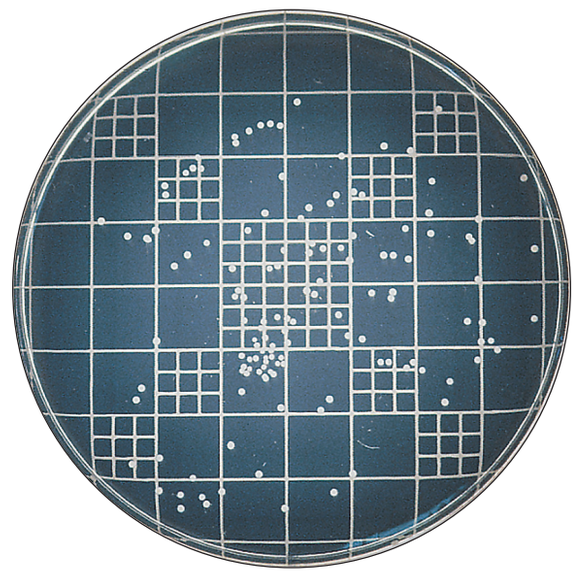


FIGURE **10.2** **Countable Plate** • A countable plate, like the one shown here on a colony counting grid, has between 30 and 300 colonies. Plates with fewer than 30 colonies are TFTC ("too few to count"). Plates with more than 300 colonies are TNTC ("too numerous to count").

Clinical Applications

The standard plate count method is one of the most common methods used to obtain an estimate of living CFU density in samples. This method is especially important in keeping our food, drug, cosmetics, and water supplies safe. For example, when you see a "boil water advisory," it means that the water management company has detected an unsafe level of bacteria in our water and we must boil the water to render it safe to drink. While keeping our water safe to drink may seem like a small matter, it is a huge global concern. The World Health Organization estimates that each year about 3.575 million people die from water-related infections, making this the leading cause of death in the world.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Micropipettes (10–100 µL and 100–1,000 µL) with sterile tips
- ❑ Sterile microtubes (5)
- ❑ 50 mL flask with about 10 mL of sterile water or sterile saline
- ❑ Nutrient agar (NA) plates (8)
- ❑ Jar (and its lid) containing ethanol, cotton in the bottom, and a bent glass rod
- ❑ Hand-tally counter
- ❑ Colony counter (optional)
- ❑ 24-hour broth culture of *Escherichia coli* (contains 2×10^7 to 2×10^{10} CFU/mL)

PROCEDURE

In this exercise, you will perform a dilution series and determine the population density of an *Escherichia coli* broth culture. To accomplish this, you'll inoculate the plates using the spread plate technique, as described in Exercise 5. As illustrated in Figure 5.4, page 82, the inoculum will be evenly dispersed over the agar surface with a bent glass rod. ***Safety Note:*** You will be sterilizing the glass rod between inoculations by immersing it in alcohol and then igniting it to dry off the alcohol. Organize your work area so the flame is kept away from the alcohol jar. If a fire starts in the jar, replace the jar's lid to extinguish the flame.

Refer to the procedural diagram in Figure 10.1 as needed. Appendix E provides an alternative procedure using glass pipettes and mL volumes.

1. **Situate Your Workspace:** Obtain all required materials (to include an *E. coli* sample and a flask of sterile saline or sterile water) and situate your workspace so it's not cluttered.
2. **Prepare Five Dilution Tubes:** Label your five microtubes 1–5. Once you have done this, aseptically add 990 µL sterile water/saline to Dilution Tubes 1 and 2. Close the caps when finished. Aseptically add 900 µL sterile water/saline to Dilution Tubes 3, 4, and 5. Close the caps when finished.
3. **Add Bacteria to Dilution Tube 1:** Carefully, but thoroughly, mix the stock bacterial culture and aseptically transfer 10 µL of the stock to Dilution Tube 1; mix well. This is a 10^{-2} dilution.
4. **Add Bacteria to Dilution Tube 2:** Aseptically transfer 10 µL from Dilution Tube 1 to Dilution Tube 2; mix well. This is a 10^{-4} dilution.
5. **Add Bacteria to Dilution Tube 3:** Aseptically transfer **100 µL** (***Note:*** This is *not* **10 µl** as you used in Step 4!) from Dilution Tube 2 to Dilution Tube 3; mix well. This is a 10^{-5} dilution.
6. **Add Bacteria to Dilution Tube 4:** Aseptically transfer 100 µL from Dilution Tube 3 to Dilution Tube 4; mix well. This is a 10^{-6} dilution.
7. **Add Bacteria to Dilution Tube 5:** Aseptically transfer 100 µL from Dilution Tube 4 to Dilution Tube 5; mix well. This is a 10^{-7} dilution.
8. **Label Eight Plates:** Obtain eight plates, organize them into four pairs, and label them A_1, A_2, B_1, B_2, etc.
9. **Inoculate Your Plates:** After mixing, aseptically transfer 100 µL from Dilution Tube 2 to Plate A_1. Using the spread plate technique (Ex. 5), disperse the inoculum evenly over the entire surface of the agar. Repeat the procedure with Plate A_2, and label both plates "10^{-5} mL original sample," or simply "10^{-5}." Following the same procedure, transfer 100 µL volumes from Dilution Tubes 3, 4, and 5 to Plates B_1 and B_2, C_1 and C_2, and D_1 and D_2, respectively. Label the plates with their appropriate original sample volumes.
10. **Incubate the Plates:** Let the liquid inoculum soak into each plate for a few minutes. Then, invert each plate and incubate them at 35 ± 2°C for 24 to 48 hours.

11 **Dispose of Waste:** Save or dispose of the original *E. coli* stock culture as your instructor directs. Tightly cap the dilution tubes and dispose of them in a biological waste container to be autoclaved.

➜ Next Lab Session

1 **Count the Colonies:** After incubation, examine the plates and determine if any are "countable" (plates with 30 to 300 colonies). Plates with less than 30 colonies are considered "too few to count" (TFTC) while those with more than 300 colonies are regarded as "too numerous to count" (TNTC). Count only the colonies on your countable plates. If available, a colony counter (Fig. 10.3) can be helpful for this.

2 **Calculate Original Cell Density (OCD):** After counting the colonies on each countable plate, average your numbers. You'll use the colony count average to determine your stock sample's OCD. The formula for this calculation was reviewed in the lab's background section and it's presented again in the data sheet.

3 **Dispose of Your Plates:** When finished, dispose of the plates in an appropriate autoclave container.

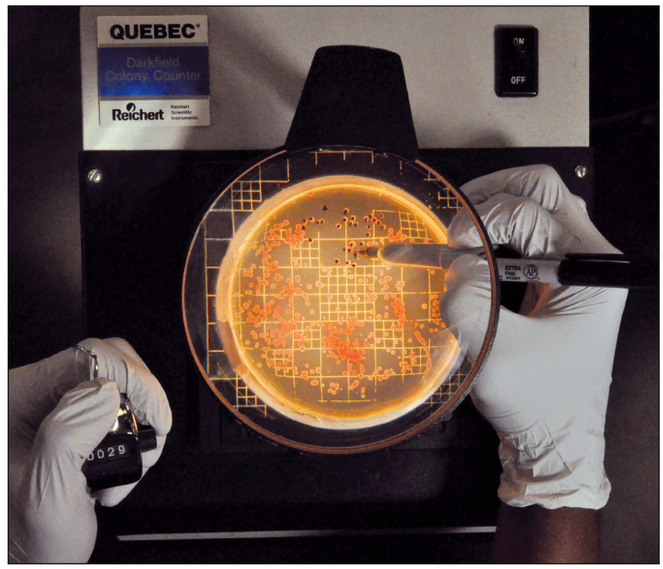


FIGURE **10.3** **Counting Bacterial Colonies** • Place the plate upside down on the colony counter. Turn on the light and adjust the magnifying glass until all the colonies are visible. Using the grid in the background as a guide, count colonies one section at a time. Mark each colony with a felt-tip marker as you record with a hand-tally counter.

— Lab Notes —

2

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 10
DATA SHEET

Standard Plate Count

During the Lab

1 Count the number of colonies for each countable plate. For any plate containing more than 300 colonies, enter TNTC ("too numerous to count"). For plates containing fewer than 30 colonies, enter TFTC ("too few to count"). If no plates are countable, use the TFTC plate closest to 30 colonies for practice. If you must use a TFTC plate to practice the calculation, make a note of that in the table and in Question 2.

Plate	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2
State if the plate was countable, TNTC, or TFTC								
For your countable plates, state how many colonies were counted*								

* If you did not get any countable plates, you may count your TFTC plates and use those numbers to practice all calculations.

2 Calculate the average number of colonies from the countable plates.

Average based on countable plates = _____ colonies

Did you need to use TFTC plates to practice this calculation? (Yes or No) _____

3 Calculate the original density in CFU/mL using the following formula:

$$\text{OCD} = \frac{\text{CFU}}{\text{Original sample volume}}$$

Original density of the *E. coli* stock culture (***Note:*** Express your answer as cells/mL)	

Lab Related Questions

1 The standard plate count is specifically used to _____.

a. enumerate the number of live cells in a sample
b. determine if a culture is pure or mixed
c. enumerate the total number of cells in a sample (living or dead)
d. identify selected pathogens in a sample
e. enumerate all live microbes in a sample

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The standard plate count method is considered an indirect enumeration technique.

_____ The standard plate count method effectively enumerates all live bacteria in a sample.

_____ To perform the standard plate count method, a glass rod is used to spread a sample on an agar plate.

_____ A 100-fold dilution is the same as a 10^{-3} dilution.

_____ In the standard plate count method, a plate with 30 colonies would be considered countable.

3 In the standard plate count method, what do the following abbreviations mean?

OCD: _____

TNTC: _____

TFTC: _____

CFU: _____

4 A sample has a density of 1.37×10^5 CFU/mL. What sample volume(s) should yield a countable plate? (Select all that apply.)

a. 1.0 mL
b. 0.1 mL
c. 0.001 mL
d. 0.2 mL
e. 0.002 mL

5 Which of the following is a limitation of the standard plate count method? (Select all that apply.)

a. This method can only be used on pure cultures.
b. This method will not enumerate all bacteria in a sample.
c. This method cannot differentiate between living or dead cells in a sample.
d. This method can only count bacteria that we can grow in the laboratory setting.
e. This method is expensive and requires highly specialized equipment.

6 Assume you added 1 mL of a non-diluted sample into 999 mL of water. What is the final dilution?

a. This is a 1-fold dilution.
b. This is a 10-fold dilution.
c. This is a 100-fold dilution.
d. This is a 1,000-fold dilution.
e. There is not enough information provided to determine the final dilution.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 10
DATA SHEET

(continued)

Critical Thinking

1 You are testing two different wound dressing materials to determine if one reduces bacterial counts over the other. You obtain the standard plate count data in the provided table. Calculate the OCD for each material and decide if one material decreased bacterial growth as compared to the other. Show your work for each calculation and clearly indicate the calculated final OCD for each material. Assume that 0.001 mL of each sample was inoculated onto each plate.

Dilution	Colony Counts after 12 Hours of Incubation Material A	Colony Counts after 12 Hours of Incubation Material B
10^{-2}	Plate 1: 300	Plate 1: 301
	Plate 2: 214	Plate 2: 315
10^{-3}	Plate 1: 28	Plate 1: 30
	Plate 2: 26	Plate 2: 32

a. OCD of Material A (calculation):

b. OCD of Material B (calculation):

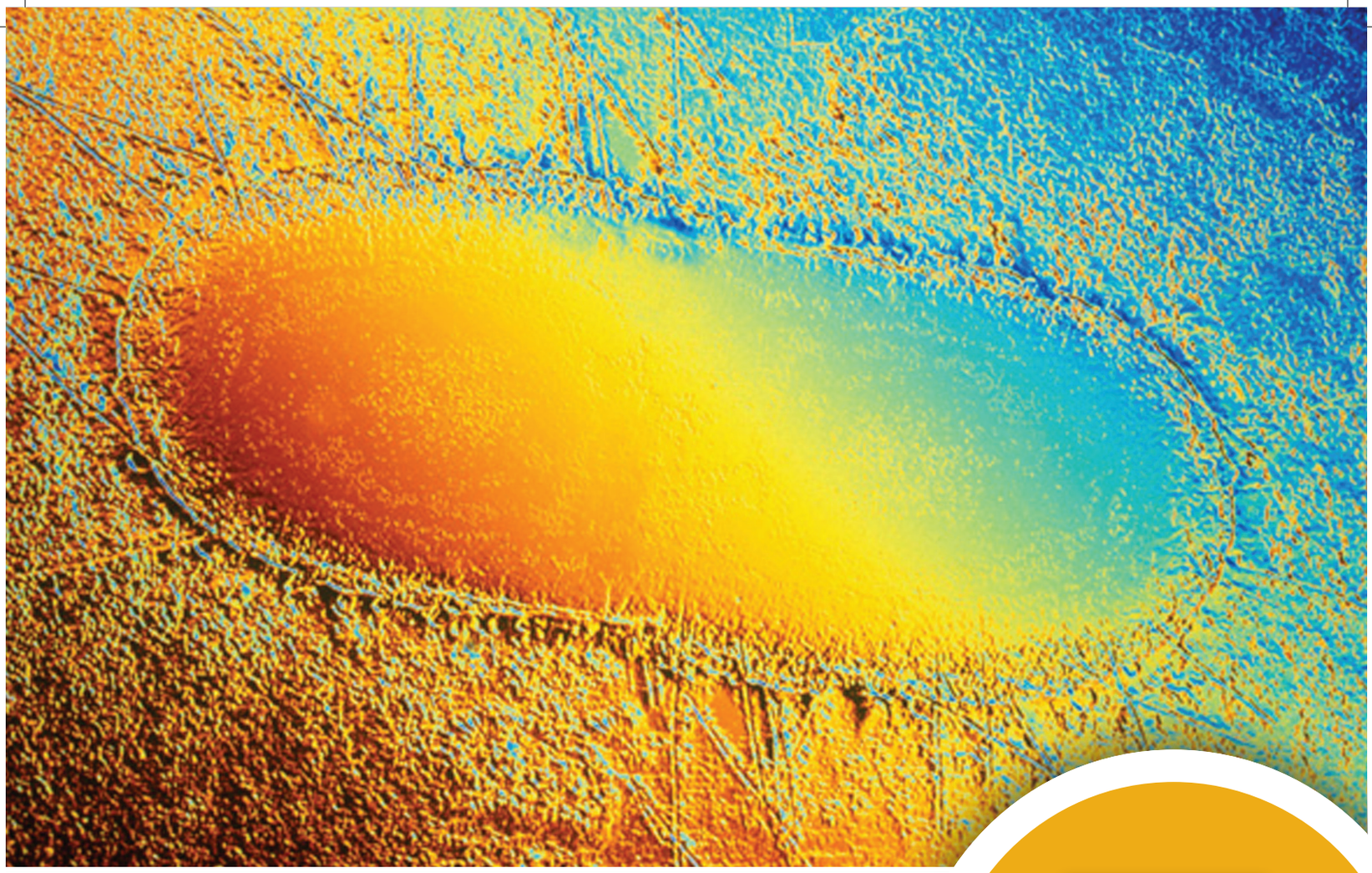
c. Did one material reduce bacterial counts over the other? If so, which one was "best"?

2 An ice-cream manufacturer, Sweet Treat, Inc., was sued because eight people died of a newly identified foodborne infection that was linked back to their Melon-Cream products. Sweet Treat, Inc., had a comprehensive microbiology quality control system in place and all evidence indicated that at the time the patients became ill, the manufacturer was in full compliance with the U.S. Food and Drug Administration's safety standard, which states:

7 CFR 58.648 — Microbiological requirements for ice cream.

"The finished product shall contain not more than 50,000 bacteria per gram as determined by the standard plate count, and shall contain not more than 10 coliform organisms per gram for plain and not more than 20 coliform per gram in chocolate, fruit, nut or other flavors in three out of five samples tested."

Sweet Treat's products were confirmed as the infection source, but the company was absolved of all liability for the infections because they were in full compliance with the FDA's regulations. Based on the case information and the information presented in the lab background about the standard plate count method, why does this ruling (even if it seems socially unfair) make sense?



SECTION

3

Staining Bacteria

In This Section

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— Lab Notes —

INSTRUCTIONAL STAINING ATLAS

These figures represent some typical staining results for a variety of species. As you perform various staining labs in your microbiology course, you may wish to refer to these images for some guidance on interpreting stain results as well as deciphering cellular shapes and arrangements.

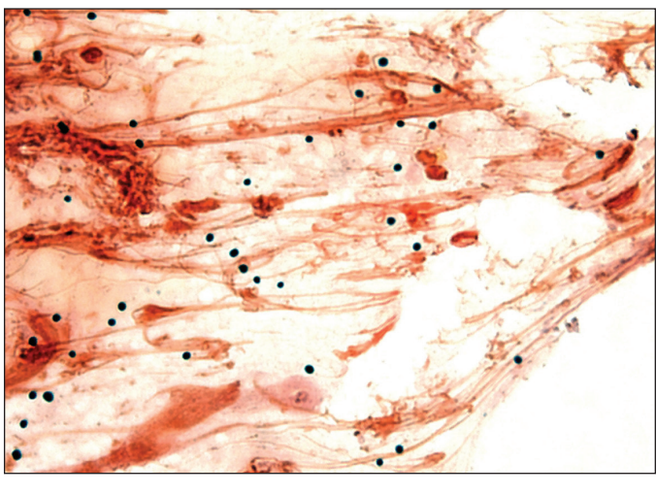


FIGURE IA.1 **Single Cocci from a Nasal Swab (Gram Stain)** • This direct smear of a nasal swab shows cocci (**dark circles**) stained with crystal violet. Each cell is about 1 µm in diameter. The red-orange background material is mostly mucus.

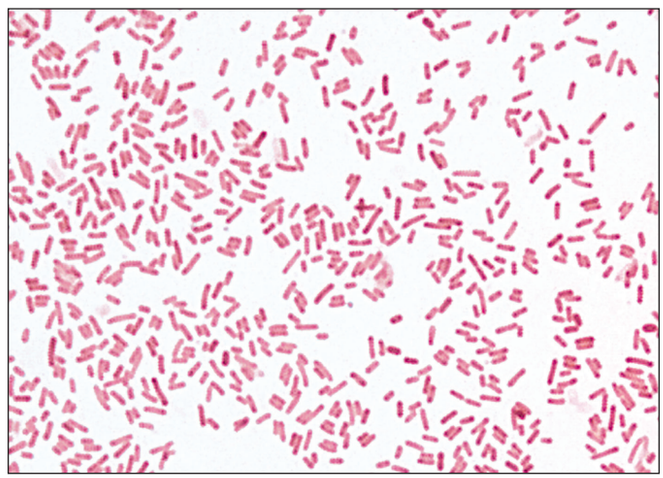


FIGURE IA.2 **Gram Stain of *Escherichia coli*, a Common Species in the Family *Enterobacteriaceae*** • All *Enterobacteriaceae* are Gram-negative, oxidase-negative rods. They also ferment glucose to acid end products.

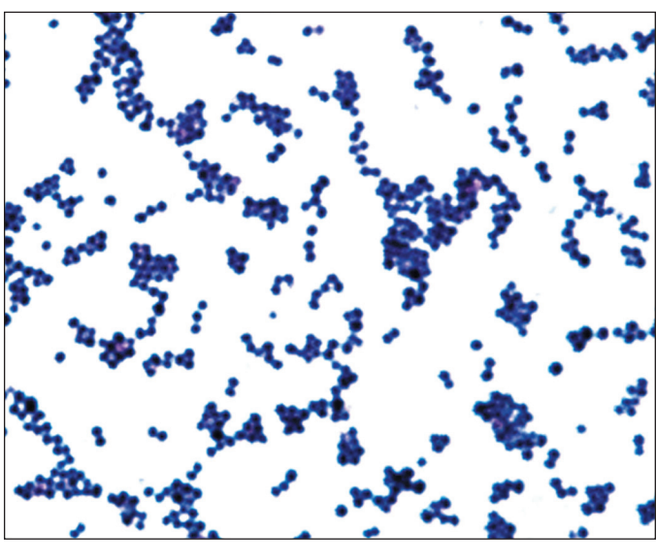


FIGURE IA.3 **Gram Stain of *Staphylococcus aureus*** • This specimen grown in broth illustrates the grapelike cell clusters characteristic of the genus. Specimens grown on solid media may not as clearly show cluster arrangements.

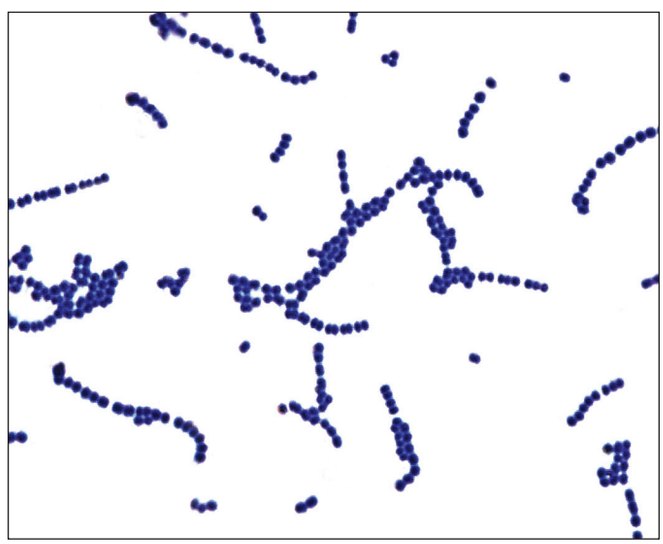


FIGURE IA.4 **Gram Stain of *Streptococcus salivarius*** • This specimen grown in broth illustrates the chainlike (streptococcal) cellular arrangement characteristic of the genus. Specimens grown on solid media may not as clearly show this cellular arrangement.

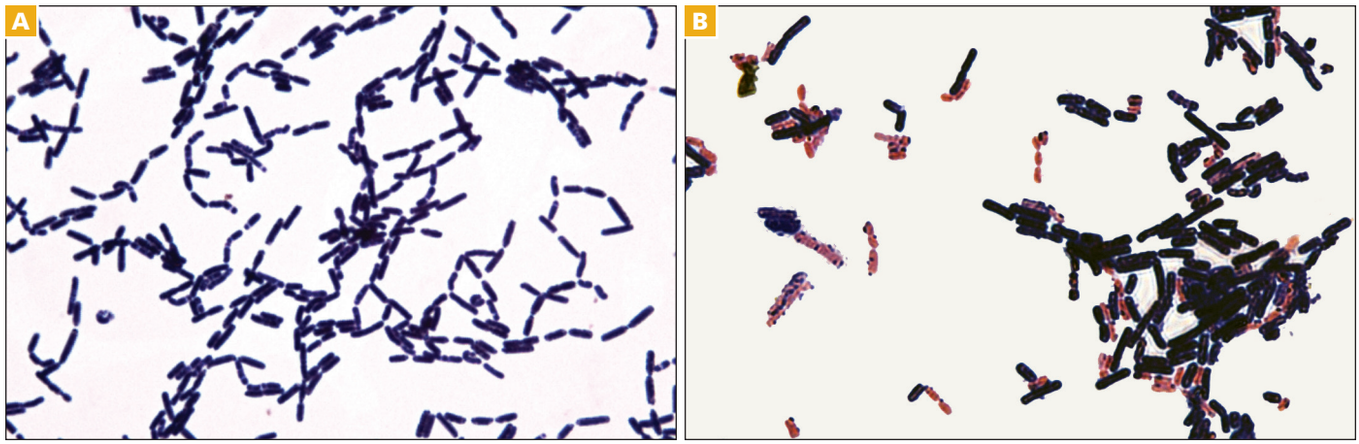


FIGURE IA.5 **Gram Stains of *Bacillus cereus*** • (A) This Gram stain of a young (<24 hours old) *B. cereus* culture stains uniformly Gram-positive. (B) With age, *Bacillus* species may undergo endospore formation and exhibit Gram variability, a situation in which some cells stain purple (Gram positive) and others appear pink (Gram negative). In this case, sporulating cells break down their cell wall and therefore do not retain crystal violet-iodine complexes during Gram staining.

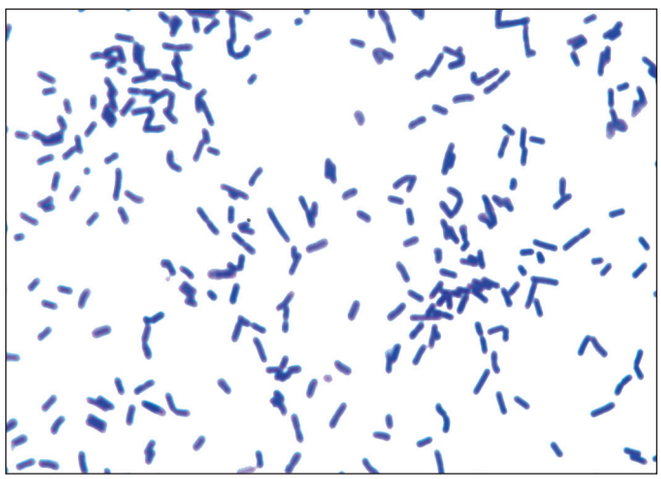


FIGURE IA.6 **Gram Stain of *Lactobacillus* spp.** • Cells in the genus *Lactobacillus* range from long, slender rods to coccobacilli.

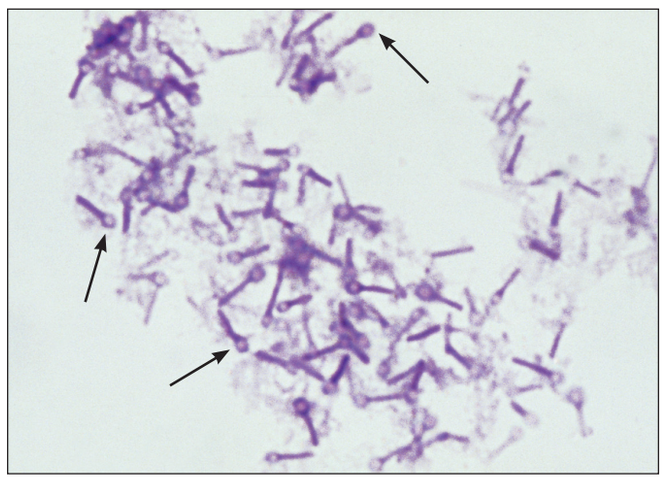


FIGURE IA.7 **Gram Stain of *Clostridium tetani*** • Note the round, terminal endospores (**arrows**) that distend the cells.

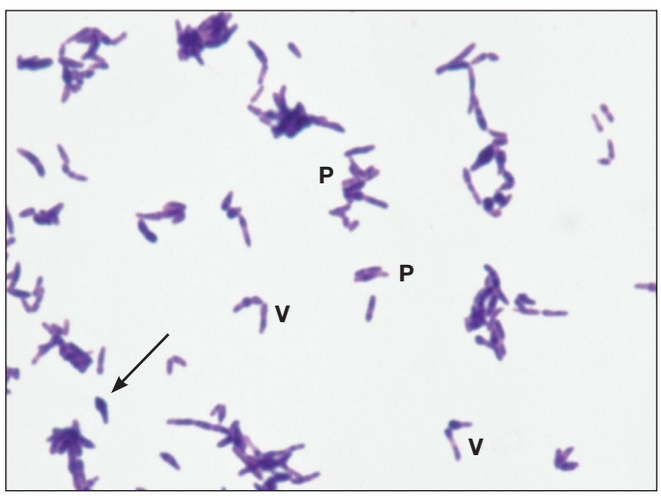


FIGURE IA.8 **Gram Stain of *Corynebacterium diphtheriae*** • Note the club-shaped cells that might be mistaken for spore producers (**arrow**). Cells of this genus often appear as "V-shaped" pairs (**V**) or palisades (**P**).

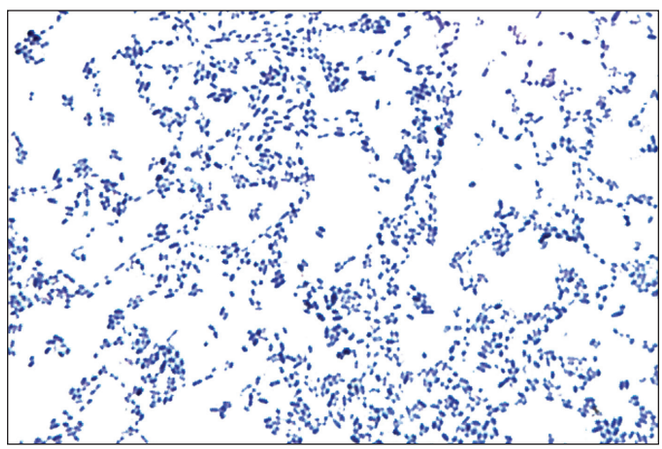


FIGURE IA.9 **Ovoid Cocci (Gram Stain)** • *Lactococcus lactis* is an elongated coccus that a beginning microbiologist might confuse with a rod. Notice the slight elongation of the cells, and that most cells are not more than twice as long as they are wide. *L. lactis* is found naturally in raw milk and milk products, but these cells were grown in culture. Cell dimensions are about 1–2 µm long by 1 µm wide.

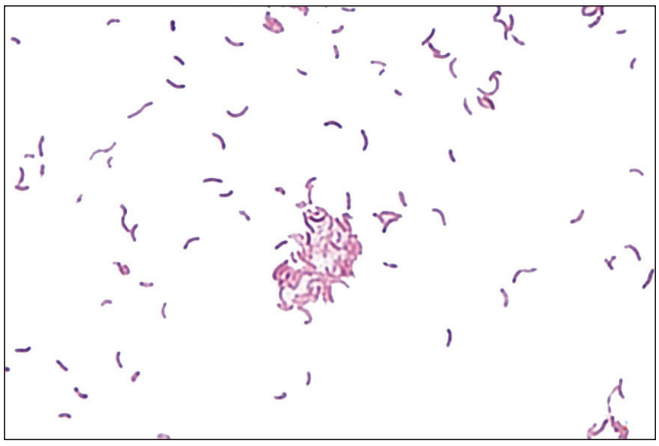


FIGURE **IA.10** **Vibrio (Gram Stain)** • *Vibrio cholerae* is the causative agent of cholera. These cells often look like curved rods, hence the generic name, *Vibrio*. They range in size from 1–3 µm long by less than 1 µm in diameter.

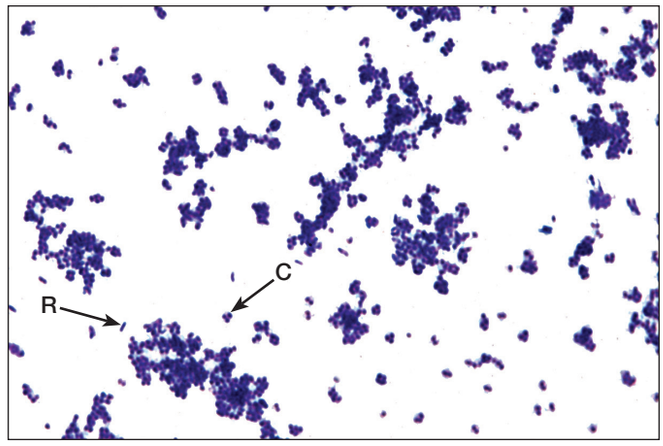


FIGURE **IA.11** **Bacterial Pleomorphism (Gram Stain)** • Some bacterial species grow in a variety of shapes and are therefore described as pleomorphic (pleo = more than one; morphic = form). Here the rods of *Corynebacterium xerosis* range from plump cocci (**C**) to short, slender rods (**R**). *C. xerosis* is a normal inhabitant of skin and mucous membranes and is generally not considered a pathogen.

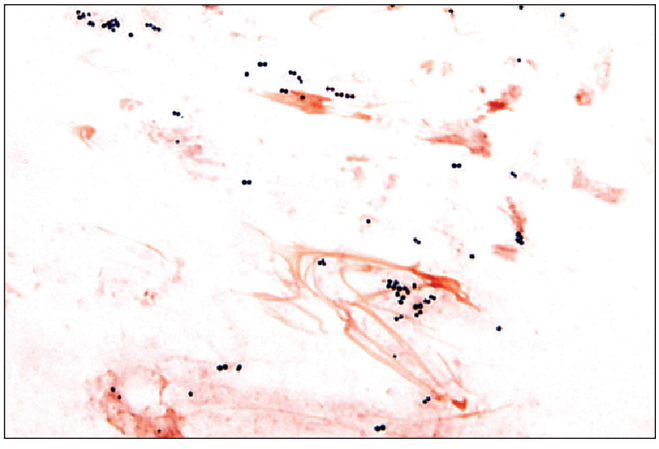


FIGURE **IA.12** **Diplococcus Arrangement (Gram Stain)** • Most cells are in pairs, but a few are singles because they have yet to divide. Expect to see a mixture of cell arrangements representing stages before the "final" one.

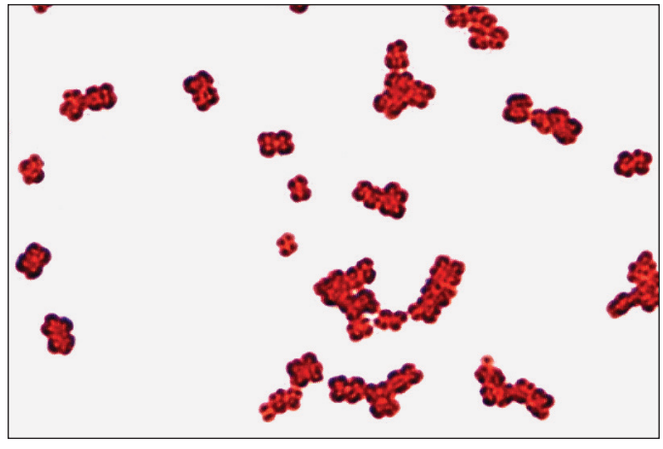


FIGURE **IA.13** **Tetrad Arrangement (Gram Stain)** • *Neisseria sicca* frequently grows in tetrads, as shown in this Gram stain of a trypticase soy agar culture. Cell arrangement (especially for cocci) is usually easier to determine from a specimen grown in a broth medium. This is because groups of cells separate from one another in liquid whereas they may artificially clump together when the bacteria are grown on a solid medium. That said, tetrads are usually easy to spot even when the bacteria are grown on a solid medium. Look at the bigger clumps and you will see tetrads within them. *N. sicca* is a normal inhabitant of the human nasopharynx, saliva, and sputum, and is an opportunistic pathogen. Cells are between 1 and 2 µm in diameter.

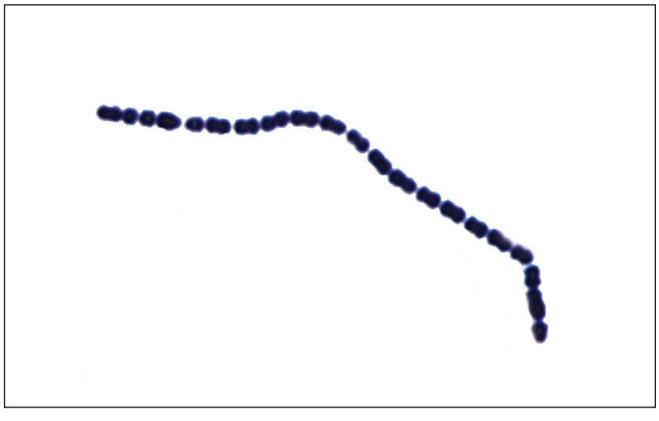


FIGURE **IA.14** **Streptococcus Arrangement (Gram Stain)** • As its name suggests, *Streptococcus salivarius* forms long chains of spherical cells and inhabits the oral cavity (abundant in saliva and in the tongue). This specimen is from a broth culture. Individual cells are approximately 1 µm in diameter.

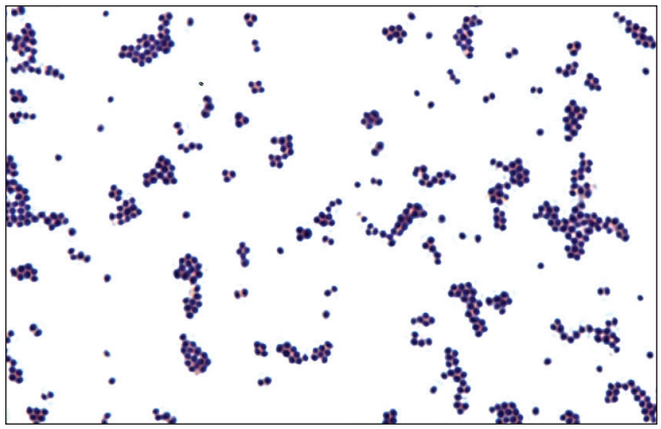


FIGURE **IA.15** **Staphylococcus Arrangement (Gram Stain)** • *Staphylococcus aureus* cells grown in broth culture are shown. Note that the staphylococcal groups are rarely bigger than a dozen cells or so. Larger clusters are generally two or more adjacent groups. This "bunching" can be especially problematic when making a preparation of cells grown on a solid medium. Be sure to emulsify them thoroughly to spread clusters out as much as possible. *S. aureus* is a common opportunistic pathogen of humans. Cells are approximately 1 µm in diameter.

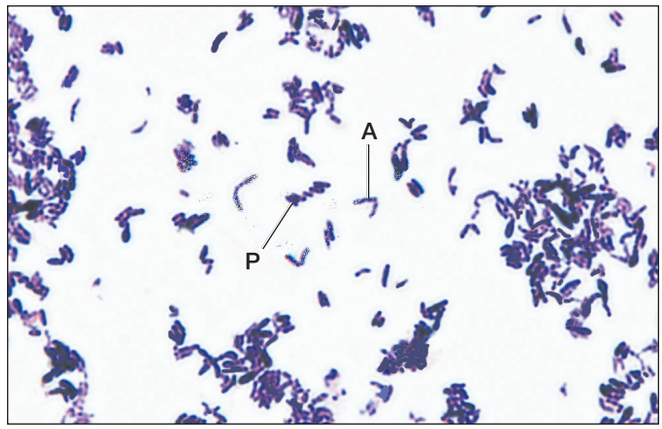


FIGURE **IA.16** **Palisade and Angular Arrangements of *Arthrobacter* (Gram Stain)** • Stacking of rod-shaped cells side-by-side is the palisade arrangement (**P**). An angular arrangement (**A**) is when a pair of rods is bent where the cells join. Both arrangements are due to "snapping division" in which the cells divide lengthwise and remain more or less attached. *Corynebacterium* also shows these arrangements.

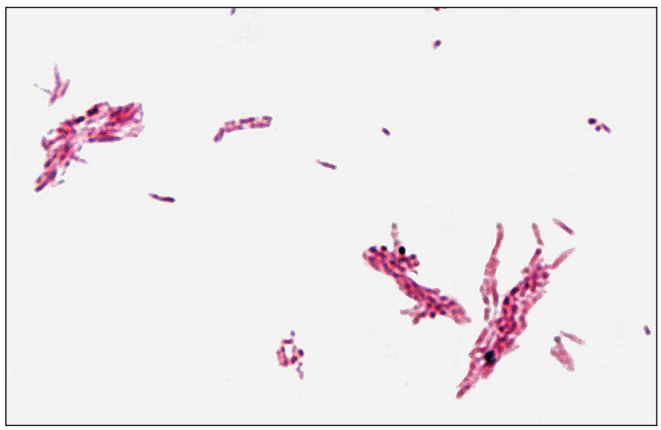


FIGURE **IA.17** **Acid-Fast Stain of *Mycobacterium nonchromogenicum*** • *Mycobacterium* species are typically composed of slender rods with a "beaded" appearance (note the dark spots). Because of their waxy walls, they are difficult to emulsify and tend to clump.

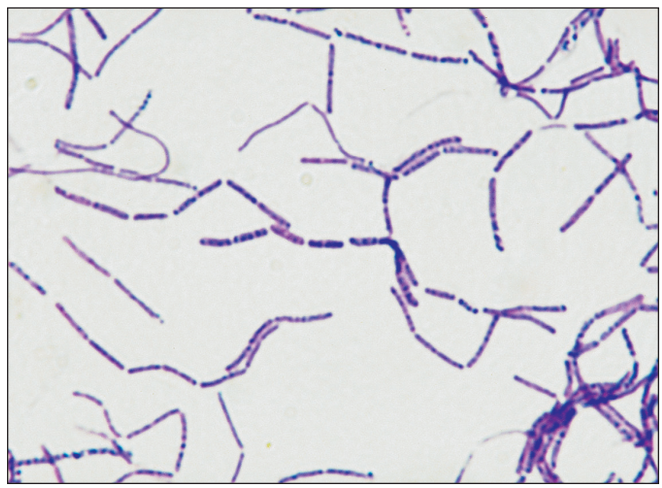


FIGURE **IA.18** **Streptobacillus Arrangement (Crystal Violet Stain)** • *Bacillus megaterium* is a streptobacillus. These cells are 2–5 µm long by 1.2–1.5 µm wide.

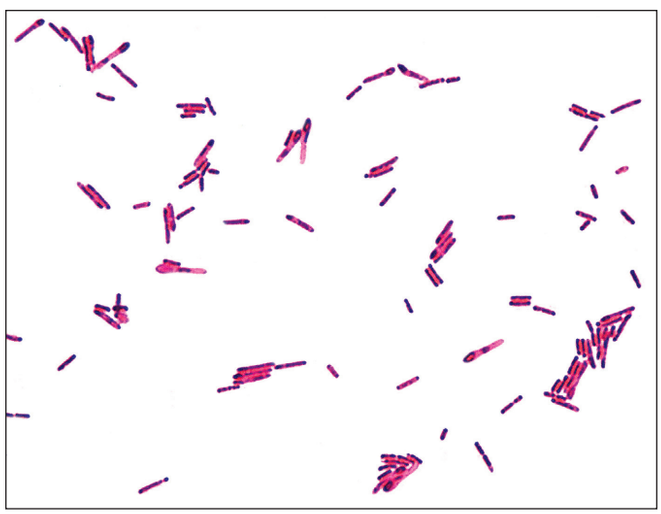


FIGURE **IA.19** **Single Bacilli (Carbolfuchsin Stain)** • *Clostridium butyricum* tend to appear as single bacilli. It is the type species for the genus (meaning it was the first described *Clostridium* species back in 1880!). It is found in soil and aquatic environments, as well as in human feces and many other habitats. It produces endospores, which are visible in this image as bulges toward the ends of some cells. The cells range in size from 0.2–1.7 µm in diameter by 2.5–7 µm long.

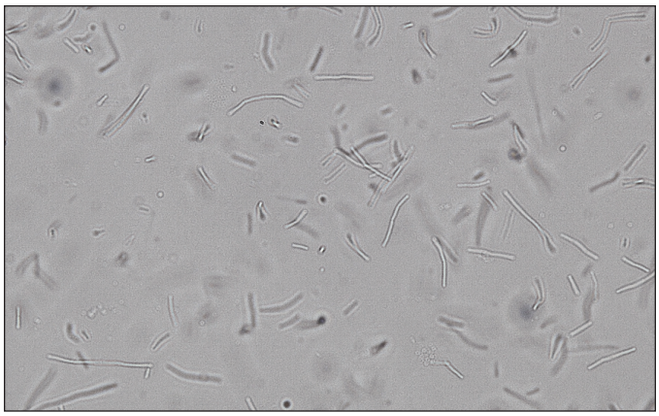


FIGURE **IA.20** **Long, Thin Bacillus (Phase Contrast Wet Mount)** ● This *Bacillus subtilis* culture was grown in trypticase soy broth for 24 hours and is shown in a wet mount using high-dry (4× lens) for magnification. Cell morphologies can vary depending on the medium used (solid vs. liquid) and culture age. The longer "individuals" you see here are actually short chains of two or three cells, as evidenced by transverse constrictions or bends where the cells are attached. The water layer of this wet mount is considerably thicker than the cells, therefore cells above or below the focal plane appear out of focus.

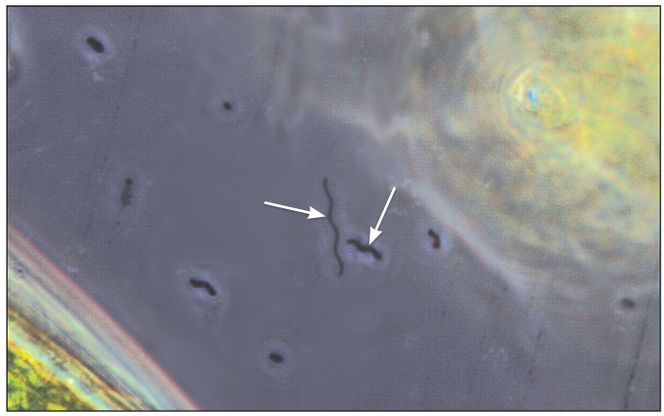


FIGURE **IA.21** **Two Different Spirilla (Phase Contrast Wet Mount)** ● Based on their different cell morphologies, these spirilla (**arrows**) are undoubtedly different species. One is long and slender with loose spirals; the other is shorter and fatter with tighter coils. Cell dimensions are less than 1 µm wide and up to 35 µm long.

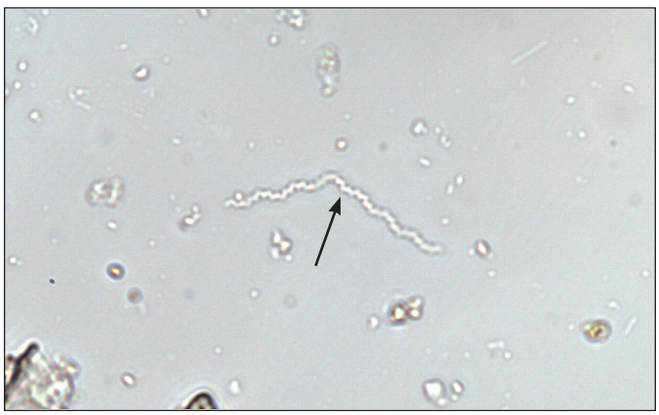


FIGURE **IA.22** **A Spirochete (Bright-Field Wet Mount)** ● Spirochetes (or spirochaetes) are tightly coiled, flexible rods. This bright-field micrograph shows a marine species of *Spirochaeta* (**arrow**). Notice the bend in the center of the cell. Cell dimensions of the genus are 5–500 µm long by less than 1 µm wide.

— Lab Notes —

EXERCISE 11

Simple Stains and Bacterial Cell Morphology

After doing this exercise, you should be able to:

1. Perform a simple stain of bacterial smears.
2. Describe and identify bacterial shapes and arrangements.
3. List the features of basic versus acidic dyes.
4. Discuss the role of fixation in staining procedures.
5. Perform oil-immersion microscopy to view your own stained bacterial samples.

Background

It can be difficult to see a clear microscopic specimen on a glass slide, so we use stains to add contrast to specimens. While microbes of all types can be stained, here we focus on how we can stain bacteria. There are a variety of bacterial staining procedures available. Some are classified as **structural stains**, which highlight specific structural features of a cell (e.g., the presence of endospores, a flagellum, or a capsule). Others serve as **differential stains** and allow us to separate one category of cell from another (e.g., differentiate between Gram-positive and Gram-negative cells). Then we have **simple stains**, which are the fastest and easiest way to add contrast to a specimen so it is easier to observe under the microscope. Simple stains can only reveal elementary morphological information, such as the bacterium's shape, arrangement, and size (Fig. 11.1).

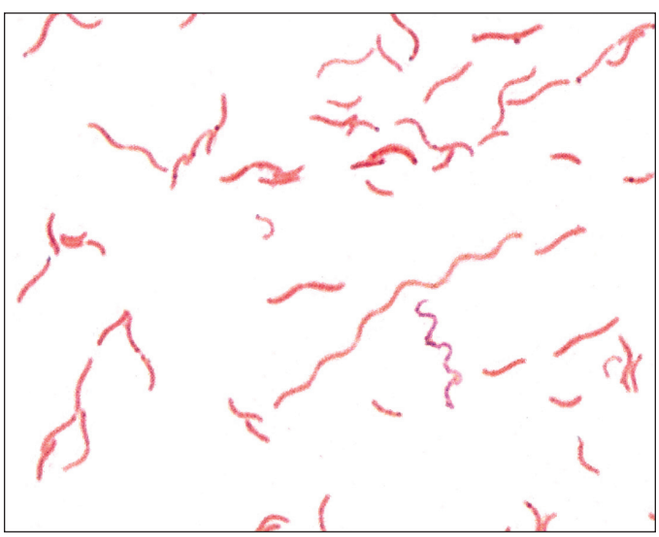


FIGURE **11.1** **Simple Stained Bacterium** • This is a simple stain of the spiral-shaped bacterium *Rhodospirillum rubrum*. Notice that the stain is associated with the cells as opposed to staining the background. The organism was grown in a broth culture, stained using the basic stain safranin, and viewed with bright-field microscopy. This photograph was taken at 1,000× (oil immersion).

Bacterial Morphology: Shapes and Arrangements

Bacteria come in diverse shapes, arrangements, and sizes. Determining cell shape is an important first step in identifying a bacterial species. Cells may be spheres (**cocci**, singular **coccus**), rods (**bacilli**, singular **bacillus**), or spirals (**spirilla**, singular **spirillum**). Variations of these shapes include slightly curved rods (**vibrios**), short rods (**coccobacilli**), and flexible spirals (**spirochetes**). You can refer to Figure 11.2 and the Instructional Staining Atlas (pp. 131–135) for an overview of bacterial shapes. Although we do not fully understand why certain bacteria have a particular shape, we do know shape is a genetic trait.

In addition to describing bacterial shapes, we also note bacterial **cell arrangements** (the higher order arrangement of cells with respect to one another). Some species do not form higher order arrangements, in which case we describe them as **single**. Spirilla are almost always single cells, but cocci and bacilli often form multicellular associations.

If two daughter cells remain attached after a coccus divides, a **diplococcus** is formed. The same process happens in bacilli that produce **diplobacilli**. If the cells continue to divide in the same plane and remain attached, they form a chain and exhibit a **streptococcus** or **streptobacillus** arrangement. Cocci and bacilli can also divide to form clusters of cells. Cocci can divide to make **tetrads** (four cells clumped together), **sarcina** (groups of eight cells in a cubelike configuration), and **staph** arrangements (irregular grapelike clusters). In contrast, when bacillus-shaped bacteria form clusters we describe them as having a **palisade** arrangement. Figure 11.2 shows the main arrangements that bacterial cells assume.

When examining a specimen that forms higher-order cellular arrangements, it's common to see various stages of these arrangements. For example, if you are viewing a smear of staphylococci, you'll see single cells that broke off a larger cluster and have not yet divided into a staph arrangement; some cells that have divided into pairs; groups of four cells (tetrads); and staph arrangements, the final arrangement format. Be patient and look for the

most common morphology and the most complex arrangement to determine the organism's most likely true arrangement. If you have difficulty identifying cell shapes and/or arrangement, consider transferring the organism to a broth culture, incubating it (even for an hour or so), and trying again. Sometimes bacterial arrangements are easier to determine when the organisms are grown in a broth rather than on a solid medium.

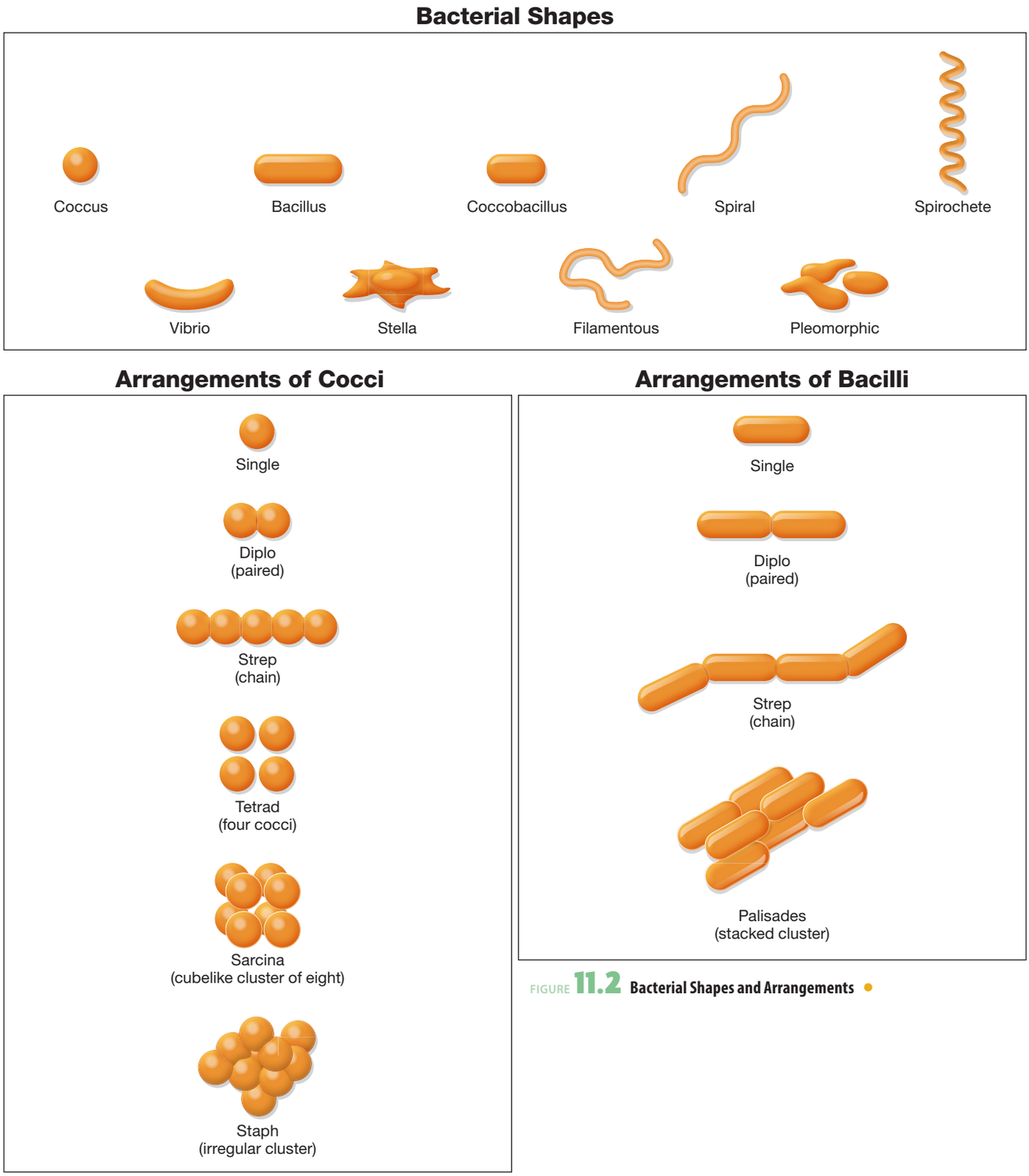


FIGURE **11.2** **Bacterial Shapes and Arrangements**

Basic Versus Acidic Dyes

No matter what type of staining procedure is performed, a colored solution (stain) is added to the specimen. A staining solution contains a dye (or **chromagen**) dissolved in a solvent (usually water or ethanol). Simple stains are considered simple because they employ only one dye. The dye's chemical nature, especially its charge, affects how it interacts with cells. The adage "opposites attract" rings true in staining, namely a substance with a positive charge is attracted to a substance with a negative charge. Many common dyes such as methylene blue, crystal violet, and safranin are alkaline (are **basic**, or have a pH above 7) and have a positive charge. These positively charged dyes are readily attracted to and bound inside bacterial cells, which tend to have an overall negative charge (Fig. 11.3). In contrast, **acidic dyes** carry a negative charge and are repealed by bacterial cells, so rather than entering cells, such dyes instead stain the background. (We discuss acidic dyes more in the next lab, which covers negative staining.)

Fixation

Most bacterial staining techniques begin by smearing a small amount of a sample onto a glass slide and then exposing the smear to heat (heat fixing or **heat fixation**) (Fig. 11.4). In some procedures, a chemical reagent may be used as a fixative (**chemical fixation**). Fixation, whether chemical or heat based, adheres the sample to the slide, so it is not as easily washed away during the staining process, and it kills most of the cells in the specimen, making it safer to handle. In simple staining, we typically use heat fixation, which is easily performed by passing the slide through the flame of a Bunsen burner or holding the slide near the opening of a benchtop incinerator (Fig. 11.5).

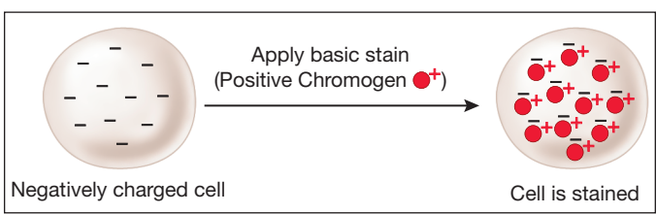
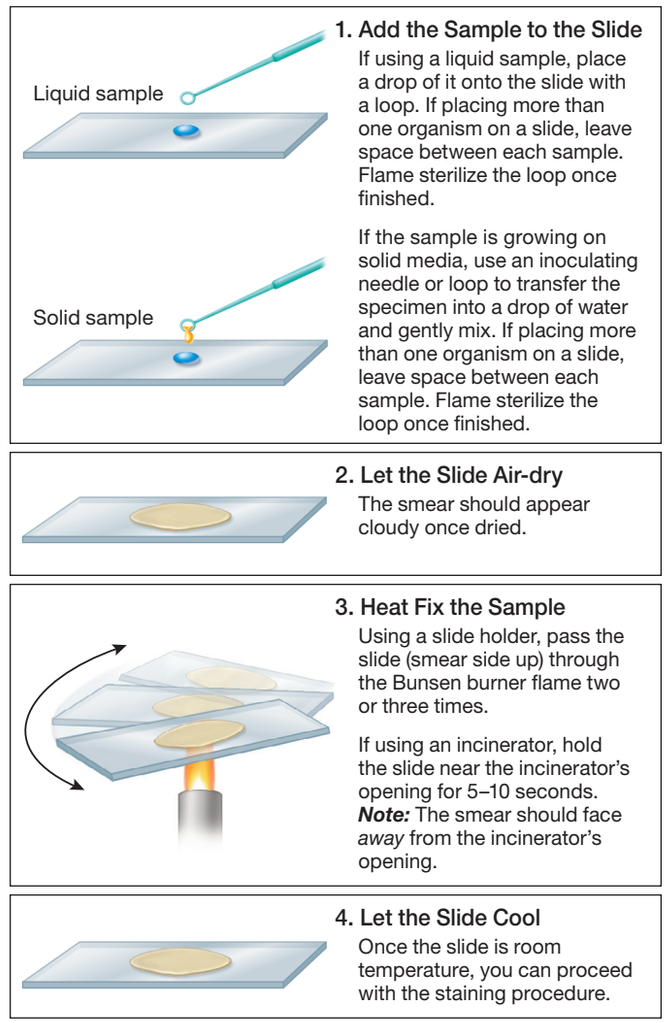


FIGURE **11.3** **Chemistry of Basic Stains** • Basic stains have a positively charged chromogen, which readily associates with the negatively charged bacterial cell to colorize it.



1. Add the Sample to the Slide

If using a liquid sample, place a drop of it onto the slide with a loop. If placing more than one organism on a slide, leave space between each sample. Flame sterilize the loop once finished.

If the sample is growing on solid media, use an inoculating needle or loop to transfer the specimen into a drop of water and gently mix. If placing more than one organism on a slide, leave space between each sample. Flame sterilize the loop once finished.

2. Let the Slide Air-dry

The smear should appear cloudy once dried.

3. Heat Fix the Sample

Using a slide holder, pass the slide (smear side up) through the Bunsen burner flame two or three times.

If using an incinerator, hold the slide near the incinerator's opening for 5–10 seconds. ***Note:*** The smear should face *away* from the incinerator's opening.

4. Let the Slide Cool

Once the slide is room temperature, you can proceed with the staining procedure.

FIGURE **11.4** **Preparing a Bacterial Smear** • Most staining procedures start with preparing and heat fixing a bacterial smear. Preparing uniform bacterial smears will make it easier to obtain consistent staining results.

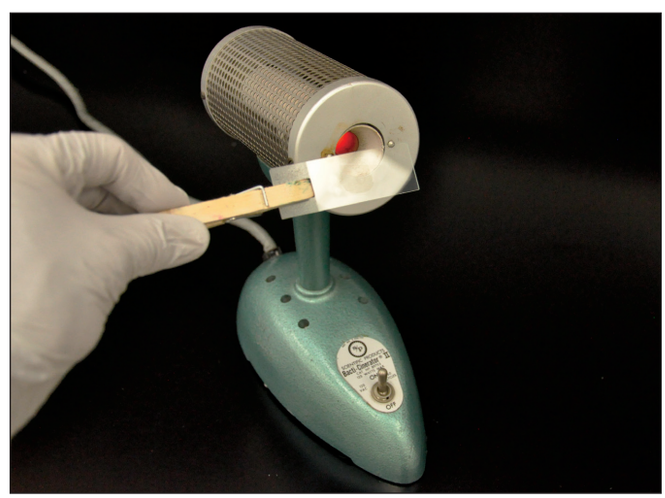


FIGURE **11.5** **Heat Fixation Using an Incinerator** • To heat fix a slide using an incinerator, hold the slide near the microincinerator's opening (with the smear facing away from the opening) for 5–10 seconds.

Clinical Applications

Bacterial cells are tiny and often clear, which makes them difficult to see on a slide. Therefore, cells are typically stained with a colored dye to make them more visible under the microscope. Stained samples are then observed so that cell morphology, size, and arrangement can be determined. In a medical laboratory, noting morphological features is helpful for bacterial identification and bacterial identity in turn guides treatment options.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Clean glass microscope slides
- ❑ Basic dyes (at least one of the following):
  - ☐ Methylene blue
  - ☐ Safranin
  - ☐ Crystal violet
- ❑ Squirt bottle with water
- ❑ Staining tray or a glass beaker to collect dye rinsed off slides
- ❑ Bibulous paper (or paper towels)
- ❑ Slide holder
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Fresh broth or slant cultures of these recommended organisms:
  - ☐ *Bacillus cereus* or any *Bacillus* species
  - ☐ *Micrococcus luteus*
  - ☐ *Neisseria sicca* or *Moraxella catarrhalis*
  - ☐ *Rhodospirillum rubrum* or *Aquaspirillum* species
  - ☐ *Staphylococcus epidermidis*
  - ☐ *Vibrio* species

3

PROCEDURE

Today you will learn how to prepare a bacterial emulsion (smear) and perform simple stains. Your instructor may supply several different bacterial species or tell you to stain one organism. Whether you stain different bacterial species or not, you can refer to the Instructional Staining Atlas (pp. 131–135) to see the variety of cell morphologies and arrangements in the bacterial world. While performing the staining procedure, wear a lab coat, gloves, and chemical eye protection. You may remove your gloves and eye protection when using the microscope.

1. **Make Your Bacterial Smears:** A bacterial smear (emulsion) is made prior to most staining procedures. Follow the procedural diagram in Figure 11.4 to prepare smears of three organisms on a slide. Be sure not to mix them. At the same time, make a second slide with the remaining three organisms. Emulsions should be about the size of a dime.
2. **Heat Fix the Smears:** Heat fix each slide as illustrated in Figure 11.4. If you are using a bacterial incinerator, hold the slide (with a slide holder) near the opening for a few seconds (Fig. 11.5).
3. **Stain the Smears:** Following the staining procedure illustrated in Figure 11.6, stain your slides with a basic stain (crystal violet, safranin, or methylene blue) for 1 minute.
4. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape and arrangement. Record your observations in the data sheet at the end of this lab.
5. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.



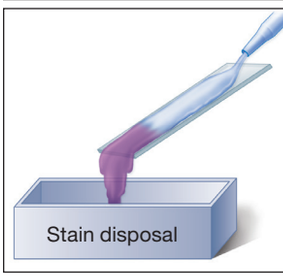
1. Prepare a Bacterial Smear/ Emulsion

See Fig. 11.3 for details on preparing a bacterial smear. ***Note:*** More than one organism can be put on a slide.



2. Stain the Sample

Place the slide over a staining tray or lay it across a beaker. The tray or beaker collects any excess dye that drips off the slide. Cover the smear with dye. There is no need to cover the whole slide, just cover the smear(s). Allow the dye to interact with the bacteria for about a minute.



3. Rinse the Stained Sample

Grasp the slide with a slide holder or a gloved hand and tilt it over a staining tray or collection beaker. Then, use a squirt bottle to *gently* rinse the stained sample to remove excess dye. Rinse until the bulk of the dye is washed away and only the cells seem stained.



4. Dry the Slide

Use bibulous paper or paper towels to blot the slide dry. Do not rub the paper over the slide. Your instructor may or may not have you add a cover slip to the specimen before you view it.

FIGURE **11.6** **Simple Staining Procedure**

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 11

DATA SHEET

Simple Stains and Bacterial Cell Morphology

During the Lab

1 Record your observations from the exercise.

Organism Name	Dye Used	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)

Lab Related Questions

1 What can a simple stain reveal? (Select all that apply.)

a. Cell shape
b. Structural features such as the presence of a capsule
c. Cell size
d. Gram property
e. Cellular arrangement

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The simple stain technique employs two or more dyes.

_____ The simple stain technique is performed on live bacteria.

_____ The simple stain technique involves a heat-fixation step.

_____ During simple staining, the bacterium is exposed to the dye for about 10 minutes.

3 Place the following steps of the simple stain in order from first to last; note some steps may not be used at all.

I. Heat fix the smear.
II. Add the chemical fixative.
III. Add the dye.
IV. Make a bacterial smear.
V. Rinse off excess dye.
VI. Blot the slide dry.
VII. Allow the smear to air dry.
VIII. Heat the dye.

Proper order: _____

4 Select the feature of a basic dye (select all that apply).

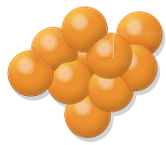
a. Alkaline
b. Negative charge
c. Stains the interior of cells
d. Includes methylene blue
e. Includes a chromagen

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 11
DATA SHEET
(continued)

5 Assume you are viewing the following bacteria. What shapes and arrangements are represented?



Shape: _____ Arrangement: _____



Shape: _____ Arrangement: _____



Shape: _____ Arrangement: _____



Shape: _____ Arrangement: _____



Shape: _____ Arrangement: _____

Critical Thinking

1 Assume you are staining a bacterial sample, and at the end of the procedure there are barely any stained cells on the slide. Interestingly, your lab partner, who used the same stock bacterial sample as you, as well as the same reagents, sees plenty of cells on their slide. Provide three possible ways you may have performed the staining procedure wrong to obtain the described results.

2 Why would growing a bacterium in liquid culture potentially make it easier for you to determine the bacterium's natural cellular arrangement?

EXERCISE 12

Negative Stains

After doing this exercise, you should be able to:

1. Perform a negative stain of bacterial smears.
2. Describe and identify bacterial shapes and arrangements.
3. List the features of basic versus acidic dyes.
4. Discuss the differences between the simple stain and the negative stain.
5. Perform oil-immersion microscopy to view your own stained bacterial samples.

Background

To avoid confusion, it's worth noting that because the negative staining procedure employs only one dye, it can technically be considered a type of simple staining protocol. That said, most microbiologists use the phrase "simple stain" to refer to staining a sample with a basic dye and reserve the phrase "negative staining" to refer to procedures where a single dye is used to color the background of the sample versus the specimen itself (Fig. 12.1). We follow this tradition and encourage you to also consider these procedures as distinct from one another; they are performed differently and the dyes used are chemically different.

The negative staining technique uses a dye with an acidic chromogen. Recall from Exercise 11 that acidic chromogens carry a negative charge. The negative charge on the bacterial surface repels the negatively charged chromogen, so the cell remains unstained against a colored background (Fig. 12.2).

As with simple staining, the negative stain only reveals basic morphological information about a bacterium, namely cell shape, cellular arrangements, and size. The distinct advantage of a negative stain over the simple stain is the negative stain does *not* include a fixation step (no chemical or heat fixation is applied). Since we do not expose the specimen to harsh chemicals or heat that can distort cell features, the negative staining procedure can reveal a species' truest size, shape, and cellular arrangement. As a case in point, you can compare how *Rhodospirillum rubrum* appears less "plump" in Figure 11.1 as compared to its appearance in Figure 12.1.

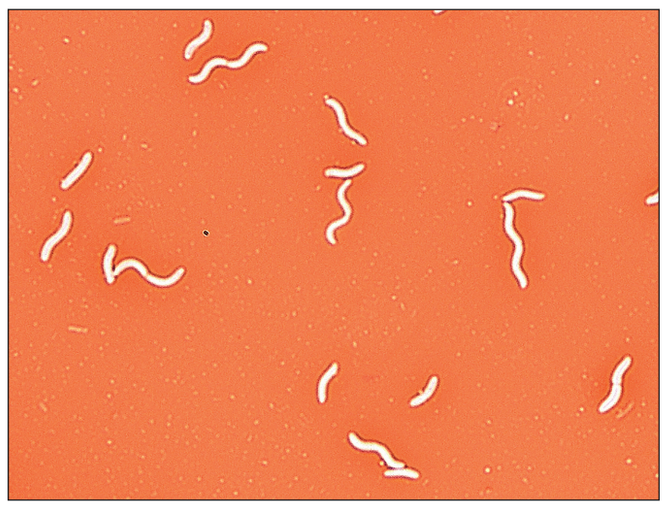


FIGURE **12.1** **Negative Stained Bacterium** • This is a negative stain of the spiral-shaped bacterium *Rhodospirillum rubrum*. Notice that the stain is associated with the background versus staining the cells. The organism was grown in a broth culture, stained using Congo red, and viewed with bright-field microscopy. This photograph was taken at 1,000× (oil immersion).

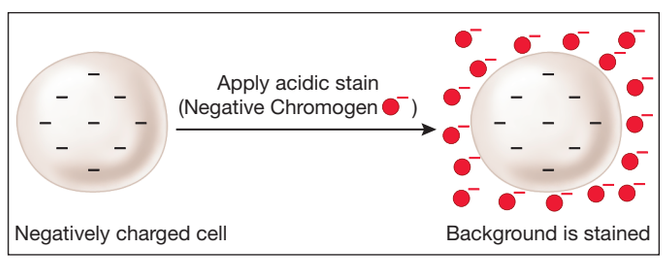


FIGURE **12.2** **Chemistry of Acidic Stains** • Acidic stains have a negatively charged chromogen that negatively charged cells repel. Thus, the background is colored and the cell remains transparent.

Clinical Applications

The negative staining technique is used to determine morphology and cellular arrangement in bacteria that are too delicate to withstand heat fixing. A primary example is the spirochete *Treponema pallidum*, which causes syphilis. This bacterium is easily distorted by the heat fixing of other staining techniques. Also, where determining the accurate size is crucial, a negative stain can be used because it produces minimal cell shrinkage.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Clean glass microscope slides
- ❑ Glass cover slips (as an optional way to drag the dye across the slide)
- ❑ Acidic dyes (at least one of the following):
  - □ Nigrosin
  - □ India ink
- ❑ Paper towels
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Fresh broth or slant cultures of these recommended organisms:
  - □ *Bacillus cereus* or any *Bacillus* species (ideally the same used in Ex. 11)
  - □ *Micrococcus luteus*
  - □ *Rhodospirillum rubrum* or *Aquaspirillum* species (ideally the same used in Ex. 11)

PROCEDURE

Today you will perform negative stains on three different organisms. You also will have the opportunity to compare the sizes of *Bacillus cereus*, *Micrococcus luteus*, and *Rhodospirillum rubrum* (or their substitutes) measured with the negative stain to their sizes as determined using a simple stain. You may wish to divide the workload with your lab partners so all specimens are stained by someone within your group. Then, if you don't have time to stain all the specimens yourself, you can examine slides prepared by your lab partner(s). You should refer to the Instructional Staining Atlas (pp. 131–135) to study the variety of cell morphologies and arrangements that bacteria assume; this will help you tackle the Lab Related Questions at the end of this exercise. While performing the staining procedure, wear a lab coat, gloves, and chemical eye protection. You may remove your gloves and eye protection when using the microscope.

1. **Stain your Sample:** Following the staining procedure illustrated in Figure 12.3, stain your slides with an acidic stain (nigrosin or India ink). ***Note:*** In this procedure, we add only one specimen per slide and there is not a heat-fixation step.
2. **Dispose of the Spreader:** Dispose of the slide or cover slip you used to drag the sample across the slide. A biohazardous sharps container is typically recommended for this.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1, page 21. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape and arrangement. Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides according to your laboratory's policy. Used slides are typically discarded into a biological sharps container.



1. Add Dye to the Slide

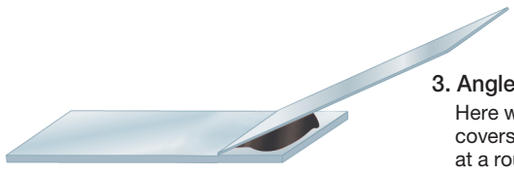
Place the slide on a paper towel on the bench space.

Place a drop of acidic stain (nigrosin in this example) at one end of a clean slide.



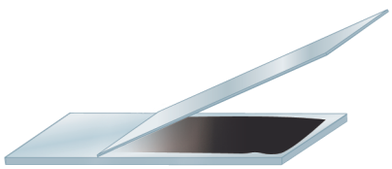
2. Add Bacteria to the Slide

With the slide resting on the paper towel-covered bench space, use an inoculating loop to transfer a small amount of bacteria into the drop of dye. Don't over-inoculate and avoid spattering the mixture. Sterilize the loop after emulsifying.



3. Angle a Spreader to Contact the Emulsion

Here we use a second clean slide (or a glass coverslip) as a "spreader." Place the spreader at a roughly 30° angle so it contacts the emulsion.



4. Drag the Emulsion Across the Slide

Using a gentle and smooth motion, drag the spreader across the slide so the emulsion is thinly spread over the slide. This should be one uninterrupted and fluid motion—do not pick up the spreader until the end of the slide is reached or you will have a choppy preparation.



5. Air-dry the Slide

Allow the sample to air-dry—it will appear matte (non-shiny) when fully dry. Once it's dry, observe the sample under the microscope.

FIGURE **12.3** **Negative Staining Procedure**

— Lab Notes —

3

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 12

DATA SHEET

Negative Stains

During the Lab

1 Record your observations from the exercise.

Organism Name	Dye Used	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)

Lab Related Questions

1 What can a negative stain reveal? (Select all that apply.)

a. Cell shape
b. Cellular arrangement
c. Cell size
d. Cell wall features
e. Structural features such as the presence of endospores

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The negative stain technique employs a single dye.

_____ The negative stain technique is performed on heat-fixed bacteria.

_____ The negative stain technique is better than a simple stain for revealing the organism's truest size.

_____ A negative stain is considered a type of differential stain.

3 Place the following steps of the negative stain in order from first to last; note some steps may not be used at all.

I. Cover the smear with dye.
II. Drag the emulsion across the smear.
III. Heat fix the smear.
IV. Warm the dye.
V. Rinse off excess dye.
VI. Add bacteria to the slide.
VII. Add dye to the slide.

Proper order: _____

4 Select the feature(s) of an acidic dye (select all that apply).

a. Alkaline
b. Basic
c. Negatively charged
d. Includes India ink
e. Lacks a chromogen

5 Why doesn't a negative stain colorize the cells in a bacterial smear?

Critical Thinking

1 Compare the sizes of *Bacillus cereus, Micrococcus luteus*, and *Rhodospirillum rubrum* cells (or their substitutes) as measured using a basic stain (Ex. 11) and an acidic stain. What might account for any difference(s)?

2 Congo red is a red acidic stain, and methylene blue is a blue basic stain. What should be the result of staining a bacterial smear with a mixture of eosin and methylene blue?

EXERCISE 13

Gram Stain

After doing this exercise, you should be able to:

1. Perform a Gram stain on bacterial smears.
2. Describe and identify bacterial shapes, arrangements, and Gram reactions.
3. Explain how the Gram stain differentiates between Gram-positive and Gram-negative bacteria.
4. Understand the difference between differential, simple, and negative stains.
5. Discuss why clinicians consider the Gram status of an infectious agent when deciding on a drug treatment.
6. Perform oil-immersion microscopy to view your own Gram-stained bacterial samples.

Background

Unlike simple and negative stains, which only reveal information about cell morphology (size, shape, and cellular arrangement), **differential stains** allow us to categorize cells (or differentiate one group from another) based on physical features. The **Gram stain** is the most commonly used differential stain in microbiology; it differentiates between bacterial cells based on cell wall composition. Another common differential stain is the acid-fast stain, which we'll review in the next lab (Ex. 14).

How the Gram Stain Works

Although there are several minor variations of the Gram-stain procedure, chemically they all work the same way, they all affect the bacterial cell wall in a manner that allows us to differentiate between Gram-positive and Gram-negative bacteria. By the end of a Gram-staining procedure, Gram-positive bacteria appear purple while Gram-negative bacteria appear pink/red (Fig. 13.1).

It's important to note that Gram staining does not *make* bacteria Gram positive or Gram negative any more than a pregnancy test makes a person pregnant. Rather, the procedure chemically highlights the natural differences between the cell walls of these bacteria. For example, Gram-negative bacteria have a thin peptidoglycan layer in their cell wall while Gram-positive bacteria have a thick peptidoglycan layer. In addition, Gram-negative bacteria have an outer membrane while Gram-positive bacteria do not. There are other important differences between these two bacterial groups, but electron microscopy and other evidence show the aforementioned features are the most central to the Gram-stain's chemistry. As we progress through the procedure, Gram-positive cells are dyed purple and remain purple the entire time, while Gram-negative cells go through a variety of color states (Fig. 13.2).

The first step in the procedure is to apply **crystal violet,** a basic (or positively charged) dye, to the bacterial smear. Because crystal violet is the first dye added in this double-staining procedure, it is sometimes referred to as the

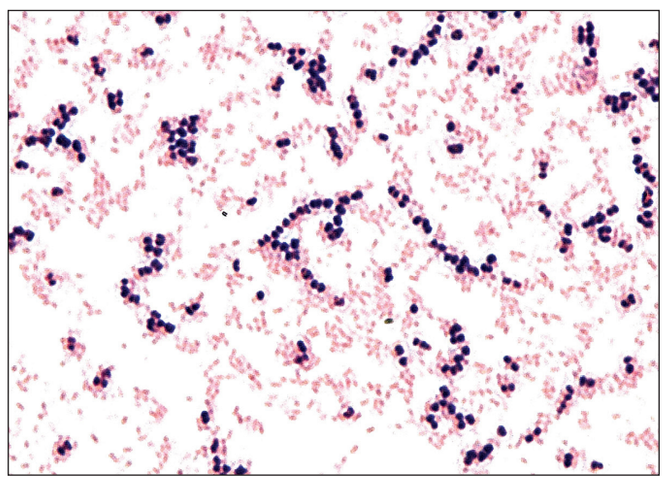


FIGURE **13.1** **Gram-Stained *Streptococcus salivarius* and *Erwinia amylovora*** ● Gram-positive bacteria, such as *S. salivarius*, appear purple at the end of the Gram-stain procedure. Gram-negative bacteria, such as *E. amylovora*, appear pink/red at the end of the procedure.

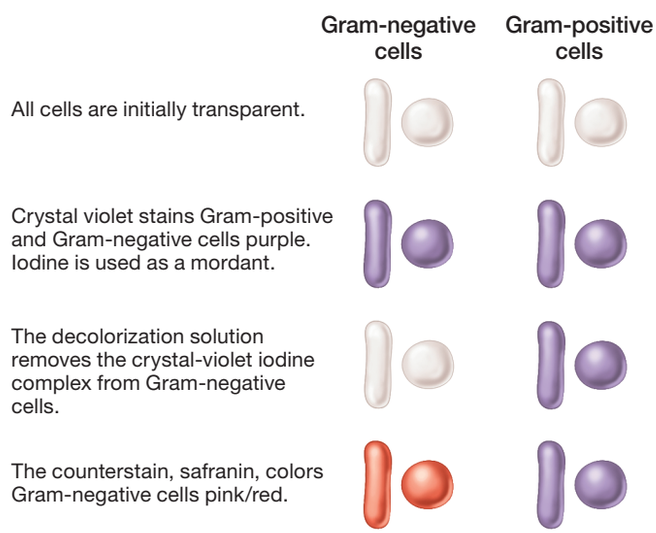


FIGURE **13.2** **Gram-Stain Overview** ● Gram-positive cells remain purple throughout the Gram-staining procedure. Gram-negative cells change colors throughout the procedure, but at the end they appear pink/red.

primary stain. As you likely deduced from its name, crystal violet has a purple color. As such, by the end of the primary staining process, all cells in the sample (regardless of their Gram property) will appear purple. Next, we add Gram iodine, which serves as a **mordant** by bonding to the crystal violet to form a **crystal violet–iodine complex** (or **CV-I complex**). After using water to rinse the excess iodine off the bacterial smear, we add the **decolorizing agent** (usually an acetone-alcohol solution). This is the most critical step in the procedure because it is here where the procedure's chemistry helps us differentiate between Gram-positive and Gram-negative bacteria (Fig. 13.3). After this step, Gram-positive cells remain purple, but the CV-I complex rinses out of Gram-negative cells, rendering them colorless.

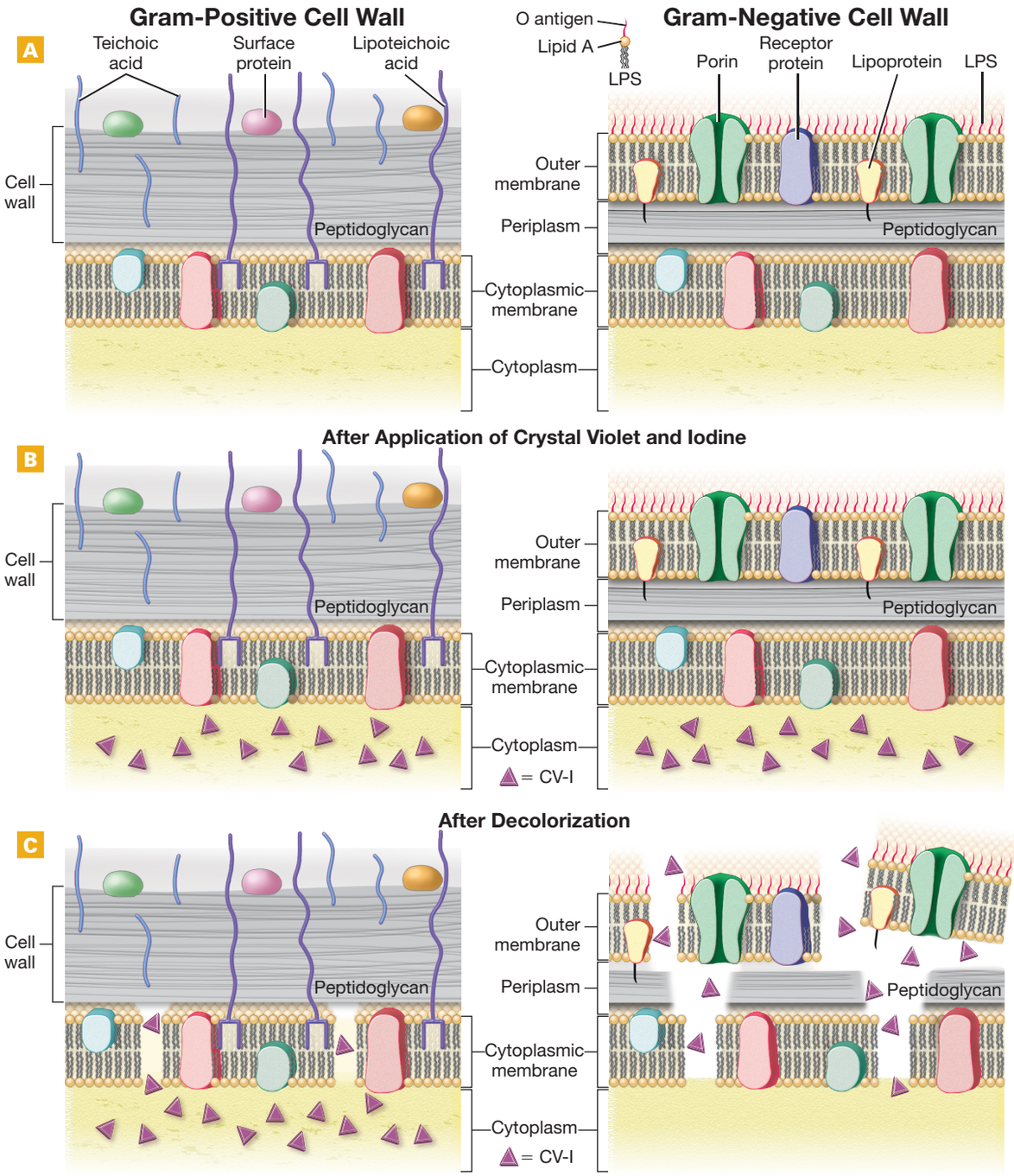


FIGURE **13.3** **Chemistry of the Gram Stain** • The Gram stain differentiates between Gram-positive and Gram-negative bacteria based on cell wall features. (A) As compared to the Gram-positive bacterial cell wall (**left**), the Gram-negative wall (**right**) has a thin peptidoglycan layer and more lipid (due to the outer membrane). (B) Relatively large crystal violet-iodine complexes (purple triangles) form in the cytoplasm of both Gram-positive and Gram-negative cells. (C) The thicker Gram-positive peptidoglycan layer dehydrates when decolorizer is added, trapping the crystal violet-iodine complexes. The lipid in the Gram-negative cell wall is dissolved by the decolorizer and the thinner peptidoglycan is incapable of preventing extraction of the crystal violet–iodine complex.

The change here is related to the cell wall differences between Gram-positive and Gram-negative cells. The alcohol/acetone in the decolorizer extracts lipids from the Gram-negative cell's outer membrane, which renders the cell wall porous and incapable of retaining the CV-I complex. However, the decolorizer has a different chemical effect on Gram-positive bacteria. In Gram-positive cells, the decolorizer dehydrates the thicker peptidoglycan layer, thereby trapping the CV-I complex inside the cells so the cells remain purple. It's difficult to see colorless cells on glass, so to ensure we don't overlook any of the decolorized Gram-negative cells, we add a second dye called **safranin** as a **counterstain**. This pink/red dye enters the colorless Gram-negative cells so that upon successful completion of the procedure, Gram-negative cells appear reddish-pink.

Gram-Variable Results and Other Considerations

The Gram-staining procedure requires some practice to get right. One source of error is emulsion preparation inconsistency. Remember, a good emulsion is about the size of a dime and dries to a faint haze on the slide. If the bacterial smear is too dense, then you can expect unclear results. As an analogy, consider overstuffing a washing machine. If you pack too many clothes into the machine, then the clothes don't get properly washed because the ratio of clothes to water and detergent is not optimal. Similarly, if you have too many cells in the bacterial smear, they will be layered on top of one another and limit the interaction of the reagents with each of the cells.

A second source of inconsistent Gram-stain results can be the organisms themselves. Some Gram-positive bacteria naturally lose their ability to retain the crystal violet–iodine complex as they age and/or are stressed. *Bacillus* species are especially prone to this effect because they form endospores when stressed. We review endospore formation more in Exercise 16, but suffice it to say here that sporulation (which only certain bacteria can do) damages the bacterial cell wall and can thereby lead to false-negative results (meaning some cells will appear pink, or Gram-negative, when the species is in fact a Gram-positive bacterium). Also, when Gram-positive cell cultures are older, some of the cells will be dying and will therefore have damaged cell walls. If a cell has a damaged cell wall, it won't retain the CV-I complex and will thereby appear pink at the end of Gram staining despite the species technically being Gram positive. To limit such errors, always use cultures that are less than 24 hours old (Fig. 13.4).

Although some organisms like *Bacillus* species may naturally appear Gram variable, most variable results are a consequence of poor technique. The decolorization step is the most crucial step in the procedure and therefore the most likely source of Gram-stain inconsistency. It is possible to **over-decolorize** by leaving the decolorizer on too long and get reddish Gram-*positive* cells (Fig. 13.5). It also is possible to **under-decolorize**, in which case one will see purple Gram-*negative* cells (Fig. 13.6). Neither of these situations changes the actual Gram property of the organism being stained, rather, these are inaccurate results due to poor technique.

In addition to procedural errors and bacterial features like Gram variability, nonbacterial elements can also complicate staining results. For instance, stain crystals from an old or improperly made stain solution can disrupt the field of view (Fig. 13.7) or stain precipitate can be mistaken for bacteria (Fig. 13.8).

Whenever you do a Gram stain it's a good idea to include control smears of known Gram-positive and

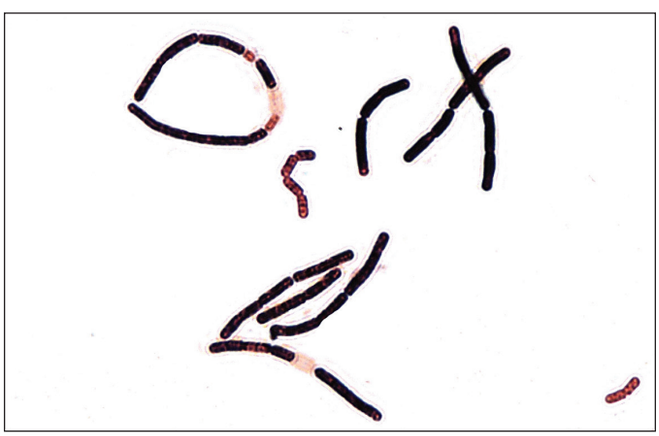


FIGURE **13.4** **Variable Gram Reaction in *Bacillus* Species** • This *Bacillus* species was isolated from aerobic estuarine mud and stained within 24 hours of growing a pure culture. Notice some cells are pink even though *Bacillus* species truly have a Gram-positive cell wall. Because they can form endospores, *Bacillus* species often appear Gram variable.

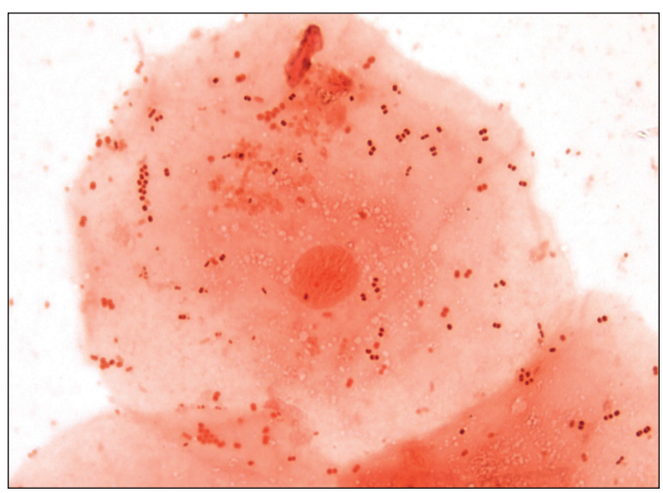


FIGURE **13.5** **Over-Decolorized Gram Stain** • This is a direct smear from the gumline. Notice the near absence of any purple cells, a certain indication of over-decolorization since the mouth harbors Gram-positive and Gram-negative bacteria.

Gram-negative organisms alongside the test organism (Fig.13.9). As an alternative control, you can also use a gumline smear, which will naturally include Gram-positive and Gram-negative organisms (Fig. 13.10).

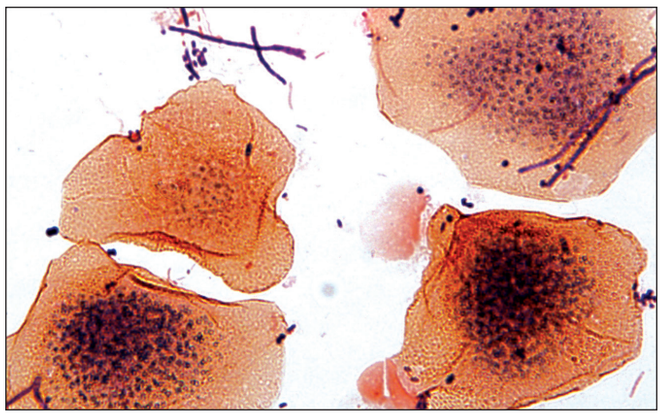


FIGURE **13.6** **Under-Decolorized Gram Stain** • This is a direct smear from the gumline. Notice the purple patches of stain on the epithelial cells. Also notice the variable quality of this stain; the epithelial cell to the left of center stained better than the others.

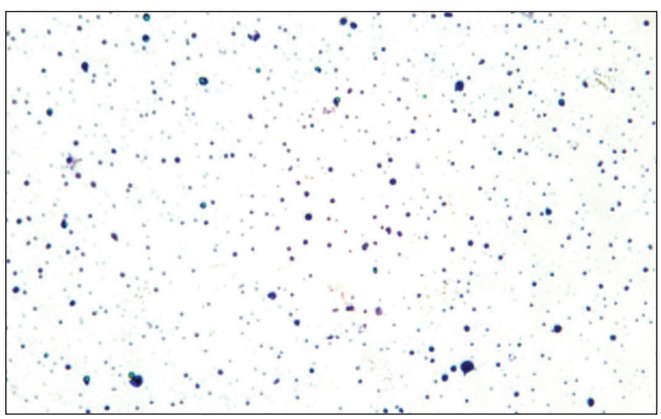


FIGURE **13.8** **Stain Precipitate (Gram Stain)** • If the slide is not rinsed thoroughly or if the stain is allowed to dry on the slide, stain precipitate may form and may be confused with bacterial cells. Their size variability is a clue that they are not bacteria.

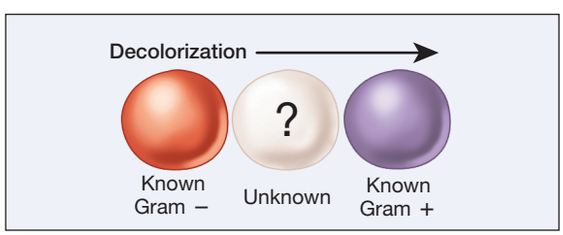


FIGURE **13.9** **Controls to Check Your Gram-Staining Technique** • It's a good idea to include known Gram-positive and Gram-negative organisms as controls on either side of your unknown organism. Try to place the smears as close to one another as possible because spreading them out across the slide makes it difficult to stain and decolorize them equally. It is also beneficial to put the Gram-negative control at the end where you will apply the decolorizer so you can more easily monitor the decolorizing solution runoff and stop decolorizing just as the runoff starts to appear clear.

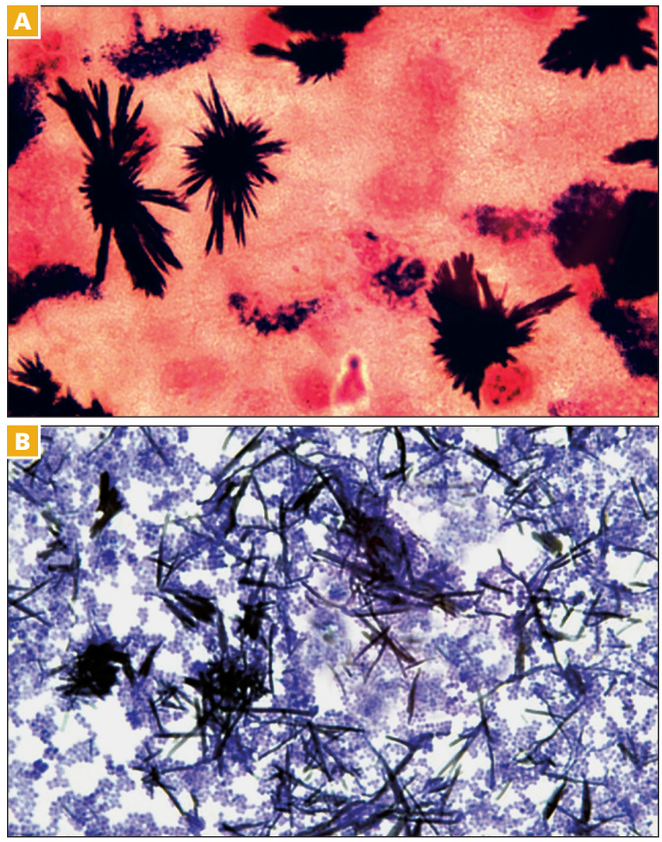


FIGURE **13.7** **Crystal-Violet Crystals (Gram Stain)** • Crystals from two different Gram stains are shown here. (A) This is a gumline direct smear. The bacterial cells are the pink material in the background (this emulsion was far too thick) and the crystals are the large, purple "snowflakes." (B) In this Gram stain of *Micrococcus roseus*, the cells are the lighter purple in the background (can you detect any tetrads?) and the crystals are the darker spikes.

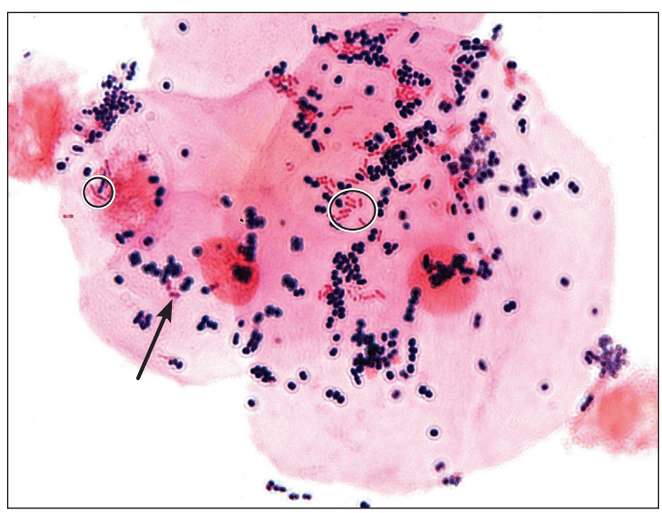


FIGURE **13.10** **A Gumline Smear as a Gram-Stain Control** • A direct smear from the gumline will include Gram-positive bacteria (especially cocci), some Gram-negative cells, and your own nucleated epithelial cells. In this micrograph, different sized Gram-positive cocci predominate, but Gram-negative cells are also present, including two Gram-negative rods (shorter and broader vs. longer and narrower in the two **circles**) and Gram-negative diplococci **(arrow)**. Notice that most bacterial cells are on the epithelial cell's surface, which is where they reside in the mouth.

Clinical Applications

The Gram stain, which distinguishes between Gram-positive and Gram-negative bacteria, is the most important and widely used microbiological differential stain. In addition to Gram reaction, this stain also reveals bacterial morphology (shape, size, and arrangement). It is often the first differential test run on a clinical specimen brought into the laboratory for identification. For example, it's an early confirmation tool for diagnosing bacteremia, a bloodstream infection with a mortality rate of about 20% in hospitalized patients. Gram-positive or Gram-negative bacteria can cause bacteremia, but knowing which is the culprit influences the treatment choice. This is because some drugs are more effective against one Gram class of bacteria over another. Many penicillin family drugs are more effective against Gram-positive bacteria than they are against Gram-negative bacteria.

While our drug pharmacopeia includes **broad-spectrum** antimicrobial drugs (agents that are effective against Gram-positive and Gram-negative bacteria), whenever possible it is best to prescribe a **narrow-spectrum** drug that will more specifically target either Gram-positive or Gram-negative bacteria. Any antibiotic can disrupt our normal microbiota, the bacteria that naturally reside in and on our bodies. However, prescribing drugs that more specifically target the infectious agent can reduce normal microbiota disruption and thereby reduce the risks of several undesirable consequences, to include *Clostridioides difficile* (or *C. diff*) diarrhea and **colitis** (inflammation of the colon). Research has shown that a prior antibiotic treatment—especially a broad-spectrum antibiotic therapy—is a key risk factor for developing *C. difficile* colitis. This is because the bacterium is less likely to establish a symptomatic infection when it must compete with the normal microbiota inhabiting the intestinal tract.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Clean glass microscope slides
- ❑ Sterile toothpick
- ❑ Squirt bottle with water
- ❑ Bibulous paper and/or paper towels
- ❑ Staining tray or glass beaker to collect dye waste rinsed off slides
- ❑ Slide holder
- ❑ Gram-stain solutions (commercial kits are available):
  - ☐ Crystal violet
  - ☐ Gram iodine solution
  - ☐ 95% ethanol (or ethanol/acetone solution)
  - ☐ Safranin
- ❑ Recommended organisms (overnight cultures grown on agar slants or turbid broth cultures):
  - ☐ *Staphylococcus epidermidis* or *Micrococcus luteus*
  - ☐ *Escherichia coli* or *Rhodospirillum rubrum*
  - ☐ *Neisseria sicca* or *Moraxella catarrhalis*
  - ☐ *Corynebacterium xerosis*

3

PROCEDURE

As stated previously (but worth repeating), the Gram stain is the single most important differential stain in microbiology. Therefore, you will probably need to practice it multiple times to get good results. Use the organisms in combinations so you will have one Gram-positive sample and one Gram-negative sample on each slide. Of the listed organisms, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Corynebacterium xerosis* have the same Gram reaction. Call these "Group A." *Escherichia coli*, *Rhodospirillum rubrum*, *Neisseria sicca*, and *Moraxella catarrhalis* have the other Gram reaction. Call these "Group B." You should place one "Group A" microbe and one "Group B" microbe on each slide along with whatever control your instructor indicates.

While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Make Your Bacterial Smears:** Follow the smear and heat-fixing procedure you used in the simple staining lab (review Fig. 11.4, p. 139, if needed). Place one organism from Group A and one from Group B *immediately next to one another* on the same slide. If you make the emulsions at opposite ends of the slide, you may find it difficult to equally stain and decolorize the smears. Strive to prepare smears of uniform thickness, because thick smears risk being under-decolorized. Also add any control sample that your instructor indicates. Do not exceed three smears per slide. Your instructor may have you prepare only one slide, or several slides with selected Gram-positive and/or Gram-negative bacteria.

2 **Make a Gumline Smear:** (Depending on institutional lab safety regulations, your instructor may not have you prepare a gumline smear.) Wash your hands thoroughly, and then use a sterile toothpick to obtain a sample from your teeth at the gumline. (Do not draw blood! What you want is easy to collect from the gingival pocket, the space between your teeth and gums.) Transfer the sample to a drop of water on a clean glass slide, air dry, and heat fix.

3 **Stain the Smears:** Follow the procedure illustrated in Figure 13.11 to stain your slides. Start by staining the pure cultures you placed on slides in Step 1. Once you have evaluated your technique and find it is consistent, then you may stain the gumline sample you prepared.

If your preparations do not give "correct" results, the most likely source of error is in the decolorization step. When the runoff is clear (generally within 10 seconds, shorter if the acetone concentration is high), *immediately* rinse with distilled H_2O. The decolorizer continues decolorizing until you have rinsed it off with water, so anticipate this and compensate accordingly so you do not over-decolorize.

4 **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and Gram reaction. Record your observations in the data sheet at the end of this lab.

5 **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

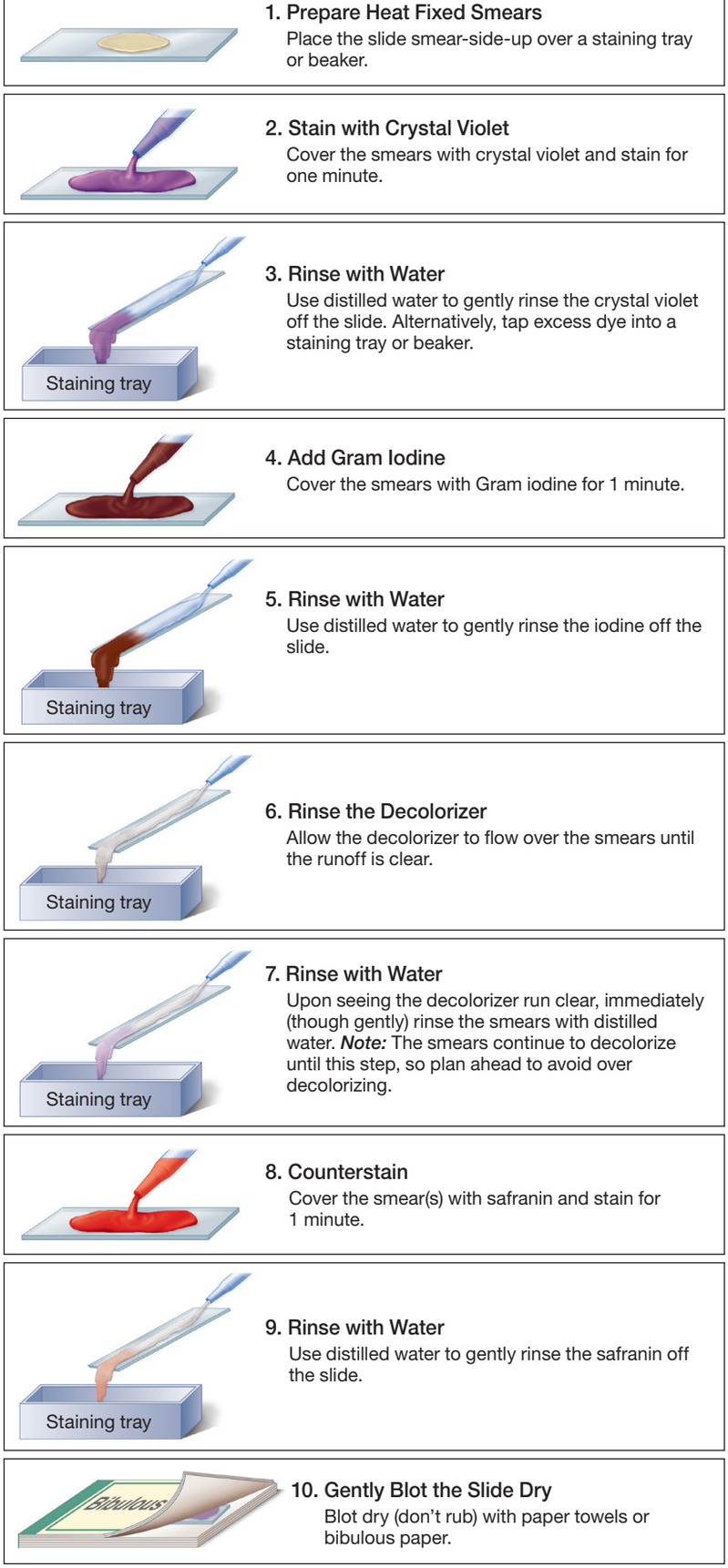


FIGURE **13.11** **The Gram Stain Procedure**

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 13

DATA SHEET

Gram Stain

During the Lab

1 Record your observations from the exercise.

Organism (or source)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Color	Gram Reaction (+/–)

Lab Related Questions

1 The Gram stain is an example of a(n):

a. Simple stain.
b. Negative stain.
c. Acidic stain.
d. Differential stain.
e. Structural stain.

2 What color would Gram-negative bacteria be if you forgot to add the *counterstain* in the Gram-stain procedure?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

3 What color would Gram-negative bacteria be if you forgot to add the *iodine* in the Gram-stain procedure?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

4 Some of the following steps are used in the Gram-staining procedure, but some of the listed steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Heat fix the samples on the slide.

_____ Add crystal violet to the slide.

_____ Add methylene blue to the slide.

_____ Rinse the smears with decolorizer solution.

_____ Add iodine to the smears.

_____ Add bacteria to the slide.

_____ Cover the smears with safranin.

_____ Heat the iodine while on the smears.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The Gram-staining technique requires one dye.

_____ The Gram-staining technique is best performed on cultures more than 48-hours old.

_____ The Gram-staining technique requires a mordant called iodine.

_____ Gram-positive bacteria should appear purple at the end of the Gram staining procedure.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 13
DATA SHEET
(continued)

6 The Gram stain differentiates between Gram-positive and Gram-negative bacterial cells based on:

a. Cell size.
b. Cell wall differences.
c. Cell shape.
d. Cell density.
e. Cell charge.

7 Why is the Gram stain clinically useful?

8 Assume you perform a Gram stain on a culture you know contains both Gram-positive and Gram-negative bacteria. Upon completing the procedure, all the cells appear pink. What errors may lead to this result? (Select all that apply.)

a. You over-decolorized.
b. You forgot to add iodine.
c. You forgot to heat fix the bacterial smears.
d. You forgot to add the counterstain.
e. You under-decolorized.

Critical Thinking

1 Both crystal violet and safranin are basic stains used to perform simple stains on Gram-positive and Gram-negative cells. This being the case, explain how they end up staining Gram-positive and Gram-negative cells differently in the Gram stain.

2 Your lab partner included Gram-positive and Gram-negative control organisms on their Gram stain of an unknown bacterial species. Their chosen controls were *Escherichia coli* and *Bacillus subtilis*. They try the procedure several times and each time they conclude they are decolorizing too long because both controls end up with mostly pink cells. What might you suggest they try and why?

EXERCISE 14

Acid-Fast Stains

After doing this exercise, you should be able to:

1. Perform an acid-fast stain on bacterial smears.
2. Describe and identify bacterial shapes, arrangements, and acid-fast reactions.
3. Explain how the acid-fast stain differentiates between acid-fast and non-acid-fast bacteria.
4. Understand the difference between the Ziehl-Neelsen and the Kinyoun staining methods.
5. Discuss the clinical significance of the acid-fast stain.
6. Perform oil-immersion microscopy to view your own acid-fast stained bacterial samples.

Background

Like the Gram stain, the acid-fast stain is a differential stain that helps us categorize cells based on cell wall properties. Just as we describe fabrics that resist fading when washed as "colorfast," we can describe cells that resist decolorizing when washed with an acid-alcohol solution as acid-fast. Acid-fast bacteria contain mycolic acid in their cell walls. This waxy substance helps the cells resist decolorization when exposed to an acid-alcohol solution (3% HCl in 95% ethanol). Non-acid-fast bacteria do not have mycolic acid in their cell walls and therefore easily decolorize when rinsed with an acid-alcohol solution (Fig. 14.1). By the end of acid-fast staining, acid-fast bacteria appear red/magenta and non-acid-fast bacteria are the color of the counterstain (usually blue) (Fig. 14.2).

The two main acid-fast staining procedures are the Ziehl-Neelsen (ZN) method (Fig. 14.3) and the Kinyoun (K) method (Fig. 14.4). The choice of method comes down to lab conventions and personal preference. In both methods, the phenolic compound carbolfuchsin is the primary stain because it is lipid soluble and penetrates the waxy cell wall. The main difference between the ZN and K methods is that we use heat in the ZN method to drive the primary stain into cells. Steam heating the preparation softens the waxy mycolic acid layer in acid-fast cells so the carbolfuchsin more readily enters

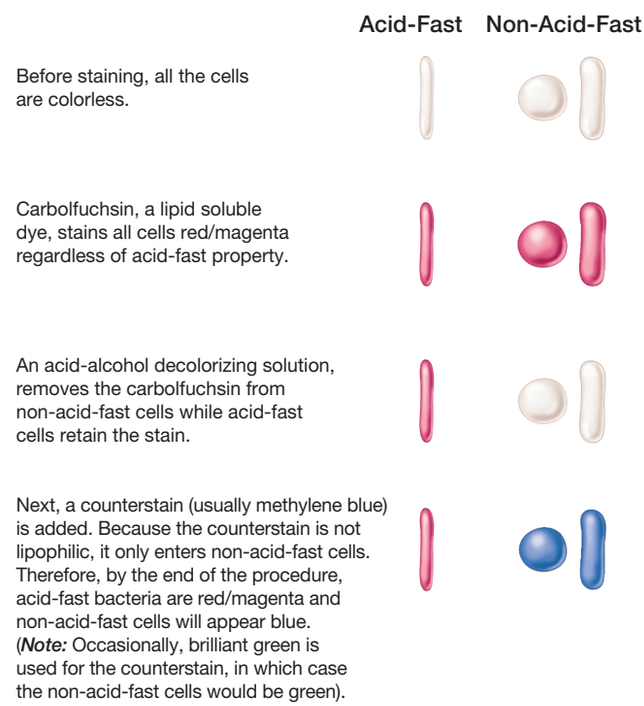


FIGURE **14.1** **Acid-Fast and Non-Acid-Fast Cell Coloration during the ZN and K Methods** • Acid-fast cells stain red/magenta; non-acid-fast cells take on the color of the counterstain, usually blue.

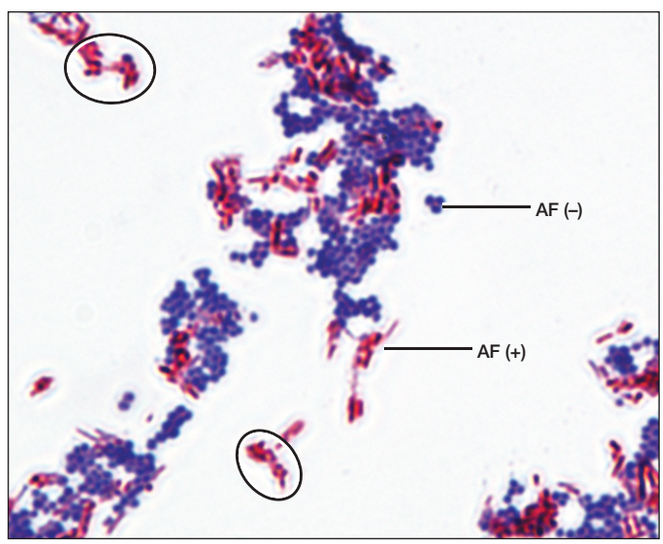


FIGURE **14.2** **Acid-Fast Staining Results** • Notice how most of the *Mycobacterium phlei* (acid-fast, or AF+) cells are in clumps; this is an unusual cell arrangement for bacillus-shaped bacteria. They do this because their waxy cell walls make them sticky. The *Staphylococcus epidermidis* cells (non-acid-fast, or AF–) are also in clumps, but that is because they grow as grapelike clusters. Also notice the characteristic beaded appearance of some acid-fast cells (**circled**).

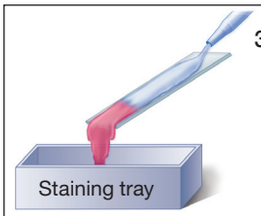


1. Prepare Heat-Fixed Smears
Up to three smears can be added per slide. Place the slide smear-side-up over a beaker of steaming water.

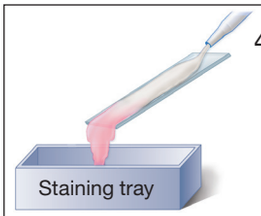


2. Steam the Smears with Carbolfuchsin
Do this step in a well-ventilated area—preferably under a fume hood. Carbolfuchsin is hazardous, so wear gloves and chemical eye protection and avoid inhaling the fumes. Place the slide smear-side-up over a steaming apparatus and then cover the smear(s) with a strip of bibulous paper or paper towel cut slightly smaller than the slide. Apply the ZN carbolfuchsin stain so it soaks the paper. Heat the slide to steaming for 5 minutes (as shown in Fig. 14.5). During the 5 minutes of steaming, keep the paper moist with stain.

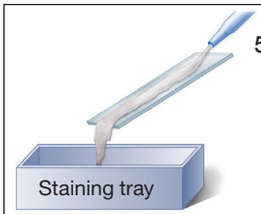
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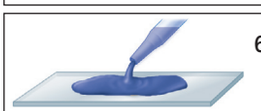
3. Rinse with Water
Dispose of the paper with forceps (to prevent stain from getting on your gloves). Then, hold the slide at an angle and use distilled water to gently rinse the carbolfuchsin off the slide. Collect the runoff into a staining tray or collection beaker. Then, if not already there, return to your lab station carrying your slide in a petri plate.



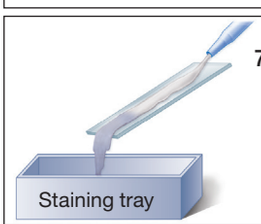
4. Rinse with Decolorizer
Allow the acid-alcohol decolorizer to flow over the smears until the runoff is clear. ***Use increased caution with this reagent***. Collect the runoff into a collection beaker or staining tray.



5. Rinse with Water
Holding the slide at an angle, gently rinse the smears with distilled water. Catch the runoff in a collection tray or beaker.



6. Counterstain
Cover the smear(s) with methylene blue and stain for 1 minute.



7. Rinse with Water
Use distilled water to gently rinse the methylene blue off the slide. Collect the runoff into a collection beaker or staining tray.



8. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

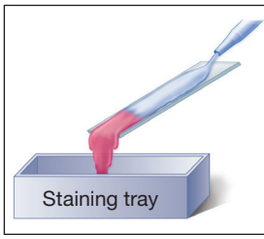
FIGURE **14.3** **Ziehl-Neelsen (ZN) Acid-Fast Staining Method**



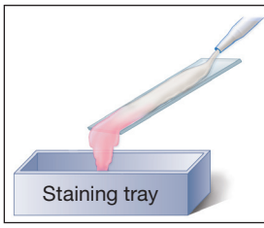
1. Prepare Heat-Fixed Smears
Up to three smears can be added per slide. Place the slide smear-side-up over a beaker or staining tray.



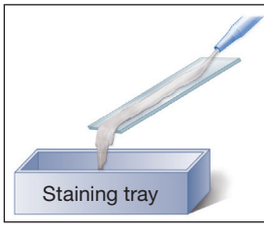
2. Stain with Carbolfuchsin (Do Not Heat)
Do this step in a well-ventilated area—preferably under a fume hood. Carbolfuchsin is hazardous, so wear gloves and chemical eye protection and avoid inhaling the fumes. Place the slide smear-side-up over a collection beaker or staining tray. Cover the smears with Kinyoun carbolfuchsin for 5–10 minutes. Do not heat the slide.



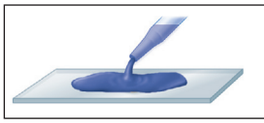
3. Rinse with Water
Hold the slide at an angle and use distilled water to gently rinse the carbolfuchsin off the slide. Collect the runoff into a staining tray or collection beaker. Then, if not already there, return to your lab station carrying your slide in a petri plate.



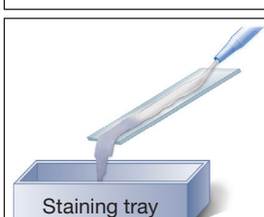
4. Rinse with Decolorizer
Allow the acid-alcohol decolorizer to flow over the smears until the runoff is clear. ***Use increased caution with this reagent***. Collect the runoff into a collection beaker or staining tray.



5. Rinse with Water
Holding the slide at an angle, gently rinse the smears with distilled water. Catch the runoff into a collection tray or beaker.



6. Counterstain
Cover the smear(s) with methylene blue and stain for 1 minute.



7. Rinse with Water
Use distilled water to gently rinse the methylene blue off the slide. Collect the runoff into a collection beaker or staining tray.



8. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

FIGURE **14.4** **Kinyoun (K) Acid-Fast Staining Method**

these typically difficult-to-stain cells (Fig. 14.5). The K method gets around the heating requirement by using a more concentrated carbolfuchsin solution.

A Note About Gram Property and Acid-Fast Bacteria

The waxy wall of acid-fast cells repels typical aqueous stains. As a result, most acid-fast organisms only appear weakly Gram positive when subjected to the Gram-staining procedure. That said, they are still classified as Gram-positive bacteria.



FIGURE **14.5** **Steaming Slides During the Ziehl-Neelsen (ZN) Acid-Fast Staining Procedure** ● Place your slide (smear-side up) over a beaker of steaming water. Cut a piece of bibulous paper or paper towel so it covers the smears but is smaller than the slide. Add carbolfuchsin to moisten the paper. Keep the paper moist with stain for the entire 5 minutes of steaming. ***Caution!*** Preferably perform this procedure in a fume hood; do not inhale the fumes.

Clinical Applications

The acid-fast stain is an important differential stain used to identify bacteria in the genus *Mycobacterium*, some of which are pathogens (e.g., *M. leprae* and *M. tuberculosis*, causative agents of leprosy and tuberculosis, respectively). Members of the actinomycete genus *Nocardia* (*N. brasiliensis* and *N. asteroides* are opportunistic pathogens) are partially acid-fast. Oocysts of coccidian parasites, such as *Cryptosporidium* and *Isospora*, are also acid-fast. Although several potentially infectious agents are acid-fast, the procedures you perform in this lab are mainly used to identify **acid-fast bacilli** (**AFB**) as a rapid, preliminary diagnosis of tuberculosis (with greater than 90% predictive value from sputum samples). It also is performed on tuberculosis patient samples to track the progress of antibiotic therapy and determine the degree of contagiousness. A prescribed number of microscopic fields are examined and the number of AFB is determined and reported using a standard scoring system.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Clean glass microscope slides
- ❑ Squirt bottle of distilled water
- ❑ Bibulous paper and/or paper towels
- ❑ Staining tray or glass beaker to collect dye waste rinsed off slides
- ❑ Slide holder
- ❑ Sterile wooden sticks, disposable plastic loops, or metal inoculating loops
- ❑ Heating apparatus (beaker of water on a hot plate) for Ziehl-Neelsen procedure only
- ❑ Sheep serum (optional)
- ❑ Acid-fast staining solutions (Ziehl-Neelsen and Kinyoun staining kits are available):
  - ☐ Ziehl-Neelsen staining method
    - ▪ Methylene blue stain
    - ▪ Ziehl's carbolfuchsin stain
    - ▪ Acid alcohol (95% ethanol + 3% HCl)
  - ☐ Kinyoun staining method (complete kits are commercially available)
    - ▪ Kinyoun carbolfuchsin
    - ▪ Acid alcohol (95% ethanol + 3% HCl)
    - ▪ Methylene blue stain, or some kits come with brilliant green as a counterstain
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Mycobacterium smegmatis* (BSL-1 strains are available; e.g., ATCC 27203 or ATCC 700084)
  - ☐ *Staphylococcus epidermidis* (BSL-1)

PROCEDURE

Today you will perform an acid-fast stain using the Ziehl-Neelsen (ZN) Method and/or the Kinyoun Method. Both methods are designed to identify members of the genus *Mycobacterium*. While performing the staining procedure(s), wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

Ziehl-Neelsen (ZN) Method

1. **Make Your Bacterial Smears:** Follow the smear and heat-fixing procedure you used in the simple staining lab (review Fig. 11.4, p. 139, if needed). Make a smear of an acid-fast bacterium and a non-acid-fast bacterium. ***Note:*** In some labs, sheep serum might be suggested instead of water as the liquid for smear preparation. When preparing the smears, gently but thoroughly mix the *Mycobacterium* smear to reduce cell clumping.
2. **Stain the Smears:** Follow the procedure illustrated in Figure 14.3 to stain your slides. Use a steaming apparatus (such as the one shown in Fig. 14.5) to heat the slide. ***Caution!*** Do not inhale the carbolfuchsin fumes; ideally perform this procedure in a fume hood or, at the very least, perform this in a well-ventilated space and keep your distance from the fumes.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and acid-fast reaction. Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

Kinyoun (K) Method

1. **Make Your Bacterial Smears:** Follow the smear and heat-fixing procedure you used in the simple staining lab (review Fig. 11.4, p. 139, if needed). Make a smear of an acid-fast bacterium and a non-acid-fast bacterium. ***Note:*** In some labs, sheep serum might be suggested instead of water as the liquid for smear preparation. When preparing the smears, gently but thoroughly mix the *Mycobacterium* smear to reduce cell clumping.
2. **Stain the Smears:** Follow the procedure illustrated in Figure 14.4 to stain your slides. In this procedure you use Kinyoun carbolfuchsin, which is more concentrated than the carbolfuchsin used in the ZN method. Also, we do not heat the smears with the dye in this procedure. ***Caution!*** Even though we do not heat the carbolfuchsin in this method, it still is a volatile reagent that generates fumes. Do not inhale the carbolfuchsin fumes; ideally perform this procedure in a fume hood or at the very least perform this in a well-ventilated space and keep your distance from the fumes.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and Gram reaction. Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 14

DATA SHEET

Acid-Fast Stains

During the Lab

1 Record your observations from the exercise.

Organism	Staining Method (ZN or K)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Color	Acid-Fast Reaction (+/–)

Lab Related Questions

1 The acid-fast stain is an example of a(n):

a. Simple stain.
b. Negative stain.
c. Acidic stain.
d. Structural stain.
e. Differential stain.

2 What color would non-acid-fast bacteria be if you forgot to add the counterstain in the procedure?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

3 What color would non-acid-fast bacteria be if you used a green counterstain?

a. Colorless
b. Purple
c. Pink
d. Green
e. Blue
f. Not enough information

4 Some of the following steps are used in the acid-fast Ziehl-Neelsen staining procedure, but some of the listed steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Heat fix the samples on the slide.

_____ Add bacteria to the slide.

_____ Add methylene blue to the slide.

_____ Steam the smears with carbolfuchsin.

_____ Add acid-alcohol to the slide.

_____ Heat the methylene blue.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The acid-fast stain helps clinicians diagnose tuberculosis.

_____ The acid-fast staining technique requires two dyes.

_____ The Ziehl-Neelsen acid-fast staining technique can be performed without heat.

_____ Acid-fast bacteria are considered Gram negative.

6 The acid-fast differentiates between acid-fast and non-acid-fast bacterial cells based on:

a. Cell wall differences.
b. Cell size.
c. Cell shape.
d. Cell density.
e. Cell charge.

7 Why is the acid-fast stain clinically useful?

8 The presence of _____ in the bacterial cell wall is what makes a cell acid-fast.

Critical Thinking

1 Assume you put *Mycobacterium smegmatis* and *Staphylococcus aureus* on the same slide and perform the ZN acid-fast staining procedure on the samples. While decolorizing, you realize the clock you are watching is not working properly. Assuming you over-decolorized during the procedure, what would you expect to see as a result? Explain your reasoning.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 14
DATA SHEET
(continued)

2 The mycolic acid layer in acid-fast bacteria is a waxy and somewhat hard to penetrate substance. Knowing this, what assumptions can you make about the timeline for infections caused by acid-fast bacteria and the treatment regimens used to manage such infections?

— Lab Notes —

EXERCISE 15

Capsule Stain

After doing this exercise, you should be able to:

1. Perform a capsule stain on bacterial smears.
2. Describe the structure and function of a bacterial capsule.
3. Explain how the capsule stain works.
4. Understand the clinical significance of the bacterial capsule.
5. Perform oil-immersion microscopy to view your own capsule stained bacterial samples.

Background

The capsule stain is a structural stain that allows us to view bacterial **capsules**, a sticky extracellular polysaccharide or peptide layer that certain bacteria rely on for adhesion and protection. Because bacterial capsules have a neutral charge, they do not readily take up acidic or basic dyes. The capsule stain technique takes advantage of this characteristic by staining *around* the cells with an acidic stain such as Congo red or nigrosin and then staining the cell itself with a basic dye. Provided the bacterial species you stained makes a capsule, the final effect is quite pretty; you'll see a colored slide background dotted with stained cells surrounded by a white halo (the unstained capsule) (Fig. 15.1).

The capsule stain incorporates some aspects of negative staining, namely we stain the background of the slide and we do *not* heat fix the sample. Avoiding heat fixation is essential because applying heat will cause cell shrinkage, which can leave an artifactual white halo around cells that might be interpreted as a capsule. Plus, capsules are fragile, so heating the preparation can distort the structure. In place of heat fixing, cells are emulsified in a drop of serum to promote their adhering to the glass slide. The serum can also enhance the size of the capsule so that it is easier to visualize.

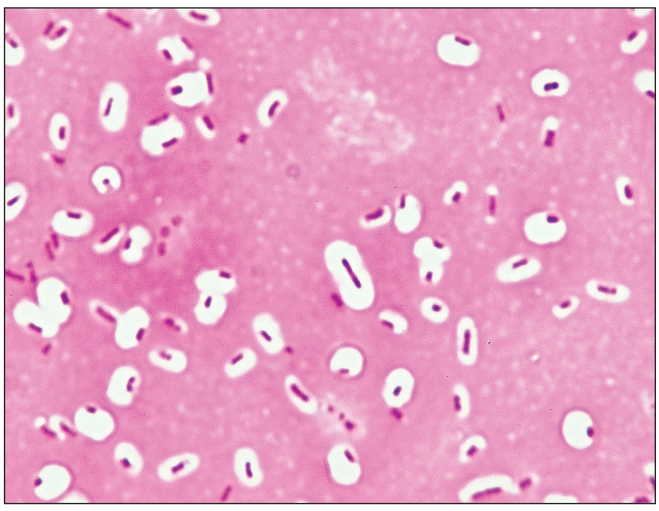


FIGURE **15.1** **Capsule Stain** • The acidic stain colorizes the background while the basic stain colorizes the cell, leaving the capsules as unstained, white clearings around the cells. Notice the lack of uniform capsule size, and even the absence of a capsule in some cells. There are several variations of the capsule stain. The capsule stain you will perform in this exercise produces different colors than shown in this micrograph, but the principle is the same, capsules appear as white clearings around cells.

Clinical Applications

A number of important Gram-positive and Gram-negative bacterial pathogens make capsules, examples include: *Bacillus anthracis* (anthrax), *Streptococcus pneumoniae* (bacterial pneumonia), *Haemophilus influenzae* (bacteremia, epiglottitis, pneumonia, and meningitis), *Neisseria meningitidis* (meningococcal meningitis), *Klebsiella pneumoniae* (pneumonia), *Salmonella typhi* (typhoid fever), and others. For these bacteria, having a capsule is an important **virulence factor** (a feature that helps an infectious agent cause disease). Not only does having a capsule help a pathogen adhere to surfaces, this extracellular structure also renders bacteria less vulnerable to phagocytosis, an important process that certain white blood cells use to clear invaders from the body. The capsule stain allows us to detect cells capable of producing an extracellular capsule and can therefore reveal information about the bacterium in question.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Clean glass microscope slides
- ❑ Squirt bottle of distilled water
- ❑ Bibulous paper and/or paper towels
- ❑ Staining tray or glass beaker to collect dye waste rinsed off slides
- ❑ Slide holder
- ❑ Sterile toothpicks
- ❑ Sheep serum (optional)
- ❑ Maneval's stain
- ❑ Congo red stain
- ❑ Coplin staining jar (or a beaker) with distilled water
- ❑ Recommended organisms (18- to 24-hour skim milk or tryptic soy agar [TSA] slant pure cultures):
  - ❑ *Aeromonas sobria*
  - ❑ *Rhizobium leguminosarum*
  - ❑ *Klebsiella pneumoniae* (BSL-2) (optional)



PROCEDURE

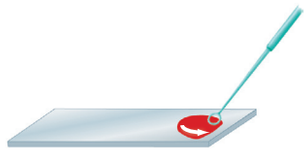
The capsule stain allows you to visualize an extracellular capsule, if present. Be careful to distinguish between a tiny white halo (a result of cell shrinkage) and a true capsule. While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Stain the Bacteria:** Follow the procedure illustrated in Figure 15.2 to stain your bacteria. Use a separate slide for each specimen. ***Note:*** Unlike our prior procedures, in this procedure we do not prepare heat-fixed bacterial smears. We do not apply heat at any point in the procedure. Instead we place the bacteria into a drop of sheep serum and dye on the slide and drag it across the slide with a second slide. This procedure is similar to the negative stain procedure in Exercise 12. ***Caution!*** Use BSL-2 precautions if *K. pneumoniae* is stained.
2. **Prepare a Gumline Sample:** Remove your gloves and wash your hands. Then, using a sterile toothpick, obtain a sample from below the gumline in your mouth. (Do not draw blood!) Mix the sample into a drop of water or serum on a slide, and then perform a capsule stain on it.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and capsule features (if present). Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.



1. Add Dye to the Slide

Place the slide on a paper towel on the bench space. Place a drop of sheep serum at one end of a clean slide. Then, add a drop or loopful of Congo red stain. Be sure to wear gloves.



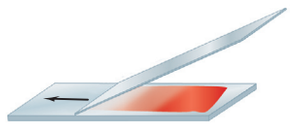
2. Add Bacteria to the Slide

With the slide resting on the paper towel-covered bench space, use an inoculating loop to transfer a small amount of bacteria into the serum-dye mixture. Don't over-inoculate and avoid spattering the mixture. Sterilize the loop after emulsifying. ***Note:*** If transferring a BSL-2 organism, use a sterile wooden stick or disposable loop/needle and dispose of it properly when finished.



3. Angle a Spreader to Contact the Emulsion

Here we use a second clean slide (or a glass coverslip) as a "spreader." Place the spreader at a roughly 30° angle so it contacts the emulsion.



4. Drag the Emulsion Across the Slide

Using a gentle and smooth motion, drag the spreader across the slide so the emulsion is thinly spread over the slide. This should be one uninterrupted and fluid motion; do not pick up the spreader until the end of the slide is reached or you will have a choppy preparation. Dispose of the spreader slide in a jar of disinfectant or sharps container.



5. Air-Dry the Slide

Allow the sample to air-dry, do not heat it at all.



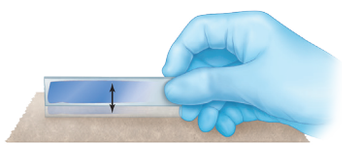
6. Add the Second Dye

Place the slide over a staining tray or beaker and cover the spread with Maneval's stain for 1 minute.



7. Rinse Off the Maneval's Stain

Grasp the slide with a slide holder and dip it into a jar of distilled water two or three times to rinse off the Maneval's stain. Change the water in the jar frequently.



8. Remove Excess Water

Gently tap the edge of the slide on a paper towel to remove excess water, then set it down to air-dry. When dry, observe under oil immersion.

FIGURE **15.2** **Capsule Stain Procedure**

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 15

DATA SHEET

Capsule Stain

During the Lab

1 Record your observations in the provided table. Use a separate line for each organism from your gumline sample that illustrates a capsule or an unusual cell morphology (cell shape and/or arrangement).

Organism	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Capsule (+/–)

Lab Related Questions

1 How many dyes are used in the capsule stain?

a. 0
b. 1
c. 2
d. 3
e. 4

2 Choose the true statement(s) about capsules (select all that apply).

a. Capsules appear colorless after a properly performed capsule stain.
b. All bacteria make capsules.
c. Capsules are a virulence factor for some bacteria.
d. Capsules are made of polysaccharides.
e. Capsules are only made by select Gram-positive bacteria.

3 The capsule stain is considered a(n) _____ stain.

a. selective
b. structural
c. basic
d. acidic
e. simple

4 Some of the following steps are used in the capsule staining procedure, but some of the steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Decolorize the slide.

_____ Add Maneval's stain to the slide.

_____ Add Congo red to the slide.

_____ Add carbolfuchsin to the slide.

_____ Heat fix the samples on the slide.

_____ Add bacteria to the slide.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The capsule stain requires steaming the dye into the capsule.

_____ The capsule staining technique incorporates aspects of the negative staining technique.

_____ The capsule stain involves an acidic dye and a mordant.

_____ Bacteria that have a capsule are resistant to phagocytosis.

6 Capsules are a(n) _____ structure.

a. intracellular
b. extracellular
c. cell wall
d. cellulose
e. heat-resistant

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 15
DATA SHEET
(continued)

Critical Thinking

1 Some oral bacteria produce an extracellular capsule. Why may a capsule benefit these cells?

2 If having a capsule is an important advantage for certain pathogens, then why wouldn't evolution favor all pathogens being encapsulated?

— Lab Notes —

EXERCISE 16

Endospore Stain

After doing this exercise, you should be able to:

1. Perform an endospore stain on bacterial smears.
2. Describe the structure and function of bacterial endospores.
3. Explain how the endospore stain works.
4. Understand the clinical significance of bacterial endospores.
5. Perform oil-immersion microscopy to view your own endospore stained bacterial samples.

Background

In this lab exercise, you will perform a structural stain called the **Schaeffer-Fulton method** to stain **endospores**, dormant structures that some bacterial species can make in response to harsh environmental conditions (e.g., nutrient depletion, unfavorable temperatures [from boiling to freezing], radiation, high population density, etc.). Because endospores are highly resistant to heat and chemicals (including disinfectants and drugs), the capacity to convert into this dormant form (undergo **sporulation**) allows bacteria to survive adverse conditions for long periods. The record for longevity goes to bacterial spores that were trapped in salt crystals about 250 million years ago and revived in a lab in the year 2000.

Endospores' resistance is due to a combination of factors, including a tough outer covering made of the protein **keratin**, its dehydrated state; DNA protective proteins; and other adaptations. When conditions are suitable again, endospores germinate into metabolically active **vegetative cells**. The keratin in the spore coat also resists staining; therefore, in the Schaeffer-Fulton method (Fig. 16.1), we use steam to drive the primary stain, malachite green, into spores. Alternatively, malachite green can be left on the slide for 15 minutes or more to stain the spores. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells and **spore mother cells** (which are responsible for producing the endospore) are easily decolorized with water and then counterstained with safranin (Fig. 16.2).

Endospores may be in the middle of the cell (**central**), at the end of the cell (**terminal**), or between the end and middle of the cell (**subterminal**). In some species, endospore location is variable, cells may exhibit a combination of terminal and subterminal, for instance. We also classify endospores by shape—either **spherical** or **elliptical** (**oval**)—and size relative to the cell (that is, whether they cause the mother cell to look swollen or not). Figures 16.3 through 16.5 show the diversity of endospore features.

The genera *Clostridioides*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Sporolactobacillus*, *Sporosarcina*, *Thermoactinomyces*, and *Oscillospira* are all examples of endospore-forming bacteria. Among these, *Bacillus*, *Clostridium*, and *Clostridioides* are the clinically important groups. In this exercise, we focus on *Bacillus* as our endospore model. Given enough time, *Bacillus* species will start to make endospores in most media that support their growth. However, we can accelerate sporulation by enriching growth media with manganese because several sporulation enzymes require manganese as a required cofactor. Your instructor may supply you either with cultures grown in sporulating agar or with older cultures not grown on such agar.

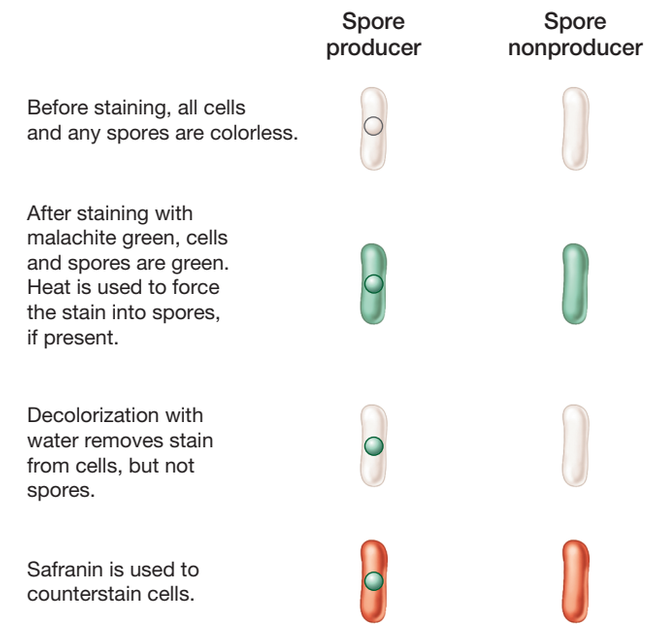


FIGURE **16.1** **Schaeffer-Fulton Spore Stain Overview** • Upon completion, endospores are green, vegetative and spore mother cells are red.

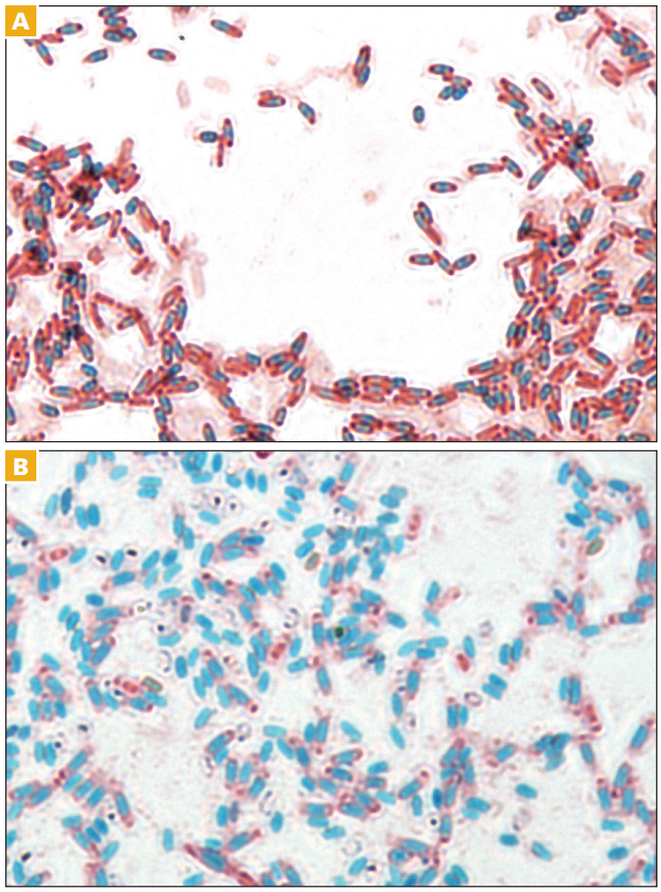


FIGURE **16.2** **Culture Age Can Affect Sporulation** • Bacteria that can form endospores do so in response to environmental stress, so as nutrients are depleted in older cultures, sporulation is more common. These two cultures illustrate different degrees of sporulation. (A) Most cells in this specimen contain endospores; very few have been released. The light pink rods are probably spore mother cells that have released their spore and have died. (B) This specimen consists mostly of released endospores.

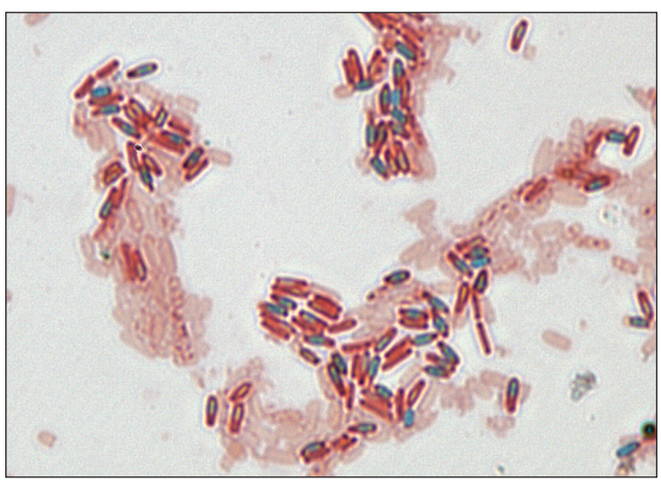


FIGURE **16.3** **Central Elliptical Endospores** • Most of these *Bacillus megaterium* endospores are centrally positioned, but some are subterminal. All are elliptical, but they do not distend the mother cell.

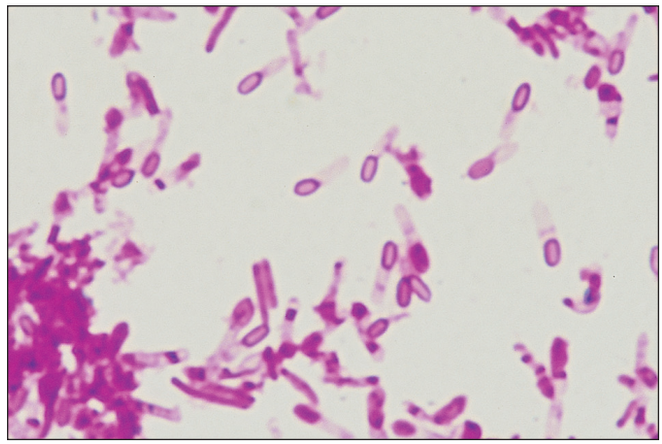


FIGURE **16.4** **Terminal Elliptical Endospores** • Shown is *Clostridium tetani* stained by a different endospore stain protocol using carbolfuchsin. Notice how the endospores have caused the ends of the cells to distend (swell).

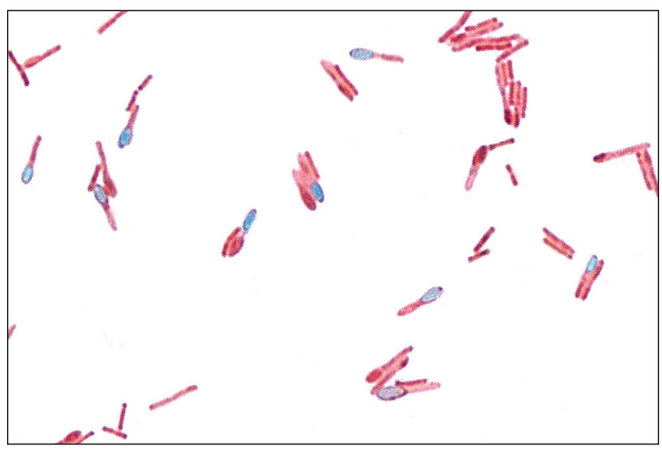


FIGURE **16.5** **Subterminal Elliptical Endospores** • In these *Clostridium butyricum* cells, most of the green endospores are "capped" by red cytosol, indicating that they are subterminal. Also notice how the spores distend the mother cell, an easily identified characteristic.

A Note on Endospore Function

Students often confuse bacterial spores with fungal spores. It's important to know that unlike fungal spores, bacterial spores do *not* have a reproductive function. A key goal of reproduction is to increase a population. Although the terminology "spore mother cell" may imply a reproductive capacity, endospore formation is not reproductive. Instead, it simply allows the original cell to enter a dormant spore state to weather out harsh conditions and germinate when the environment is more hospitable. The phrase "spore mother cell" is used to emphasize that the cell gives rise to a given spore. Upon spore completion, the mother cell (or rather, what remains of the original cell) is simply a husk that, once destroyed, releases the spore into the environment.

Clinical Applications

The endospore stain is a differential stain used to detect the presence, shape, and location of endospores in bacterial cells. Only a few genera produce spores. Most common are the genera *Bacillus* (more than 100 species) and *Clostridium* (more than 160 species). In addition, several "new" genera formerly classified as *Bacillus* species are also endospore producers. These include *Brevibacillus*, *Geobacillus*, *Paenibacillus*, and *Virgibacillus*, among others.

Most *Bacillus* species are soil, freshwater, or marine **saprophytes** (organisms that live off decaying organic matter), but two are pathogens. *B. anthracis* causes anthrax, and *B. cereus* causes two kinds of food poisoning: emetic and diarrheal. Most *Clostridium* members are harmless soil or aquatic saprophytes; some are notorious pathogens—namely, *C. tetani* (tetanus), *C. botulinum* (botulism), and *C. perfringens* (gas gangrene).

Because endospores are especially resistant to chemicals, heat, and other environmental stresses, they are quite challenging to destroy both in patients and in clinical environments. For example, the pathogen *Clostridioides difficile* (formerly *Clostridium difficile*), which causes infectious diarrhea, can enter the dormant endospore state in response to drug therapies. Thus, it is common for patients who successfully recover from a bout of *C. difficile* diarrhea to relapse within weeks as the spores germinate and reenter an actively growing vegetative state.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Clean glass microscope slides
- ❑ Squirt bottle of distilled water
- ❑ Bibulous paper and/or paper towels
- ❑ Staining tray or glass beaker to collect dye waste rinsed off slides
- ❑ Heating apparatus (beaker of water on a hot plate)
- ❑ Slide holder
- ❑ Malachite green stain
- ❑ Safranin stain
- ❑ Recommended organisms:
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus cereus*
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus coagulans*
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus megaterium*
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus subtilis*

Note: Tryptic soy agar can be substituted for sporulating agar, but incubation times should be adjusted to 48 hours and 5 days.

Recipe for Sporulating Agar

Pancreatic digest of gelatin	6.0 g
Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Agar	15.0 g
Manganous sulfate ($MnSO_4$)	0.3 g
Distilled or deionized water	1.0 L

Final pH 6.6 ± 0.2 at 25°C

PROCEDURE

Today you will visualize endospores of as many as four *Bacillus* species. To do this, you will use the Schaeffer-Fulton endospore staining method. In addition, you will be supplied with two cultures of different ages of each species to see if there is a relationship between culture age and endospore production. While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Prepare the Bacterial Smears:** For each organism, prepare and heat fix two smears on the same slide. One smear should be the 24-hour culture, and the other the 48-hour culture (or 48-hour and 5-day cultures if TSA is the medium). Divide the work of slide preparation within your lab group. Minimally, each student should prepare one slide with smears of the 24-hour and 48-hour cultures of one species. ***Caution!*** Use BSL-2 precautions if you have subcultured soil isolates for endospore staining.

2 **Stain the Bacteria:** Follow the procedure illustrated in Figure 16.6 to stain your bacteria. Use a steaming apparatus like the one shown in Figure 16.7. Perform the steaming step in a well-ventilated area (preferably inside a fume hood). Always avoid inhaling the fumes. If you must carry the slide to and from the steaming apparatus, put it in a covered petri plate.

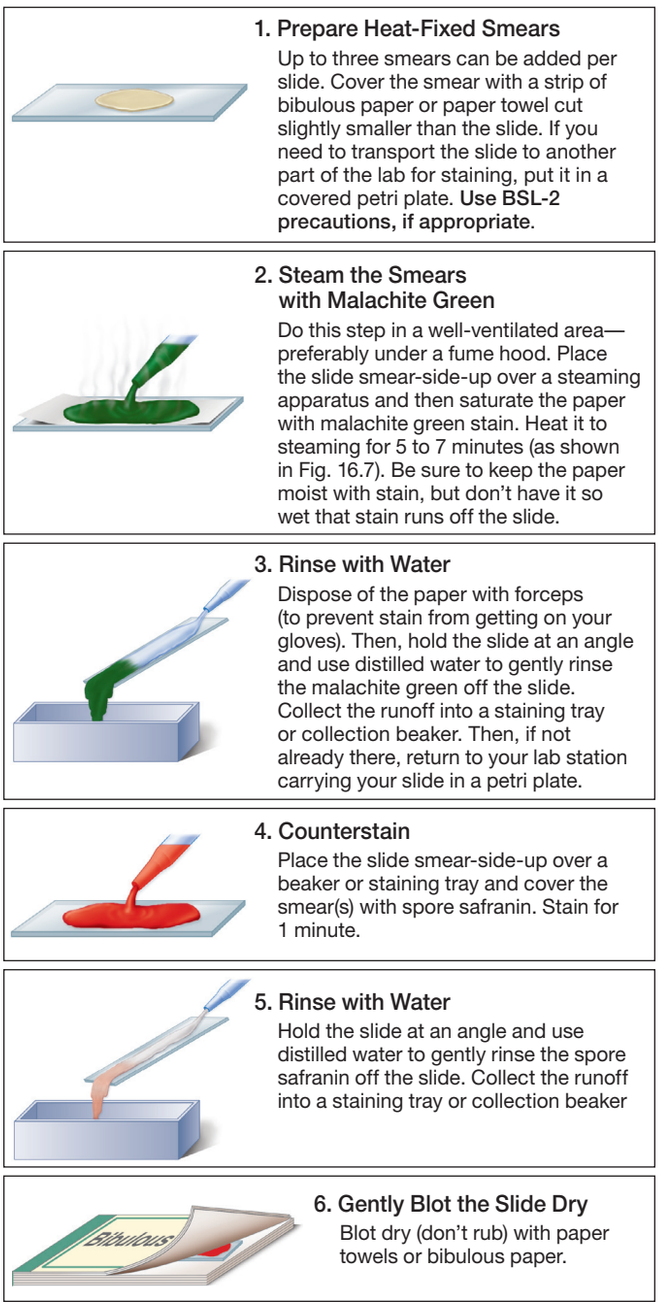


FIGURE **16.6** **Schaeffer-Fulton Endospore Staining Procedure** • Perform this procedure with adequate ventilation (preferably in a fume hood), eye protection, a lab coat, and gloves. ***Caution!*** Employ BSL-2 precautions if you have subcultured a soil isolate for endospore staining.

3 **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of visible endospores for each species. Be sure to note their shapes, locations, and relative sizes.

4 **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.



FIGURE **16.7** **Steaming the Slide During the Endospore Stain** • Carefully steam the slide to force the malachite green into the endospores. Do not boil the slide or let it dry out. Keep it moist with stain for up to 7 minutes of steaming. ***Caution!*** This should be performed in a well-ventilated area (preferably in a fume hood) while wearing a lab coat, safety glasses, and gloves.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 16

DATA SHEET

Endospore Stain

During the Lab

1 Record your observations in the provided table. Use a separate line for each bacterial smear you observed.

Organism (include culture age)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells and spores)	Spores (present or absent)	Spore Shape and Position (if present)

Lab Related Questions

1 What is the primary stain in the Schaeffer-Fulton endospore staining method?

a. Methylene blue
b. Malachite green
c. Safranin
d. Crystal violet
e. Carbolfuchsin

2 Choose the true statement(s) about endospores (select all that apply).

a. Endospores resist staining.
b. Endospores stain pink in the endospore staining procedure.
c. *Bacillus* species make endospores.
d. Endospores are dormant structures.
e. Endospores are heat sensitive.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ An endospore's heat resistance is in part due to its low water content.

_____ Endospores serve as reproductive structures.

_____ Endospores are a clinical challenge because they can resist drug treatments.

_____ Adding magnesium to growth media can encourage spore-forming bacteria to make endospores.

4 The Schaeffer-Fulton endospore staining method is considered a(n) _____ stain.

a. acidic
b. basic
c. selective
d. simple
e. structural

5 Some of the following steps are used in the Schaeffer-Fulton endospore staining method, but some of the steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Rinse the primary stain off the slide with water.

_____ Steam the slide with the primary dye for 5–7 minutes.

_____ Decolorize the slide with acid-alcohol.

_____ Add safranin dye to the slide.

_____ Add Maneval's stain to the slide.

_____ Heat fix the samples on the slide.

_____ Rinse the counterstain off the slide with water.

_____ Blot the slide dry.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 16
DATA SHEET
(continued)

6 The process of endospore formation is called _____.

7 Endospores will appear _____ while mother cells appear _____ at the end of the Schaeffer-Fulton endospore staining method.

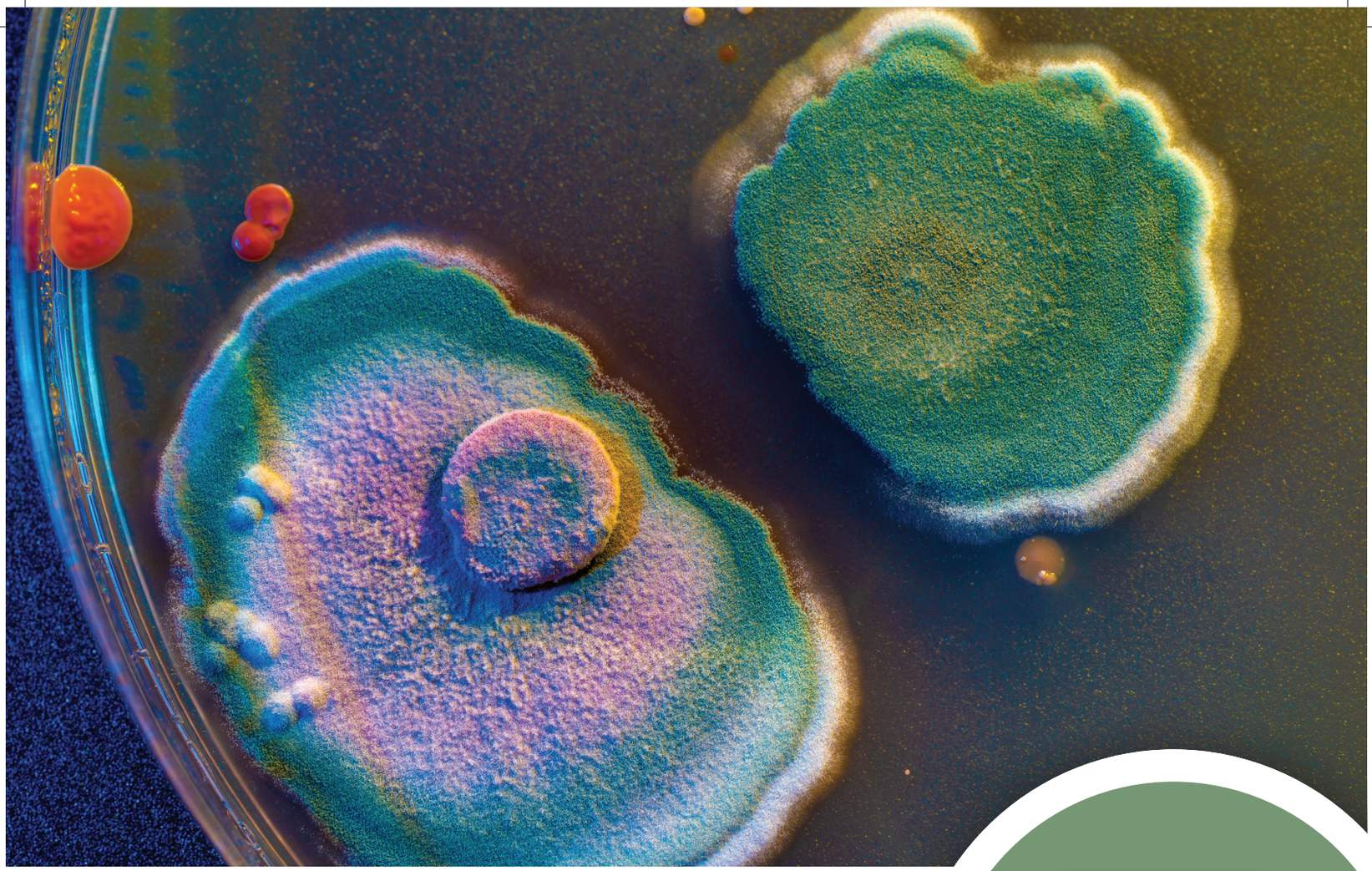
a. green; blue
b. blue; green
c. green; red
d. red; green
e. green; violet

Critical Thinking

1 Design an endospore staining procedure where carbolfuchsin and methylene blue are used as the dyes. Describe your protocol based on the fundamentals of the Schaeffer-Fulton endospore staining method.

2 Some people harbor *Clostridioides difficile* as a natural inhabitant of their gastrointestinal tract. However, this bacterium is unlikely to cause diarrhea unless the person recently underwent an antibiotic regimen (especially a broad-spectrum antibiotic treatment). Why would taking a broad-spectrum antibiotic treatment put a patient at risk for *C. difficile* diarrhea?

— Lab Notes —



SECTION

4

Media Properties and Conditions that Affect Bacterial Growth

In This Section

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EXERCISE 18 Thioglycollate Broth **195**

EXERCISE 19 Anaerobic Jar **203**

EXERCISE 20 The Effect of Temperature on Microbial Growth **211**

EXERCISE 21 The Effect of pH on Microbial Growth **219**

— Lab Notes —



EXERCISE 17

Evaluating Complex Versus Defined Media

After doing this exercise, you should be able to:

1. Differentiate between fastidious and non-fastidious bacteria.
2. Define synthetic versus complex media and describe how they are different.
3. Compare chemotrophs to phototrophs.
4. Compare heterotrophs to autotrophs.
5. Apply aseptic bacterial transfer and culture techniques.

Background

All organisms need an energy source and a carbon source. Regarding energy sources, we can classify organisms as either **chemotrophs** or **phototrophs**. Chemotrophs get their energy by breaking down nutrients, while phototrophs rely on solar energy. In terms of carbon sources, we classify organisms as either **autotrophs** ("self-feeding" organisms that convert inorganic carbon such as CO_2 into organic carbon) or **heterotrophs** (organisms that obtain their carbon from organic nutrients). Putting these categories together, plants are **photoautotrophs** while we (and most clinically important microorganisms) are **chemoheterotrophs**.

Some heterotrophs are metabolically flexible and can build most of their organic compounds from scratch. Such microbes only need a few simple organic compounds to grow and divide, which is why we classify them as **non-fastidious**. In contrast, **fastidious** microbes are less self-sufficient; they can't build their necessary organic compounds from scratch and instead they obtain those compounds from their environment. This means fastidious microbes usually require an enriched environment to grow and divide, while non-fastidious microbes can survive on a few simple organic nutrients. In general, autotrophs are the least fastidious and therefore the most self-reliant in terms of nutrients, even as compared to the most non-fastidious heterotrophs.

To successfully grow a microbe in the laboratory, we must satisfy its nutritional needs. The absence of even one required nutrient can prevent growth. The more fastidious the organism, the more ingredients a medium typically must have. **Complex media** (also called **enriched media**) contain extracts from, or digests of, plant and/or animal matter and are usually rich in nutrients. Because complex media include less precisely defined organic nutrients, one batch may vary from another. For example, the amount of glucose in the blood added to one batch of blood agar may vary from the blood used to make another batch of such agar. Complex media are useful for growing the greatest variety of culturable microbes and are therefore commonly used in clinical microbiology labs to assess clinical samples.

In contrast, **defined media** (sometimes called **synthetic media**) get their name from the fact that each organic and inorganic ingredient is precisely known and quantified, which means these media are accurately reproducible from batch to batch. As a result, defined media usually grow a narrower range of organisms, usually a specific known organism with well-characterized nutritional needs.

Regardless of a media's classification as complex or defined, we can prepare it in solid or liquid formats; that is, we can find any of these media poured as broths (which is what we'll use in this lab exercise), or as plated and slant formats. To make plated or slant formats, we would add **agar** (a solidifying agent extracted from red seaweed), to broth media. Because most clinically important bacteria do not digest agar, it's a good solidifying agent for clinical microbiology applications.

A Note on Growth Rates

In this exercise, you will assess the relative growth rates of different bacteria grown in different media. In general, all organisms increase their population the fastest when grown under optimal growth conditions. By comparing the turbidity (cloudiness) of each culture, you can determine how each media supported (or failed to support) the growth of a given microbe. By comparing how well each species grew in enriched versus defined media, you can rank the bacteria by their relative fastidiousness.

The term *growth rate*, as used in this manual, refers to reproductive rate. While inherent growth rates can differ from one species to another, we can legitimately compare growth rates of the same organism in different media. However, growth rate comparisons between *different* organisms in the *same* medium require us to assume the inherent growth rates are the same

(or similar). In this exercise we will make this assumption, with the understanding that it may not be true.

Clinical Applications

You've probably had a sample, such as a throat swab, sent to a clinical microbiology lab for a definitive diagnosis. Upon receiving that sample, a clinical lab scientist would have streaked it onto a complex media called blood agar and then checked the growth characteristics the next day to learn about the bacteria present in the sample. A bacterium's growth profile on different media helps microbiologists determine the pathogen's identity. A variety of studies suggest most bacteria are **noncultivable in vitro** (not growable in the lab setting outside of an animal model). A best guess is that we can only grow perhaps 2% of bacterial species in the environment using existing media. When we consider body sites such as the mouth, it's estimated that using currently available methods, we can only grow perhaps half of the species present. Until 2018, *Treponema pallidum* (the causative agent of the sexually transmitted infection syphilis) was not cultivable outside of an animal model. Ultimately, if we are to understand how to more effectively treat bacterial pathogens, we must be able to study them in the laboratory, and understanding what they need to grow is an important step toward that process.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile transfer pipettes (4)
- ❑ Tubes (5 each) of:
  - ☐ Brain-heart infusion broth (BHIB)
  - ☐ Glucose salts broth (GSB)
  - ☐ Nutrient broth (NB)
- ❑ Fresh broth cultures of these four recommended bacteria:
  - ☐ *Escherichia coli*
  - ☐ *Lactococcus lactis*
  - ☐ *Moraxella catarrhalis* (some strains are BSL-2)
  - ☐ *Staphylococcus epidermidis*

Medium Recipes

Nutrient Broth (NB)

Beef extract	3.0 g
Peptone*	5.0 g
Distilled or deionized water	1.0 L

*A protein extract typically made by the breakdown of milk, animal tissue, plants, or microbial cultures (e.g., yeast).

Brain-Heart Infusion Broth (BHIB)

Calf brains, infusion from 200 g	7.7 g
Beef heart, infusion from 250 g	9.8 g
Proteose peptone	10.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	2.0 g
Sodium chloride (NaCl)	5.0 g
Disodium phosphate (Na_2HPO_4)	2.5 g
Distilled or deionized water	1.0 L

Glucose Salts Broth (GSB)

Glucose ($C_6H_{12}O_6$)	5.0 g
Sodium chloride (NaCl)	5.0 g
Magnesium sulfate ($MgSO_4$)	0.2 g
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium phosphate (K_2HPO_4)	1.0 g
Distilled or deionized water	1.0 L

PROCEDURE

In this exercise, you will examine how media composition affects what organism(s) can grow. You will also assess the relative fastidiousness of different bacteria. While performing the lab, wear a lab coat, gloves, and safety glasses.

1. **Obtain Your Media:** Working with your group, obtain five tubes of each medium (a total of 15 tubes). Label each tube with your group name, the date, and the medium's name.

2 **Designating the Tubes for Certain Bacteria:** Divide your 15 tubes of media into five sets; each set should contain the following: one tube of nutrient broth (NB), one tube of brain-heart infusion broth (BHIB), and one tube of glucose salts broth (GSB). Next, label each media set with a different organism name (*E. coli*, *L. lactis*, *M. catarrhalis*, and *S. epidermidis*); label the last set of media tubes "uninoculated."

3 **Inoculate Your Media with the Designated Bacteria:** Mix each stock bacterial tube thoroughly, but be careful not to splash into the cap. ***Caution!*** Use BSL-2 precautions when transferring *Moraxella catarrhalis*. Use a sterile transfer pipette to transfer 1 drop of the given organism into each type of media (e.g., add 1 drop of *S. epidermidis* into the NB media, 1 drop into the BHIB media, and 1 drop into the GSB media); repeat this process until each organism has been inoculated into each medium. Instead of repeatedly going into the stock sample tube, which will increase the chances of contamination, you may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to each tube without flaming them. In your group, leave one of each tube uninoculated.

CAUTION!

- When you go to inoculate the media, insert the pipette well into the tube (but avoid touching the glass or the medium).
- Be careful not to spatter as you deliver the drop(s) to each tube, or accidentally drip organism between tubes. Clean up the spill if you do.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow-out") the pipette because you will create aerosols.
- Properly dispose of the pipette when finished.

4 **Incubate the Samples:** Incubate all tubes (including the uninoculated ones) at 35 ± 2°C for 24–48 hours.

5 **Clean up:** Save or dispose of the original cultures as directed by your instructor.

➜ Next Lab Session

1 **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples) and organize your tubes into groups by organism in one direction and by medium in the other.

2 **Mix and Observe the Samples:** Mix all of your tubes well and examine them for turbidity (cloudiness). Avoid splashing broth into the caps. Score relative amounts of growth using "0" for no growth, and "1," "2," and "3" for successively greater degrees of growth. (***Note:*** Increased cloudiness or turbidity reflects greater growth.) Choose the tube (regardless of medium) with the most turbidity to act as a "3." And use the uninoculated tubes as your "0" reference point.

3 **Record your results**: Note all observations and results on the data sheet at the end of this lab. (***Note:*** Results may vary. Record what you see, not what you expect or what other groups are getting for results.)

4 **Clean up:** Dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 17

DATA SHEET

Evaluating Complex Versus Defined Media

During the Lab

1 In the provided table, score the relative amount of growth in each tube. Compare the five tubes of each medium with each other, as well as the amount of growth for each organism in the various media. Use "0" for no growth, "3" for abundant growth, and "1" and "2" for degrees of growth in between. Use the most turbid of the 15 broths as your "3."

Specimen	Nutrient Broth	Brain-Heart Infusion Broth	Glucose Salts Broth	Interpretation (relative fastidiousness)
Uninoculated control				**Not applicable**

2 In the provided table, identify the carbon and nitrogen source(s) in each medium and then characterize each as "defined" or "complex." If the media is complex, list the ingredient(s) that make it so. Finally, use your results to characterize each medium based on its ability to support growth of a wide range of organisms (poor, fair, good).

Media Description	Nutrient Broth	Brain-Heart Infusion Broth	Glucose Salts Broth
Carbon source(s)			
Is this media complex or defined?			
Ingredient(s) making it "complex" (if applicable)			
Ability to support a wide range of organisms (poor, fair, good)			

Lab Related Questions

1 Which of the following is (are) classified as complex media? (Select all that apply.)

a. Glucose minimal salts broth
b. Brain-heart infusion broth
c. Blood agar
d. Nutrient broth
e. Agar

2 Select the true statement(s) about clinically important microbes (select all that apply).

a. Most can use solar energy.
b. Most require an organic carbon source.
c. Most are autotrophs.
d. Most are heterotrophs.
e. Most are chemotrophs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 17
DATA SHEET
(continued)

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Defined media are commonly used for isolating an unknown pathogen from a clinical sample.

_____ Complex media are precisely defined and replicable from batch to batch.

_____ Most bacteria are non-cultivable using current laboratory media.

_____ Adding agar to a medium makes it a complex medium.

4 In this exercise, we compared the _____ of each culture to determine how each media supported (or failed to support) the growth of a given microbe.

5 Select the false statement(s) about fastidious microbes. (You may select more than one answer choice.)

a. They are more likely to grow on defined media as compared to complex media.
b. They are more likely to be heterotrophs than autotrophs.
c. They include most clinically important microbes.
d. They can make all their own complex organic compounds from scratch.
e. They do not require a carbon source.

6 Select the ingredient(s) that would not be included in defined media. (You may select more than one answer choice.)

a. Yeast extract
b. Glucose
c. Water
d. Blood
e. Salt
f. Peptone

Critical Thinking

1 Use the provided data to fill in the missing data—blanks **a** through **f**—with the correct term, *growth* or *no growth*.

	Bacterium 1	Bacterium 2
Glucose Salts Broth	No growth	Growth
Glucose Salts Broth + Tryptophan (an amino acid)	No growth	a.
Glucose Salts Broth + Histidine (an amino acid)	b.	c.
Glucose Salts Broth + Peptone	No growth	d.
Glucose Salts Broth + Peptone + Sheep Blood	e.	f.
Sheep Blood Agar	Growth	Growth

2 Explain your reasoning for the response you entered in space "a" in the table.

3 Explain your reasoning for the response you entered in space "b" in the table.

4 Based on the data in the table in Question 1, would you classify Bacterium 1 as fastidious or non-fastidious? Explain your reasoning.

EXERCISE 18

Thioglycollate Broth

After doing this exercise, you should be able to:

1. Define aerotolerance and differentiate aerotolerance by culturing microbes in thioglycollate medium.
2. Describe how thioglycollate media allows us to determine a microbe's aerotolerance.
3. State the clinical importance of determining aerotolerance for infectious agents.
4. Apply aseptic bacterial transfer and culture techniques.

Background

Thioglycollate broth is an enriched (or complex) medium that usually contains yeast extract and pancreatic digest of casein. We use this generalized enriched recipe for this lab. However, if we were trying to grow especially fastidious bacteria, we would add a variety of supplements like hemin (an iron-containing compound extracted from red blood cells) and vitamin K to support their growth; recall that many pathogens are particularly fastidious.

A Note on Microbial Oxygen Tolerance

Most microorganisms survive within a range of environmental conditions, but not surprisingly, growth is optimal when conditions are ideal. Oxygen (O_2) levels especially influence microbial growth. **Aerotolerance** is the ability or inability to live in the presence of oxygen.

Some organisms, called **obligate aerobes**, require oxygen for their metabolic needs. These organisms, like us, require oxygen to extract energy from nutrients (to carry out aerobic respiration). On the opposite end of the aerotolerance spectrum, we encounter **obligate** (**strict**) **anaerobes**, which don't use oxygen gas for their metabolic pathways. Furthermore, obligate anaerobes don't survive for long in the presence of oxygen gas (O_2) because they can't sufficiently protect themselves from toxic oxygen intermediates—especially superoxide (O^{-2}) and hydrogen peroxide (H_2O_2)—that arise when oxygen participates in certain chemical reactions. Some microbes called **facultative anaerobes** grow in the presence *or* absence of oxygen. When oxygen is available, they use it to carry out aerobic respiration; but if oxygen is unavailable they either carry out fermentation or respire anaerobically (reducing sulfur or nitrate instead of oxygen). (Refer to Appendix A and Section 5 for more information on anaerobic respiration and fermentation.) Still other organisms, called **aerotolerant anaerobes**, are indifferent to oxygen; they can survive when it is around, but they don't use it. Lastly, **microaerophiles**, as the name suggests, survive only in environments containing lower than atmospheric oxygen levels. Specific microaerophiles, called **capnophiles,** only survive if O_2 levels are reduced and carbon dioxide (CO_2) levels are elevated. Of course, bacteria, to include those that are pathogens, are diverse in their oxygen needs and span the range of these described categories.

How Thioglycollate Media Reveals an Organism's Aerotolerance

As a complex medium, thioglycollate broth nutritionally supports the growth of a broad variety of organisms, but it is particularly well adapted for cultivating organisms that can't grow in atmospheric levels of oxygen. Why is that? The answer has two parts: (1) the effects of autoclaving and (2) some specialized ingredients. Let's tackle autoclaving effects first.

As with most media, before inoculation, we autoclave the thioglycollate media to sterilize it. This ensures we are specifically studying our target sample, but importantly this process also removes most of the medium's free oxygen gas (O_2). After autoclaving, the media cools and oxygen diffuses back into the tube media in a gradient manner, leveling out at about 20–21% O_2 at the top of the tube, about 2–10% O_2 toward the middle of the tube, and no oxygen gas in the bottom portion of the tube. Autoclaving alone doesn't allow us to establish an area of the tube that is verifiably free of oxygen gas. To maintain a verifiable absence of oxygen at the bottom of the tube, specialized ingredients and oxygen indicators are added to the medium.

The sodium thioglycollate (an organic sodium salt) and L-cystine (an amino acid) in the media reduce oxygen to water, which generates an anaerobic (oxygen-free) environment deeper in the tube. An oxygen detector called **resazurin** indicates if an oxygen-free environment exists at the bottom of the tube. Resazurin appears pink when oxidized, which occurs when oxygen gas is present, and colorless when reduced—when oxygen gas is absent (Fig. 18.1). A small amount of agar is also added to the medium; while it's not enough agar to solidify the media, it is enough to slow oxygen diffusion through the media.

Based on the generated oxygen gradient in thioglycollate media, we expect the following growth patterns that reveal an organism's aerotolerance (Fig. 18.2):

- **Obligate aerobes:** These organisms require oxygen to conduct aerobic respiration and therefore only thrive at the top of the medium.
- **Facultative anaerobes:** These microbes will grow throughout the medium, but will be denser near the top of the medium because of the higher ATP yield from aerobic respiration compared to fermentation or anaerobic respiration.
- **Aerotolerant anaerobes:** Indifferent to oxygen levels, these microbes live uniformly throughout the medium.
- **Microaerophiles:** These microbes grow in the middle region of the medium.
- **Obligate (strict) anaerobes:** Because even small amounts of oxygen are lethal to these organisms, they only grow toward the bottom of the medium.

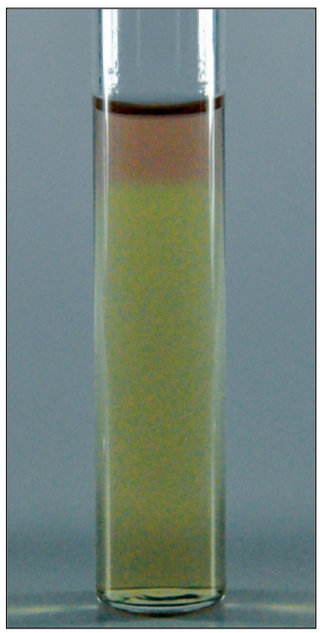


FIGURE **18.1** **Aerobic and Anaerobic Zones in Thioglycollate Broth** ● The pink region in the upper, aerobic portion of the broth results from oxidation of the indicator resazurin. In the lower, anaerobic portion of the tube, the dye is not reduced so the typical straw color of the medium shows.

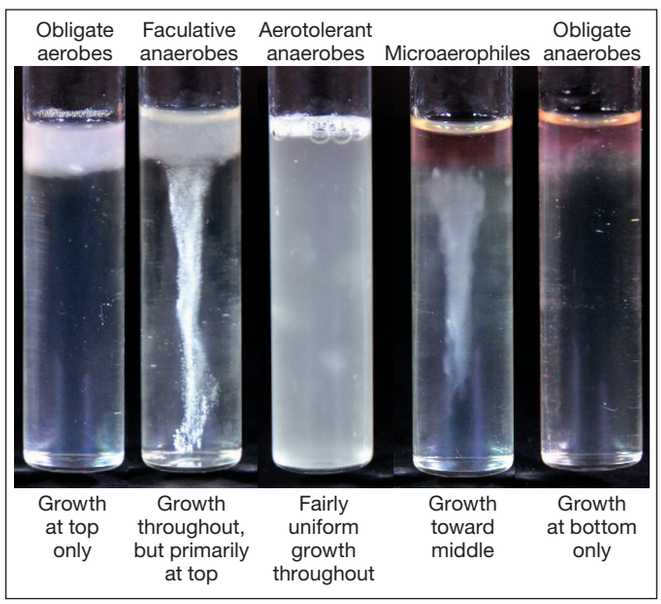


FIGURE **18.2** **Growth Patterns in Thioglycollate Broth** ● Compare these tubes to the uninoculated tube in Figure 18.1.

Clinical Applications

Thioglycollate broth is an enriched liquid medium that promotes growth of a wide variety of fastidious and nonfastidious microorganisms. While it can be used to grow and temporarily store and/or transport a pure culture, it is more often used to study an uncharacterized sample, which may or may not contain a mixture of bacteria with diverse aerotolerances. It's especially useful in diagnostic clinical microbiology because many pathogens are not only fastidious about their nutrients, but they're often particular about how much oxygen gas they encounter. Consequently, this media is primarily used to grow anaerobic and microaerophilic bacteria from blood samples. Thioglycollate media is also useful for isolating important pathogens such as *Clostridium* species. For example, *C. perfringens*, which causes gas gangrene, and *C. difficile*, which causes an infectious diarrhea that can progress to pseudomembranous colitis, are both obligate anaerobes.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Tubes of thioglycollate broth (4)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Clostridium butyricum* (BSL-1 strain ATCC 19398)
  - ☐ *Staphylococcus epidermidis*

Medium Recipe

Thioglycollate Broth

Yeast extract	5.0 g
Pancreatic digest of casein	15.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	5.5 g
Sodium chloride (NaCl)	2.5 g
Sodium thioglycollate	0.5 g
L-cystine ($C_6H_8O_4(NH_2)_2S_2$)	0.5 g
Agar	0.75 g
Resazurin	0.001 g
Distilled or deionized water	1.0 L



PROCEDURE

Today you will be inoculating three different bacteria into thioglycollate broth to determine their aerotolerance. Your instructor may have additional organisms or may have you collect environmental samples to test. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain four tubes of thioglycollate media and label them with your name, the date, and the medium's name.
2. **Label Your Tubes:** Label three of the tubes with the name of an organism (add a different organism name on each tube) and label the fourth tube "Uninoculated."
3. **Inoculate Your Media with the Designated Bacteria:** Using your loop, inoculate three broths with the organisms provided. (***Note:*** When inoculating thioglycollate broth, it helps to dip the loop all the way to the bottom of the tube and gently mix the broth with the loop as you remove it. Do not shake the tube. Finish mixing by gently rolling the tube in an upright position between your hands.) Do not inoculate the fourth tube.
4. **Incubate the Samples:** Incubate all tubes (including the uninoculated ones) at 35 ± 2°C for 24–48 hours.
5. **Clean up:** Save or dispose of the original cultures as your instructor directs.

➜ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe the Samples:** Using the uninoculated control as a comparison, examine and note the location of the growth in all tubes.
3. **Record your results:** Note all observations and results on the data sheet at the end of this lab. (***Note:*** Results may vary. Record what you see, not what you expect or what other groups are getting for results.) Also, note any changes that may have occurred as a result of incubation, especially in the colored region at the surface.
4. **Clean up:** Dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 18
DATA SHEET

Thioglycollate Broth

During the Lab

1 In the table provided, list three pieces of information for each of your four tubes:

- First, sketch each culture showing the location of growth.
- Second, indicate the amount of growth in the aerobic and anaerobic regions using the following system:
  0 = no growth
  1–2 = moderate growth
  3 = abundant growth
- Third, place each organism into an aerotolerance category.

Specimen	Location of Growth in Medium	Aerotolerance Category
Uninoculated control		Not applicable

2 Additional observation notes (e.g., media coloration).

Lab Related Questions

1 Why is it essential that you avoid mixing/agitating thioglycollate broth at all points in this lab exercise? (Select all that apply.)

a. Mixing can disrupt the oxygen gradient in the media.
b. Mixing can change the nutrient gradient in the broth.
c. Mixing increases the risk of contaminating the sample.
d. Mixing can disperse the bacteria into areas where they did not actually grow.
e. Mixing can alter the aerotolerance of the samples inoculated into the media.

2 Select the true statement(s) about thioglycollate broth (select all that apply).

a. The dye resazurin removes oxygen from the media.
b. This media is useful for growing fastidious microbes.
c. This media can grow organisms that may not tolerate atmospheric oxygen levels.
d. When fresh, this media is normally pink throughout.
e. Thioglycollate broth is used to detect the presence of pathogens in clinical blood samples.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ If growth is absent at the top of the tube, it means obligate aerobes did not grow.

_____ Agar is added to thioglycollate medium to solidify it.

_____ If growth is absent at the bottom of the tube, it means obligate anaerobes did not grow.

_____ Regarding nutrients, this medium is considered a defined media.

4 Match the terms to their descriptions. Descriptions can be used more than once, and the terms may have more than one appropriate description, in which case you should include all the relevant descriptions from the list.

Terms:

_____ Obligate anaerobe

_____ Microaerophile

_____ Facultative anaerobe

_____ Obligate aerobe

_____ Capnophile

_____ Aerotolerant anaerobe

Descriptions:

a. Cannot grow in the presence of oxygen gas.
b. Grow in the absence of oxygen.
c. Cannot grow without gaseous oxygen.
d. Require increased CO_2 levels to grow.
e. Require reduced gaseous oxygen levels to grow.
f. Grow in the presence of oxygen.
g. Include pathogens.
h. Use oxygen in their metabolic processes.
i. Do not use oxygen in their metabolic processes.

5 Old thioglycollate can give inaccurate data. What could you expect if older thioglycollate medium was used? (Select all that apply.)

a. Fastidious bacteria would be unable to grow.
b. Strict anaerobes may not be detected.
c. Obligate aerobes would grow less than expected.
d. Microaerophiles would grow less than expected.
e. Aerotolerant anaerobes would be unable to grow.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 18 DATA SHEET

(continued)

6 Thioglycollate media has an oxygen gradient and maintains it for the medium's normal shelf life due to (select all that apply):

a. The additive sodium thioglycollate.
b. The additive CO_2.
c. Resazurin.
d. L-cystine.
e. Autoclaving effects.
f. The presence of a high agar concentration.

Critical Thinking

1 Thioglycollate medium is useful for characterizing the microorganisms present in blood cultures. However, interpreting blood cultures is challenging since normal microbiota (normal flora) from the skin at the venous blood draw site can contaminate the samples. For this reason, blood is usually taken from each arm and the growth from the blood draw of the right arm is compared to that of the sample from the left arm. Imagine you obtain the following blood sample data:

Sampled Site	Growth Detected	Growth Location in Thioglycollate Medium
Right arm	Yes	Abundant bacterial growth at the top of the tube and bacterial growth isolated at the bottom of the tube
Left arm	Yes	Bacterial growth isolated at the bottom of the tube; no other growth observed

Answer the following based on the data:

a. Would you conclude normal microbiota were detected? Explain your reasoning.

b. Would you conclude the patient has an anaerobic blood infection (bacteremia)? Explain your reasoning.

2 Oxygen is usually abundant in the lungs, so why do some bacteria that cause lung infections (e.g., *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis) evolve to be facultative anaerobes?

EXERCISE 19

Anaerobic Jar

After doing this exercise, you should be able to:

1. State why anaerobic jars are useful in the microbiology lab setting.
2. Describe the different versions of anaerobic jars and how they work.
3. State the clinical usefulness of anaerobic jars.
4. Apply aseptic bacterial transfer and culture technique to grow bacteria under anaerobic conditions in an anaerobic jar.

Background

In Exercise 18, we explored thioglycollate broth and the specifics regarding bacterial classifications by aerotolerance (obligate aerobes, facultative anaerobes, aerotolerant anaerobes, microaerophiles, and strict anaerobes). We also learned that thioglycollate is an enriched liquid medium that allows us to culture fastidious bacteria that may require reduced oxygen levels, or even anaerobic conditions to grow. And while thioglycollate broth is useful for isolating an unknown bacterium present in a specimen (e.g., patient blood samples) and/or maintaining certain samples for shorter term storage and transport, the oxygen gradient dissipates over time as oxygen diffuses through the media. Another limitation of thioglycollate broth is that, aside from revealing what oxygen levels an organism can tolerate, it does not reveal much else about the microbe's metabolic features. In such a case, we'd need to grow our bacterium on specialized biochemical test media while simultaneously maintaining the culture under the bacterium's optimal atmospheric growth conditions. An aerobic jar facilitates this.

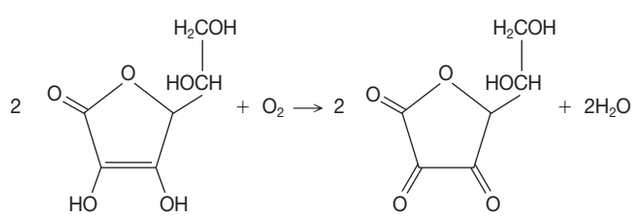
Various airtight jars and pouches allow us to culture obligately anaerobic, microaerophilic, or capnophilic microorganisms under their preferred oxygen conditions without using an anaerobic incubator and/or a CO_2 incubator. Anaerobic jars are also an ideal way to store and transport anaerobic cultures without having to first transfer the cultures to thioglycollate media. Anaerobic jar setups vary in size, but typically accommodate multiple agar plates and/or test tubes and still fit in a standard incubator. The GasPak 100 Anaerobic Jar by Becton Dickinson (BD) is an example (Fig. 19.1), it holds up to 12 petri plates.

Each system can be used with a variety of gas generating sachets (packets) that produce the desired atmosphere. For instance, BD GasPak EZ Anaerobe Sachets contain water, ascorbic acid, activated carbon, and inorganic carbonate. When removed from their wrapper and sealed in the jar, the contents are exposed to air and ascorbic acid reacts with O_2 to form dehydroascorbic acid and water (Fig. 19.2), thus making the interior of the jar anaerobic (<1% O_2). The inorganic carbonate reacts to produce an atmosphere with roughly 13% carbon dioxide (air is 0.04% CO_2).

With a slightly different composition, the BD GasPak EZ Campy Sachets ("Campy" stands for *Campylobacter jejuni*, a microaerophilic pathogen) produce microaerophilic conditions (6–16% O_2). The inorganic carbonate produces an atmosphere of 2–10% CO_2.



FIGURE **19.1** **An Anaerobic Jar** ● The large white packet inserted between the jar and the plates performs the chemistry required to produce anaerobic conditions within the jar. Condensation within the jar is an indication that the packet has performed its chemistry, but doesn't verify that anaerobic conditions have been produced. The white strip at the top contains a dye that is colorless when oxygen is absent.



Ascorbic Acid **Dehydroascorbic Acid**

FIGURE **19.2** **Reaction of Ascorbic Acid and Oxygen** ● When exposed to air in the sealed anaerobic jar, ascorbic acid reacts with oxygen to produce dehydroascorbic acid and water to make the atmosphere within the jar anaerobic. This water is often visible as condensation inside an anaerobic jar.

Oxoid AnaeroGen Atmospheric Generation Systems are an alternative to the BD systems and work in a similar way. The advantages of both systems over previous ones are that they don't require addition of water or a palladium catalyst; and, most importantly, they don't produce flammable H_2 gas as a byproduct. An indicator strip is used with the anaerobic systems to ensure that anaerobic conditions have been created. The indicator strip is soaked with methylene blue or resazurin dye that appears blue or pink, respectively, when oxidized and colorless when reduced.



Clinical Applications

Anaerobic jars are a convenient and relatively inexpensive way to grow and study anaerobic and/or microaerophilic bacteria in a standard incubator. These jars typically accommodate plated and/or broth media. This expands the types of media we can use to grow these oxygen-intolerant bacteria beyond the isolation media, thioglycollate broth (see Ex. 18). Growing microbes on diverse biochemical media is often important for isolating and better characterizing infectious agents. Also, for example, if we wished to perform an antibiotic sensitivity test on an anaerobic bacterium that was causing an infection, we'd need to incubate the sample plates under anaerobic conditions. Anaerobic jars also make it easier to transport anaerobic specimens to another laboratory, if needed.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Anaerobic jar with anaerobic gas generator packet (1)
- ❑ Nutrient agar (NA) plates (2)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Clostridium butyricum* (BSL-1 strain ATCC 19398)
  - ☐ *Staphylococcus epidermidis*



PROCEDURE

Today you will be inoculating different bacteria onto two nutrient agar plates. One plate will be incubated in an anaerobic jar and the other will be grown under standard conditions. This will allow you to make a comparison regarding which microbes grew under anaerobic versus aerobic conditions. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain two nutrient agar plates.
2. **Label Your Tubes:** Using a marking pen, divide the bottom of each plate into three sectors (or more, if your instructor has additional organisms for you to use). Label each plate with your name, the date, and organism by sector.
3. **Inoculate Your Media with the Designated Bacteria:** Using your loop, inoculate corresponding sectors of both plates with the organisms provided. (***Note:*** Inoculate with single streaks about 1 cm long.) Tape the lids in place.
4. **Place Your Anaerobic Plate in the Anaerobic Jar:** Place one of your plates into the anaerobic jar in an inverted position. Depending on your class size, more than one jar may be necessary. When all groups have placed their plates in the jar, discharge the packet as follows.

- If using a BD system, follow the packet instructions, but they should be something like this:
  - a Stick the methylene blue strip on the wall of the jar.
  - b Open the packet and place it in the jar with the label facing inward.
  - c Immediately close the jar.
  - d Examine for condensation within about 30 minutes. If none appears, start over with a new packet.
- If using an Oxoid system, follow the packet instructions, but they should be something like this:
  - a Stick the resazurin indicator strip on the wall of the jar.
  - b Open the foil packet and remove the sachet. Place the sachet in the jar.
  - c Close the jar within 1 minute.
  - d Examine for condensation within about 30 minutes. If none appears, start over with a new packet.

Once the anaerobic packet is deemed to be working, the sealed anaerobic jar is placed into a standard incubator set to 35 ± 2°C. The samples will be incubated for 24 to 48 hours.

5. **Place Your Aerobic Plate in the Incubator:** Place your second plate (the aerobic plate) in a standard incubator set to 35 ± 2°C. Incubate the samples for 24 to 48 hours.
6. **Clean up:** Save or dispose of the original cultures as your instructor directs.

Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe the Samples:** Examine and compare the growth on the plates. (***Note:*** Density of growth will be the most useful basis for comparison.)
3. **Record Your Results**: Note all observations and results on the data sheet at the end of this lab.
4. **Clean up:** Properly dispose of the packet in laboratory waste. The Oxoid packet may still be warm to the touch. Let it cool before disposing of it. Dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 19

DATA SHEET

Anaerobic Jar

During the Lab

1 In the table provided, indicate the amount of growth of each organism on each plate using the following system:

0 = no growth

1–2 = moderate growth

3 = abundant growth

Organism	Growth on Aerobic Plate	Growth on Anaerobic Plate	Aerotolerance Category

Lab Related Questions

1 Suppose that after incubation you examine the jar before opening it and see that the methylene blue strip is blue (or the resazurin strip is pink). What is the *best* conclusion about the internal jar environment in such a case?

a. It became aerobic upon being exposed to room temperature.

b. It was anaerobic for most of the incubation period.

c. It was aerobic for most of the incubation period.

d. It was aerobic for at least a portion of the incubation period.

e. It was incubated at the wrong temperature.

2 How would you expect the growth on the plates inside the jar to differ from the plates incubated outside the jar? (Select all that apply.)

a. Obligate aerobes will grow best inside the jar apparatus.

b. Obligate anaerobes will grow best inside the jar apparatus.

c. Facultative anaerobes will grow best inside the jar apparatus.

d. Obligate aerobes will grow best outside the jar apparatus.

e. Facultative anaerobes will grow best outside the jar apparatus.

3 An alternative to the anaerobic jar is a candle jar, in which a candle is placed in the jar, lit, and the lid closed to enable the flame to use the available oxygen. Typically, in this system, not all of the oxygen is used. Which organisms would most likely benefit from this environment? (Select all that apply.)

a. Obligate anaerobes

b. Aerotolerant anaerobes

c. Obligate aerobes

d. Facultative anaerobes

e. Microaerophiles

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Anaerobic jars are rendered anaerobic by a chemical reaction in which atmospheric oxygen is chemically converted to CO_2.

_____ Anaerobic jars will only permit the growth of obligate anaerobes.

_____ Aerotolerant anaerobes should grow in an anaerobic jar, but such a jar is not necessary for their cultivation.

_____ Anaerobic jars are only used with thioglycollate media.

5 Which of the following represent an advantage that anaerobic jars have over thioglycollate media?

a. Anaerobic jars are portable.
b. Anaerobic jars can allow for anaerobic incubation of diverse media formats.
c. Anaerobic jars are useful for growing anaerobes.
d. Anaerobic jars are useful for growing facultative anaerobes.
e. Anaerobic jars are useful for isolating species with an unknown aerotolerance.

6 Which of the following is (are) likely to lead to the anaerobic conditions in an anaerobic jar being disrupted? (Select all that apply.)

a. Opening the jar during incubation.
b. Improperly sealing the jar.
c. Using an expired chemical pack to deoxygenate the jar.
d. Incubating the jar outside of a 35±2°C range.
e. Moving the jar.

7 The dye _____ or _____ is used to indicate that an anaerobic environment was achieved/maintained in an anaerobic jar. When oxygen is absent, these dyes appear _____.

Critical Thinking

1 Assume you are studying a newly discovered pathogen. You obtain the following data for two different isolates of the bacteria that were inoculated onto nutrient agar and then incubated at 35±2°C under the conditions noted in the table.

Standard Incubator		Anaerobic Jar Incubation	
Observation 24-hours post-inoculation		Observation 24-hours post-inoculation	
Sample Isolate	**Growth detected?**	**Sample Isolate**	**Growth detected?**
1	Yes (abundant)	1	Yes (moderate)
2	Yes (absent)	2	Yes (abundant)
Observation 48-hours post-inoculation		Observation 48-hours post-inoculation	
Sample Isolate	**Growth detected?**	**Sample Isolate**	**Growth detected?**
1	Yes (abundant)	1	Yes (abundant)
2	Yes (absent)	2	Yes (abundant)

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 19
DATA SHEET
(continued)

Answer the following questions based on the data:

a. Based on these data, what can you conclude about Isolate 1? Support your answer by referring to specific data from the tables provided.

b. Based on these data, what can you conclude about Isolate 2? Support your answer by referring to specific data from the tables provided.

2 Considering the gaseous changes resulting from the burned candle, which group would benefit the most from a functional candle jar? (***Hint***: Think about a gas that is not involved in aerotolerance.)

— Lab Notes —

EXERCISE 20

The Effect of Temperature on Microbial Growth

After doing this exercise, you should be able to:

1. Explain why prokaryotes are especially sensitive to environmental pH and temperature shifts.
2. Define the terms *optimal temperature*, *minimum temperature*, *maximum temperature*, and *cardinal temperatures*.
3. Describe the temperature conditions that support the growth of psychrophiles, psychrotrophs, mesophiles, thermophiles, and extreme thermophiles.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the cardinal temperatures for a selection of bacteria.

Background

Even minor environmental changes can dramatically affect microbes, especially prokaryotes. Seemingly small shifts in environmental pH and/or temperature can be a life or death matter, because such factors dramatically change a prokaryote's ability to transport materials across the membrane, maintain membrane fluidity, perform complex enzymatic reactions, and maintain critical cytoplasmic pressure. This is in part why we are so detailed about incubation temperatures and the pH of the media we use in the laboratory setting.

One way to study how microbes respond to environmental changes is to alter an external factor and measure the resulting effect on the microbe's growth rate; that is, the cell density after a given incubation time. Such studies help us classify organisms by their preferred oxygen ranges (see Ex. 18), based on their preferred temperature growth range (this exercise), and according to their preferred pH growth range (covered in Ex. 21).

Prokaryotes and Temperature Tolerance

Prokaryotes have been discovered living in habitats ranging from –10°C to more than 110°C. Because microbes cannot regulate their internal temperature in response to their surroundings, they instead create proteins and membranes that function within a particular temperature range. As such, each species is characterized by a minimum, maximum, and optimum temperature, collectively known as its **cardinal temperatures** (Fig. 20.1).

Minimum and maximum temperatures are, simply, the temperatures below and above which the organism will not survive. Optimum temperature is the temperature at which an organism grows the fastest, its highest growth rate. The optimum temperature is usually presented as a tight range of temperatures within the minimum and maximum range; it is not usually defined as a single temperature point.

Classifying Prokaryotes by Temperature Growth Range

Prokaryotes that prefer to grow below 20°C are called **psychrophiles**. These are common in ocean, Arctic, and Antarctic habitats where the temperature remains permanently cold with little or no fluctuation. Organisms adapted to cold habitats that fluctuate from about 0°C to above 30°C are called **psychrotrophs.** Because psychrotrophic bacteria grow at room temperature as well as in refrigerated and frozen foods, they are associated with foodborne illness. Bacteria adapted to temperatures between 15°C and 45°C are known as **mesophiles**. Most bacterial residents in the human body (normal microbiota), as well as numerous human pathogens, are mesophiles. **Thermophiles** are organisms adapted to temperatures above 40°C. Thermophilic prokaryotes are found in composting organic material, soil surfaces subjected to direct sunlight, and silage. Prokaryotes isolated from ocean floor hydrothermal vents and other geothermal sites are called **extreme thermophiles** because they can survive temperatures in the 65°C and 110°C range (up to 122°C for *Methanopyrus*). Extreme thermophiles grow best above 80°C. Figure 20.2 illustrates typical temperature ranges and classifications for the prokaryotes (domains Bacteria and Archaea).

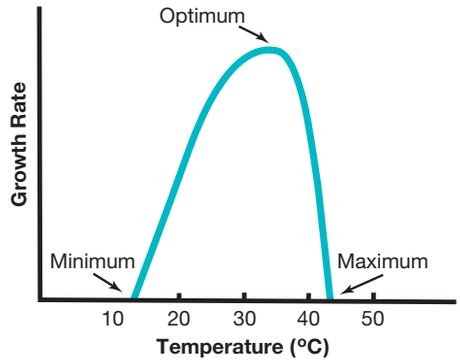


FIGURE **20.1** **Typical Temperature Growth Range for Mesophiles** ● The "minimum" and "maximum" are temperatures beyond which no growth takes place. The "optimum" is the temperature at which growth rate is highest. These are the organism's "cardinal temperatures."

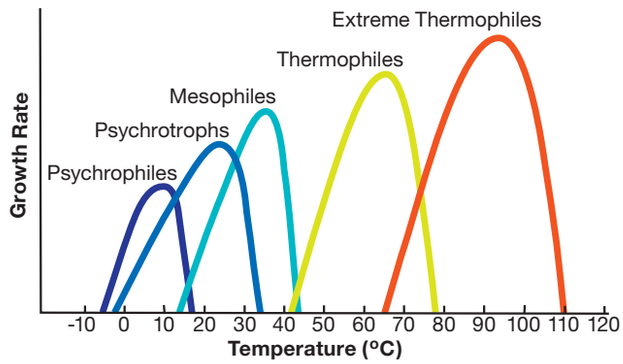


FIGURE **20.2** **Thermal Classifications of Bacteria** ● This graph shows that bacteria can be grouped into five main categories based on the temperatures at which they can grow. The temperature data represented here are generalizations for each group.

Clinical Applications

This is a qualitative procedure that allows us to study how temperature affects microbial growth. It allows us to estimate the cardinal temperatures for individual species rather than for an entire group, as shown in Figure 20.2.

Understanding the cardinal ranges for temperature growth is certainly important for culturing bacteria in the laboratory, but it also has important pathophysiological consequences. Most pathogenic bacteria are mesophiles; however, even within these groupings, we find different optimal temperatures and even growth ranges for different bacterial species.

For example, *Mycobacterium leprae*, the pathogen that causes leprosy, is a mesophile with an optimal growth temperature of 33–35°C. Because this bacterium's optimal growth temperature is below the normal core human body temperature (which is 36.5–37.5 °C), *M. leprae* mainly infects the superficial peripheral nerves, the nasal passages, and the skin, areas where the bacterium's optimal growth temperature range is met. In contrast, we find the causative agent of tuberculosis (*Mycobacterium tuberculosis*) in the same genus as *M. leprae*. It is another example of a mesophile; however, it has an optimal growth temperature that aligns with normal core human body temperature. If *M. tuberculosis* could not grow at core human temperature, it would certainly struggle to infect deep in the lungs as it does.

Further complicating matters, we find that some bacteria have an exceedingly broad temperature growth range. For example, *Listeria monocytogenes*, which causes a foodborne infection called listeriosis, has a growth range that spans -1.5–45°C. This broad temperature tolerance means it can easily grow in refrigerated foods, but its optimal growth temperature spans our normal core body temperature, making it possible for the bacterium to cause gastrointestinal infections.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile tryptic soy broths (TSB) (or nutrient broths, NB) (25 total tubes)
- ❑ Tryptic soy agar (TSA) plates (2)
- ❑ Sterile transfer pipettes (4)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Escherichia coli*
  - ☐ *Geobacillus (Bacillus) stearothermophilus*
  - ☐ *Serratia marcescens*
  - ☐ *Pseudomonas fluorescens*
- ❑ Incubating devices set at 10°C, 25°C, 35°C, 45°C, and 60°C (5 total). These five devices may be any combination of the following:
  - ☐ refrigerator
  - ☐ incubator
  - ☐ hot-water bath
  - ☐ cold-water bath

PROCEDURE

Today you will examine the growth characteristics of four organisms at five different temperatures. You'll also observe how temperature influences pigment production in *Serratia marcescens*. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain 25 tryptic soy broths (TSB) and two TSA plates.
2. **Label Your Tubes:** Label your tubes so that you have designated one broth for each organism at each temperature plus one for an uninoculated control (Fig. 20.3). Label one TSA plate for 20°C *S. marcescens* and the other for 35°C *S. marcescens*. Each student should choose one or two stock cultures from the options your instructor provides to inoculate. Divide the work load with your lab partner(s) as evenly as possible.
3. **Inoculate Your Media with the Designated Bacteria:**
   - **Plate Inoculations:** To inoculate each TSA plate with *Serratia marcescens*, use an inoculation loop and perform the quadrant streak plate isolation method as described in Exercise 4.
   - **Broth Inoculations:** Mix each stock sample thoroughly, but be careful not to splash the culture into the tube cap. Then, using a sterile transfer pipette, inoculate each set of TSB tubes with 1 drop of organism (be consistent). Refer to Figure 20.3 for guidance on this. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your set of tubes, leave one tube uninoculated.

CAUTION!

- Before delivering the inoculum, insert the pipette well into the tube (but do not touch the broth).
- Do not spatter as you deliver the drop to each tube.
- Do not accidentally drip organism between tubes. If you do, then clean up the spill with proper decontamination approaches as your instructor directs.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow out") the pipette because you will create aerosols.
- Once finished, properly dispose of the pipette.

4. **Incubate the Samples:** Incubate all the TSB tubes at their appropriate temperatures for 24 to 48 hours. Place one of your TSB plates in a 20°C incubator and the other in a 35°C incubator and incubate the samples for 24 to 48 hours. Remember to invert your plates for incubation so condensation won't drip onto the agar surface.

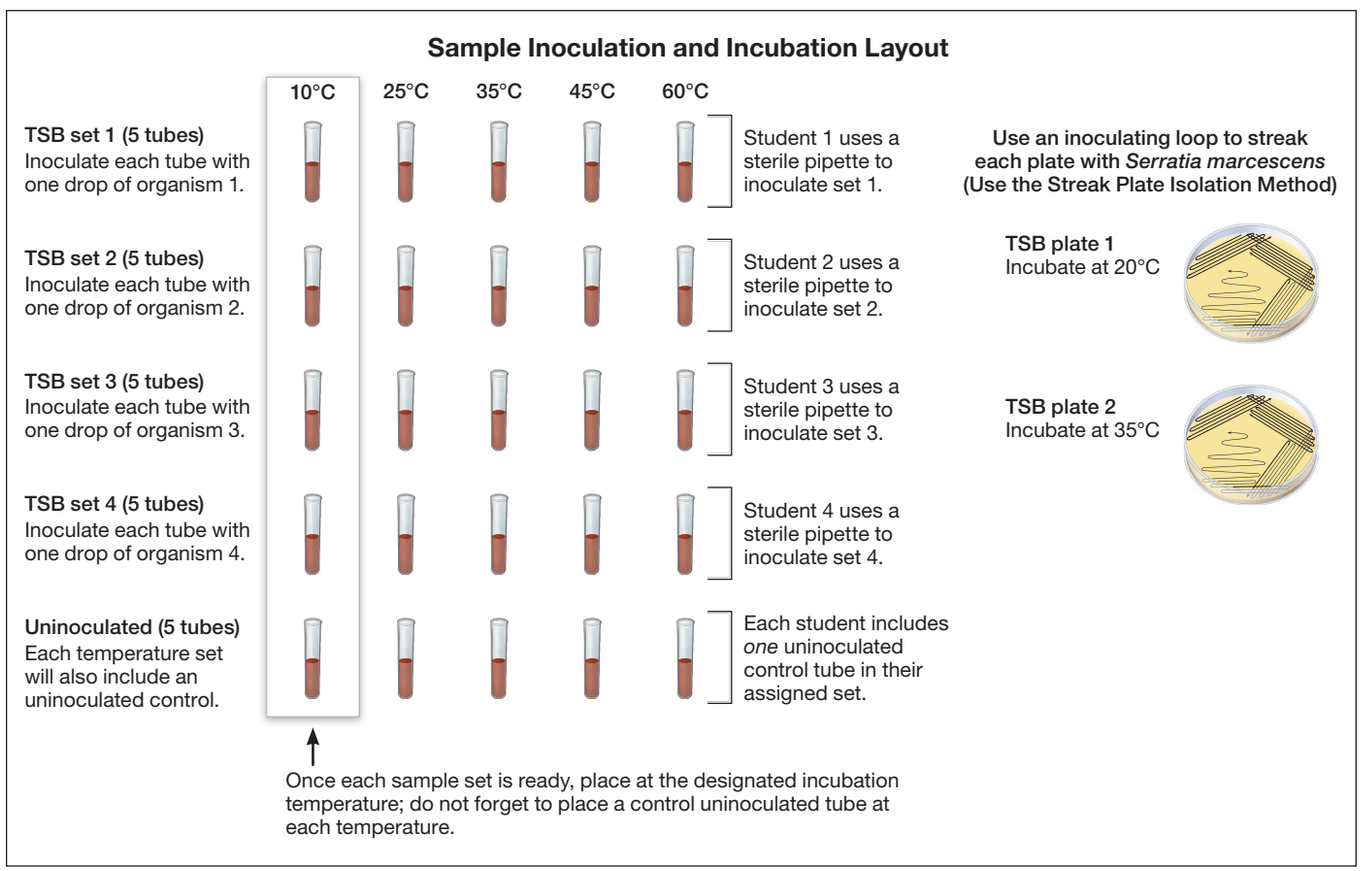


FIGURE **20.3** **Broth and Plate Sample Organization and Inoculation Process**

5 **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1 **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).

2 **Clean the Outside of Each Broth Tube:** Use a clean tissue to wipe the glass of each broth tube and place each tube in a test-tube rack organized into groups by organism.

3 **Observe Your Broth Sample Data and Record the Results:** Gently mix each tube until you obtain uniform turbidity (cloudiness). Be careful not to splash the culture into the caps. Compare all tubes of a species to each other. Rate each as 0, 1, 2, or 3, according to its turbidity (0 is clear—the uninoculated tube—and 3 is the most turbid.) Note your observations and results on the data sheet at the end of this lab.

4 **Analyze Your Broth Sample Data:** Use the data you recorded in Step 3 to make a graph. Use the graph paper provided with the data sheet at the end of this exercise. On your graph, plot the relative turbidity values versus temperature for each organism. If you need help with graphing, see pages 10–12.

5 **Develop Conclusions Based on the Broth Growth Data:** On your graph, indicate the optimum temperature for each bacterium studied. Then use brackets to indicate the temperature range for each. (***Note:*** This also indicates the minimum and maximum temperatures.) Finally, use this data to develop a conclusion about the temperature classification for each organism.

6 **Observe the Plate Samples and Record the Results:** Now, examine the *Serratia marcescens* plates that were incubated at two different temperatures. Compare the growth characteristics for each plate and enter your observations and results on the data sheet at the end of this lab.

7 **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 20

DATA SHEET

The Effect of Temperature on Microbial Growth

During the Lab

1 For each organism, in the table provided record the turbidity (cloudiness) of the sample. The cloudier the sample, the more growth. Use your uninoculated control as a basis for your "0" point.

0 = no growth (completely clear sample)

1–2 = moderate growth

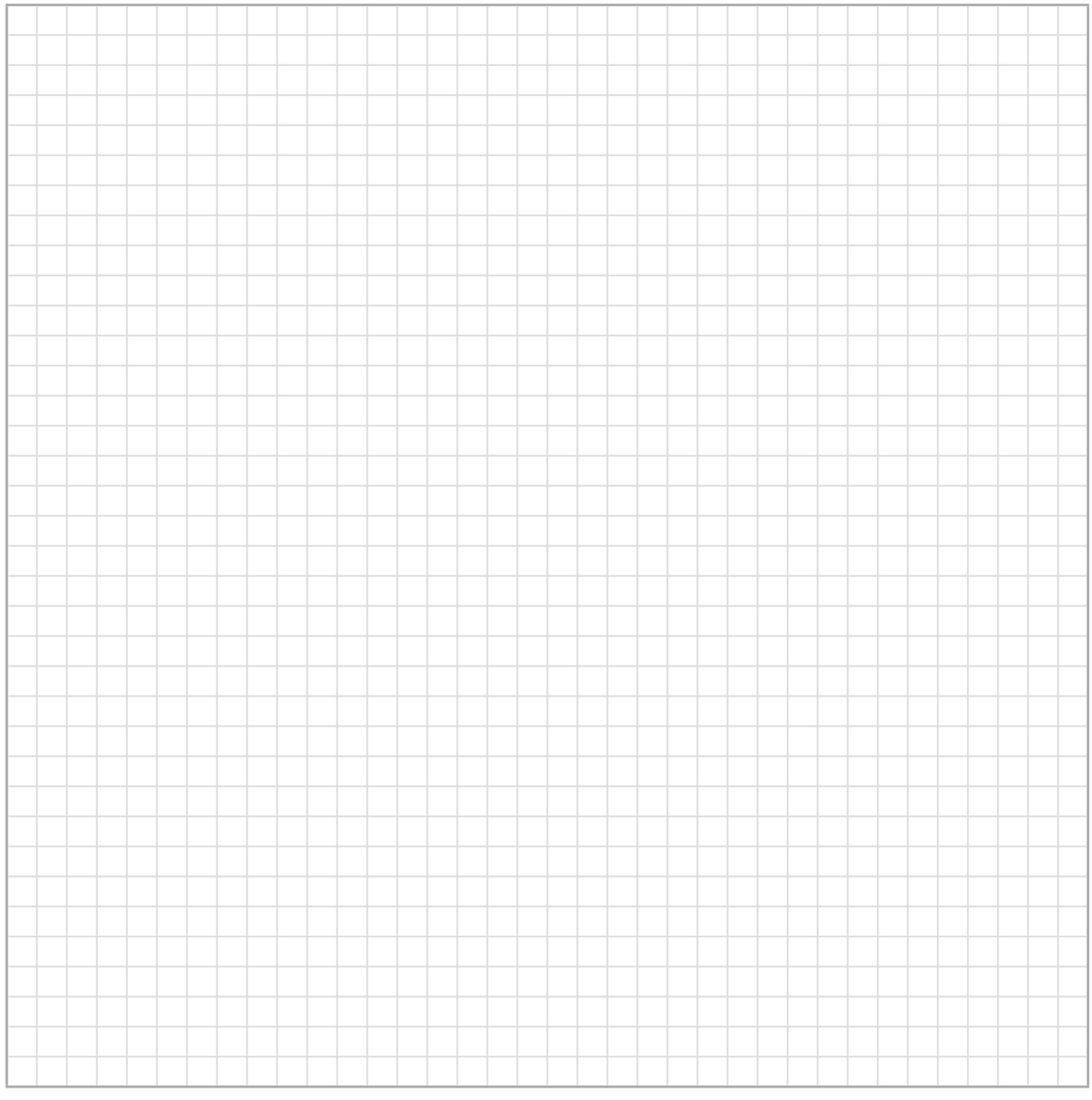
3 = abundant growth

Broth Data						
Organism	10°C	25°C	35°C	45°C	60°C	Temperature Range Classification

2 In the table provided, record the growth characteristics of the *Serratia marcescens* incubated at two temperatures.

Plate Data	
Incubation Temperature	Description of Growth
20°C	
35°C	

3 Plot relative turbidity (y-axis numeric values will be 0, 1, 2, 3) versus temperature (x-axis values will be the five tested temperatures) on the graph paper provided. If you need help with graphing, see pages 10–12.



4 Using the data in your graph, determine the cardinal temperatures for each of the four organisms. Indicate the optimum temperature for each organism on your graph and use brackets to designate the range (minimum to maximum) for each.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 20 DATA SHEET

(continued)

Lab Related Questions

1 From the following, what is the *best* conclusion about mesophiles?

a. They do not grow at room temperature.
b. They do not cause disease.
c. They are pathogens.
d. They would grow at 0°C.
e. They would not grow at 70°C.

2 In this experiment, _____ is the independent variable.

3 In this experiment, _____ is the dependent variable.

4 The organisms most likely to cause frozen food spoilage are (select all that apply):

a. Pathogens.
b. Psychrophiles.
c. Psychrotrophs.
d. Mesophiles.
e. Prokaryotes.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Mesophiles include pathogens.

_____ The optimal temperature is the warmest temperature that supports a given microbe's growth.

_____ An organism's optimum temperature is usually a tight range of temperatures within the minimum and maximum range.

_____ All mesophiles have the same optimal growth temperature.

6 Match the terms to their most appropriate descriptor.

_____ Thermophiles	a. Grow best below 0°C.
_____ Psychrophiles	b. Grow best above 80°C.
_____ Mesophiles	c. Include most human pathogens.
_____ Extreme thermophiles	d. Are likely to live in composting organic material.
_____ Psychrotrophs	e. Are adapted to habitats ranging from about 0°C to above 30°C.

7 Choose the true statement(s) about mesophiles (select all that apply).

a. They are all able to cause disease.
b. They are characterized by the same optimal growth temperature.
c. They are characterized by the same minimum and maximum growth range.
d. They include normal microbiota.
e. None of the above are true.

Critical Thinking

1 A classic Thanksgiving meal often includes a stuffed turkey. However, for food safety reasons, the recommendation is to cook the turkey separately from the stuffing. In the context of this lab, why does this advice make sense?

2 Different animals have different normal body temperatures. For example, chickens have a normal body temperature of 40.6–41.7°C (105–107°F). How might different animal body temperatures affect their susceptibility to certain bacterial pathogens?

EXERCISE 21

The Effect of pH on Microbial Growth

After doing this exercise, you should be able to:

1. Explain why prokaryotes are especially sensitive to environmental pH shifts.
2. Define the terms *optimal pH*, *minimum pH*, *maximum pH*, and *cardinal pH range*.
3. Describe the pH conditions that support bacterial growth.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how pH variation affects bacterial growth.

Background

Solution pH is an important chemical property, it reflects the concentration (or activity) of hydrogen (H^+) ions in a solution. We report pH using a logarithmic scale that ranges from 0–14. A pH of 7 is neutral, a pH less than 7 is acidic, and a pH above 7 is basic.

We can calculate pH using the following formula:

$$pH = -\log [H^+]$$

For example, let's assume we have an aqueous (water-based) solution that contains 10^{-6} moles of dissociated hydrogen ions per liter (mol/L). In this case, the concentration of H^+ (represented as $[H^+]$ in the formula) is 10^{-6} mol/L. Knowing this, we can determine the solution's pH as follows:

$$pH = -\log [H^+]$$

$$pH = -\log (10^{-6})$$

$$pH = -(-6)$$

$$pH = 6$$

Pure water contains 10^{-7} moles of hydrogen ions per liter and therefore has a pH of 7 and is neutral. As hydrogen ions increase, the pH decreases. As H^+ concentrations decrease, the solution pH increases (Fig. 21.1).

Solution Classification Acidity/Alkalinity	pH	H^+ Concentration in Moles/Liter	Common Examples	Organismal Classification
↑ Acidic	0	10^0	Nitric acid	↑
	1	10^{-1}	Stomach acid	
	2	10^{-2}	Lemon juice	
	3	10^{-3}	Vinegar, cola	
	4	10^{-4}	Tomatoes, orange juice	
	5	10^{-5}	Black coffee	Acidophiles
	6	10^{-6}	Urine	
Neutral	7	10^{-7}	Pure water	Neutralophiles
	8	10^{-8}	Seawater	
	9	10^{-9}	Baking soda	Alkaliphiles
	10	10^{-10}	Soap, milk of magnesia	
	11	10^{-11}	Ammonia	
	12	10^{-12}	Lime water [$Ca(OH)_2$]	
	13	10^{-13}	Household bleach	
↓ Alkaline	14	10^{-14}	Drain cleaner	↓

FIGURE **21.1** The pH Scale

Prokaryotes and pH Tolerance

Even small changes in pH can have dramatic biochemical consequences. We see this in human beings; even minor blood pH shifts outside of the normal 7.35–7.45 pH triggers lightheadedness and fainting (syncope), and larger shifts can be deadly. Of course, prokaryotes are also sensitive to pH shifts. Changes to pH outside the range to which they are adapted may damage cellular protein structure (a process called denaturation) and destroy necessary membrane potential (used in ATP production). You can easily see protein denaturation in response to pH changes by adding lemon juice to milk. The curdling you would witness in this scenario is a protein denaturation event; casein, a protein in milk, denatures below pH 4.6. Just as we can't uncurdle milk (we can't restore the casein to its prior form), cells are often unable to restore a normal protein structure to their proteins once they are denatured. Consequently, these adverse effects may be irreversible and therefore lethal if widespread in a cell.

Most cells, including bacteria, use substances called buffers to maintain a near-neutral intracellular environment. Without buffers, the acids that cells make as a byproduct of their metabolic activities (e.g., acids made in carbohydrate fermentation or basic byproducts generated in protein metabolism) could shift intracellular pH enough to damage cellular proteins. Just as it is essential to maintain pH within a cell, when we are growing bacteria, it can be helpful to maintain an optimal growth pH. For this reason, we sometimes add buffers to bacteriological growth media. Phosphate salts (e.g., monosodium phosphate and its conjugate base disodium phosphate) are a common choice for buffering bacteriological media because they maintain the pH growth range of most bacteria. In solution, the phosphate buffer alters between a weak acid ($H_2PO_4^-$) and conjugate base (HPO_4^{2-}) to maintain H^+/OH^- equilibrium, as shown in the following equations:

$$H^+ + HPO_4^{2-} \rightarrow H_2PO^{4-}, \text{ and}$$

$$OH^- + H_2PO^{4-} \rightarrow HPO_4^{2-} + H_2O$$

Other buffer options also exist, but the principle of how they all work is the same, they stabilize pH by ensuring there is not a sharp overall change (increase or decrease) in H^+ concentration.

Classifying Prokaryotes by pH Growth Range

Although we can find bacteria living throughout the pH spectrum, most microbes grow best at a pH between 6.5 and 8.5. Like temperature and oxygen tolerance, pH tolerance is also a useful classification criterion. Within each microbe's survivable pH range are the **minimum**, **optimum**, and **maximum pH** for growth. The minimum pH is the lowest pH where growth is supported. The maximum pH is the highest pH where growth can occur. And the optimal is the preferred pH level for growth, the pH that supports the highest rate of cell division for a given microbe.

The three major classifications are:

1. **Acidophiles**: These organisms are adapted to grow well in environments of pH 1 (or less) to pH 5. We can find them in places like sulfur hot springs.
2. **Neutralophiles** (occasionally referred to as neutrophiles): Most microorganisms (and, by extension, most pathogens) prefer pH levels between 5 and 8.
3. **Alkaliphiles**: These organisms live at an alkaline (or basic) pH of about 9 to 11. These microbes can live in places like soda lakes that are enriched with sodium carbonate.



Clinical Applications

This is a qualitative procedure used to estimate the minimum, maximum, and optimum pH for growth of microbial species.

Understanding the cardinal pH ranges is important for culturing bacteria in the laboratory and it helps us understand the conditions that prevent or support the growth of certain pathogenic bacteria. For example, the acid environment in the stomach (pH of 1.5–3.5) is an important protection against a variety of potential pathogens that we may ingest in our food and/or water. Pathogens that cause gastrointestinal infections require specific adaptations to survive their passage through the stomach as well as through the acidic first segment of the small intestine. An example of such a pathogen is *Helicobacter pylori*, which causes stomach and duodenal ulcers. Given that this bacterium successfully infects the stomach, it seems counterintuitive to say this organism is neutralophilic. However, the bacterium doesn't survive for long in stomach acid. It instead employs temporary protections against stomach acid, measures that buy it enough time to burrow into the more neutral stomach mucosa to establish an infection.

Stomach pH is such an important first-line barrier to infection that regularly taking proton pump inhibitors (PPIs) for preventing acid reflux (e.g., drugs like Prilosec, Prevacid, Nexium, etc.) has been associated with increased susceptibility to certain gastrointestinal pathogens to include *Clostridioides difficile* (the causative agent of pseudomembranous colitis, "*C. diff*" infectious diarrhea), *Vibrio cholerae* (causative agent of cholera), and

Campylobacter jejuni (a leading cause of foodborne gastrointestinal infections). PPIs reduce H^+ concentrations in the stomach and thereby raise gastric pH, making it easier for certain pathogens to survive their brief transit through the stomach and successfully establish an infection in the intestinal tract.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ pH adjusted tryptic soy broth (TSB) (or nutrient broth, NB); 5 of each as follows: pH 2, pH 4, pH 6, pH 8, and pH 10 (25 total)
- ❑ Sterile transfer pipettes (4)
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Alcaligenes faecalis* (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - ☐ *Lactococcus lactis*
  - ☐ *Staphylococcus saprophyticus*

Medium Recipe

pH-Adjusted Nutrient Broth (NB)

Beef extract	3.0 g
Peptone	5.0 g
Distilled or deionized water	1.0 L
NaOH or HCl as needed to adjust pH	

PROCEDURE

Today you will cultivate four organisms and observe how pH affects them. You will then classify the tested bacteria based on your results. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Media:** Working with your group, obtain five tubes of each pH broth—one of each pH per organism plus a control (25 tubes total).
2. **Label Your Tubes:** Label your tubes so that you have designated one broth for each organism plus one tube for an uninoculated control at each pH level (Fig. 21.2). Each student should choose one or two stock cultures from the options your instructor provides to inoculate. Divide the work load with your lab partner(s) as evenly as possible.
3. **Inoculate Your Media with the Designated Bacteria:** Mix each stock sample thoroughly, but be careful not to splash the culture into the tube cap. Then, using a sterile transfer pipette, inoculate each tube of media with 1 drop of organism (be consistent). Refer to Figure 21.2 for guidance on this. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your set of tubes, leave one tube uninoculated.

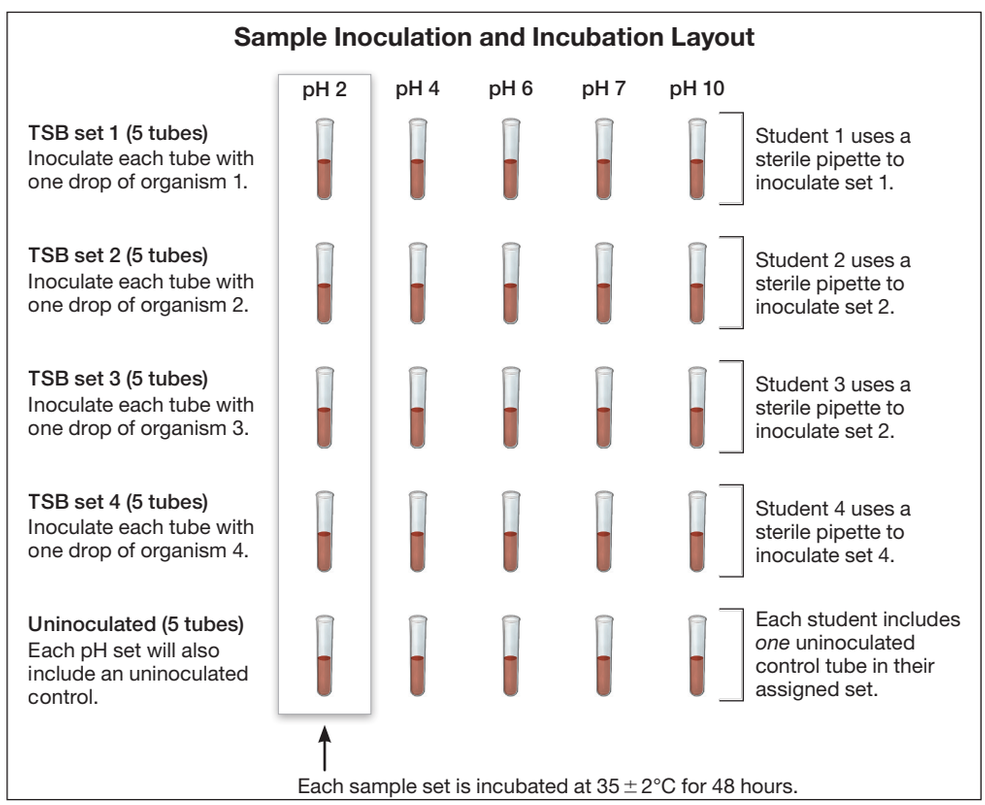


FIGURE **21.2** **Broth Sample Organization and Inoculation Process** ●

CAUTION!

- Your lab should have chemical solutions to clean up acid and base spills. If you spill any of the broths on the table or other inanimate objects, have a lab partner notify your instructor while you neutralize the spill with the appropriate solution available in your lab. For an acid spill, a sodium bicarbonate solution can be used. For a base spill, an acetic acid solution is appropriate. Be sure to know what neutralizing solutions are available in your laboratory and where they are located before you begin this laboratory exercise.
- If you spill on your skin, rinse thoroughly with water until your instructor gives you permission to stop. Do not try to neutralize acid and base spills on your skin.
- Before delivering the inoculum, insert the pipette well into the tube (but do not touch the broth).
- Do not spatter as you deliver the drop to each tube.
- Do not accidentally drip organism between tubes. If you do, then clean up the spill with proper decontamination approaches as your instructor directs.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow out") the pipette because you will create aerosols.
- Once finished, properly dispose of the pipette.

4 **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.

5 **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1 **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).

2 **Clean the Outside of Each Broth Tube:** Use a clean tissue to wipe the glass of each broth tube and place each tube in a test-tube rack organized into groups by organism.

3 **Observe Your Broth Sample Data and Record the Results:** Gently mix each tube until you obtain uniform turbidity (cloudiness). Be careful not to splash the culture into the caps. Compare all tubes of a species to each other. Rate each as 0, 1, 2, or 3, according to its turbidity (0 is clear—the uninoculated tube—and 3 is the most turbid). Note your observations and results on the data sheet at the end of this lab.

4 **Analyze Your Broth Sample Data:** Use the data you recorded in Step 3 to make a graph. Use the graph paper provided with the data sheet at the end of this exercise. On your graph, plot the relative turbidity values versus pH for each organism. If you need help with graphing, see pages 10–12.

5 **Develop Conclusions Based on the Broth Growth Data:** On your graph, indicate the optimum pH for each bacterium studied. Then use brackets to indicate the pH range for each. (***Note:*** This also indicates the minimum and maximum pH where you observed growth for a given organism.) Finally, use this data to develop a conclusion about the pH classification for each organism.

6 **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 21

DATA SHEET

The Effect of pH on Microbial Growth

During the Lab

1 For each organism, in the table provided record the turbidity (cloudiness) of the sample. The cloudier the sample, the more growth. Use your uninoculated control as a basis for your "0" point.

0 = no growth (completely clear sample)

1–2 = moderate growth

3 = abundant growth

Organism	pH 2	pH 4	pH 6	pH 8	pH 10	Classification

2 Plot relative turbidity (y-axis numeric values will be 0, 1, 2, 3) versus pH (x-axis) on the graph paper provided. If you need help with graphing, see pages 10–12.

3 Using the data in your graph, indicate the pH optimum for each organism on your graph and use brackets to designate the range (minimum to maximum) pH for each.

Lab Related Questions

1 From the following, what is the *best* conclusion about neutralophiles?

a. They thrive in relatively neutral environments.
b. They cannot survive an exposure to an acid.
c. They do not cause disease.
d. They are primarily pathogens.
e. They would not infect the stomach mucosa.

2 Which of the following describe(s) *Helicobacter pylori*? (Select all that apply.)

a. Pathogen
b. Acidophile
c. Neutralophile
d. Archaea
e. Causes stomach ulcers

3 In this experiment, _____ is the independent variable.

4 In this experiment, _____ is the dependent variable.

5 Match each term to its most appropriate descriptor(s).

_____ Alkaliphiles	a. Would grow at a pH of 3.
_____ Acidophiles	b. Would grow at a pH of 9.
_____ Neutralophiles	c. Include most human pathogens.
	d. Would grow at a pH of 6.5.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ As the concentration of H^+ increases so does pH.

_____ A pH less than 7 is considered acidic.

_____ A pH greater than 7 is considered basic.

_____ The pH scale ranges from 1–14.

7 Choose the true statement(s) about buffers (select all that apply).

a. They stabilize solution pH.
b. They may be added to bacteriological growth media.
c. They are a combination of an acid and a base.
d. They raise pH when a base is added to a solution.
e. They lower pH when an acid is added to a solution.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 21
DATA SHEET
(continued)

Critical Thinking

1 As a hobby, you make pickles. Your cousin comes over and you offer them a taste of your latest batch of locally famous "never-cooked refrigerator sweet-and-sour cucumber dills." They turn down the offer and mention to you that the CDC (Centers for Disease Control and Prevention) just issued a warning that an outbreak of foodborne *Campylobacter jejuni* infections associated with cucumbers has been tearing through the region for the last month. The cases are all linked to cucumbers purchased at a local farmer's market, the same market where you buy all your cucumbers. Your cousin reminds you that the pickle batch you just completed was made using cucumbers that were harvested and sold during the time frame in which the infections occurred. Are your pickles, which soak in a pH 3.2 vinegar brine for at least a month, safe to eat? Explain your reasoning.

2 The "alkaline diet" has gained in popularity to improve general wellness, and even "prevent the growth of harmful bacteria." The premise of the diet is that certain foods raise your body's pH to an alkaline level (basic level) and that increase in pH is protective. Apply what you learned in this lab to discuss if this is a legitimate claim or wishful thinking.



SECTION

5

Media for the Selection and/or Differentiation of Bacteria

In This Section

EXERCISE 22 Mannitol Salt Agar (MSA) **229**

EXERCISE 23 MacConkey (MAC) Agar **237**

EXERCISE 24 Eosin Methylene Blue (EMB) Agar **245**

EXERCISE 25 Blood Agar **253**

— Lab Notes —



EXERCISE 22

Mannitol Salt Agar (MSA)

After doing this exercise, you should be able to:

1. Explain why MSA is a selective media.
2. Describe how MSA differentiates bacterial species from one another based on mannitol fermentation.
3. Discuss the formulation of MSA and state specifically which ingredients allow it to be selective versus differential.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how different bacteria grow on MSA.

Background

In Exercise 17, we learned about complex (or enriched) media versus defined (or synthetic) media. As a refresher, complex media contain extracts from, or digests of, plant and/or animal matter and are rich in nutrients to support the growth of fastidious microbes. In contrast, for defined media, each organic and inorganic ingredient is precisely known and quantified. In this exercise, we start our exploration of differential and selective media. Both complex and defined media can be modified in their ingredients to take on differential and/or selective properties.

Selective media contain ingredients that prevent the growth of certain bacteria. As their name states, they select for the growth of one class of microbe over another. **Differential media** include ingredients that allow us to visually distinguish (or differentiate) one group of bacteria from another based on metabolic features. In this lab, we explore how the medium **mannitol salt agar** (**MSA**) helps us select for certain bacteria that can tolerate salty environments. And for those bacteria that can grow in a salty environment, it allows us to differentiate bacteria based on their ability to ferment **mannitol** (a carbohydrate in the medium).

Selective and Differential Aspects of MSA

MSA is an enriched media, but because MSA contains 7.5% sodium chloride (NaCl), many bacteria cannot grow on it. The relatively high salt level exerts osmotic stress on cells (draws water out of cells that lack evolutionary adaptations to grow in salty environments). Thus, the media's salt level renders it selective for the growth of salt-tolerant bacteria such as members of the genera *Staphylococcus*, *Bacillus*, *Enterococcus*, *Micrococcus*, and others.

For those bacteria that will grow on MSA, we can then differentiate between those that can ferment the carbohydrate mannitol and those that cannot. This is easy to do through a visual inspection of the growth. Bacteria that can ferment mannitol release acids into the media and lower the local pH. The pH indicator in the media, **phenol red**, changes color in response to pH changes. Phenol red appears yellow below pH 6.8, red at pH 7.4 to 8.4, and pink at pH 8.4 and above. Consequently, the media around bacteria that can ferment mannitol appears yellow while the media around bacteria that can't ferment mannitol remains red/pink.

Differentiating *Staphylococcus* Species with MSA

As mentioned earlier, a number of bacterial genera can grow on MSA. However, this media is especially useful for differentiating *Staphylococcus* species from one another. Over 40 different *Staphylococcus* species have been characterized, and while most tend to grow well on MSA, not all of them ferment mannitol. The potential pathogen *Staphylococcus aureus* ferments mannitol, which produces acids and lowers the pH of the medium (Fig. 22.1). The result is formation of bright yellow colonies usually surrounded by a yellow halo (Fig. 22.2). For more information on fermentation, refer to Appendix A (Fig. A.6 and Table A.6).

It's important that you avoid four common misconceptions about MSA. First, while the genera of bacteria that can grow on MSA are usually Gram positive, this media is *not* selecting based on Gram property, it is selecting based on salt tolerance. Second, this media is *not* completely specific for isolating *Staphylococcus* species, even though that is how it's primarily used. Simply obtaining growth on this media is not enough for you to definitively conclude you are dealing with a *Staphylococcus* species. Third, this media is mainly employed as an isolation media, meaning that if we have a mixed sample (e.g., a swab of a skin wound) we can streak the sample onto this media to start the process to

see if *S. aureus*, a common skin wound pathogen, is present. Fourth, do not think that only *S. aureus* ferments mannitol. The truth is numerous (although not all) *Staphylococcus* species can ferment mannitol.

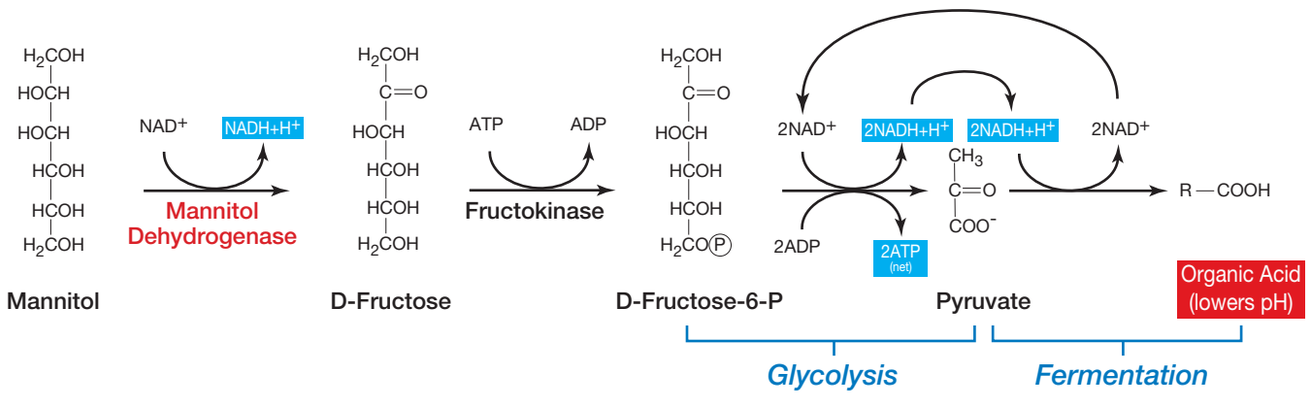


FIGURE **22.1** **Mannitol Fermentation with Acid End Products** • A key end product of mannitol fermentation is an organic acid (red box in the figure). That acid is detected by the phenol red pH indicator in MSA. For simplicity, only the relevant parts of glycolysis are shown. See Appendix A, Figure A.2 for details.

5



FIGURE **22.2** **Mannitol Slat Agar (MSA)** • Here you see two lines of inoculated bacteria. Each streak is of a different *Staphylococcus* species. The organism in the top streak fermented mannitol and therefore generated a yellow halo in the media. The bottom bacterium did not ferment mannitol, so the media remained red/pink.

Clinical Applications

Mannitol salt agar is mainly used to isolate and differentiate *Staphylococcus* species. These species are clinically significant; not only are they normal residents (normal microbiota) of the skin and mucosal surfaces that are near the skin (e.g., nasal passages, lower segment of the urethra, etc.), some are common pathogens.

S. aureus is arguably the most dangerous member of the *Staphylococcus* genus. It has unique **virulence factors** (features that make a pathogen better at establishing infection and advancing disease) that make it a versatile pathogen. It causes a wide range of infections such as sepsis, endocarditis pneumonia, toxic shock syndrome, and serious wound infections. However, other genus members are also problematic.

S. saprophyticus is one of the most common causes of urinary tract infections. *S. haemolyticus*, *S. hominis*, and *S. epidermidis* are common normal skin residents that don't typically cause disease in a healthy host, but they are problematic opportunistic pathogens, especially as causes of **health care-associated infections** (HAIs; also called **nosocomial infections**). For example, *S. haemolyticus*, *S. hominis*, and *S. epidermidis* are increasingly common causes of bacteremia (bacterial infection of the bloodstream). *S. epidermidis* is also increasingly responsible for orthopedic device-related infections. Adding to the clinical concern is the fact that we now find antibiotic resistant strains of all these bacterial species. Of these

five discussed species, *S. epidermidis* is the only one that doesn't usually ferment mannitol, the other four (*S. aureus*, *S. saprophyticus*, *S. haemolyticus*, and *S. hominis*) all ferment mannitol.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Mannitol salt agar (MSA) plate (1)
- ❑ Nutrient agar (NA) plate (1)
- ❑ Inoculating loop
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Staphylococcus aureus* (this is a BSL-2 agent)
    - Alternatively, *Bacillus subtilis* (a BSL-1 agent) could be substituted for *S. aureus* if your lab is not approved for BSL-2 samples. This bacterium demonstrates the same metabolic features on MSA as *S. aureus*.
  - ☐ *Staphylococcus epidermidis*
  - ☐ *Escherichia coli*

Medium Recipes

Mannitol Salt Agar (MSA)

Beef extract	1.0 g
Peptone	10.0 g
Sodium chloride (NaCl)	75.0 g
D-Mannitol ($C_6H_{14}O_6$)	10.0 g
Phenol red	0.025 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 7.2–7.6 at 25°C

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C



PROCEDURE

Today you will test how three organisms grow on nutrient agar versus on mannitol salt agar, a media that has selective and differential properties. MSA would normally be streaked for isolation, but in this lab, you will use a simple zigzag inoculation of pure cultures for the sole purpose of seeing what the different results look like.

1. **Obtain and Label Your Media:** Obtain your two plates of media (one MSA plate and one NA plate). Using a permanent marker, divide the bottom of each plate into three sectors. Label the base of each plate with the organisms' names (one organism in each plate section), your name, and the date. Use the same positions for each specimen on each plate.
2. **Inoculate Your Plates:** Gently mix each stock culture, being careful to not splash the culture into the tube's cap. Use an inoculating loop to perform a zigzag inoculation of each bacterium into their designated section on the plates. Inoculate three bacteria onto the MSA plate and the same three bacteria onto the NA plate. You will need to flame sterilize the loop between each organism so you do not cross-contaminate the samples. ***Caution!*** If you are not using all BSL-1 strains, then you must use BSL-2 safety practices. Follow your instructor's directions for this.
3. **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.
4. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Examine and compare the plates for color and quality of growth. Note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **22.1** Mannitol Salt Agar Results and Interpretations

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism's growth is inhibited by NaCl	Not a *Staphylococcus* species
Good growth (G)	Organism's growth is not inhibited by NaCl	*Staphylococcus* is possible, or some other bacterium known to grow well on MSA (e.g., a *Bacillus*, *Enterococcus*, *Micrococcus* species)
Yellow growth or halo (Y)	Organism produces acid from mannitol fermentation	Possible pathogenic *Staphylococcus aureus* (further testing needed to make this conclusion; many *Staphylococcus* species ferment mannitol and some *Staphylococcus* bacteria may ferment mannitol)
Red growth (no halo) (R)	Organism does not ferment mannitol	Organism other than *S. aureus*

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 22

DATA SHEET

Mannitol Salt Agar (MSA)

During the Lab

1 Record your results in the table provided, and use Table 22.1 to help you shape your interpretations.

Organism	Growth (N/P/G)		MSA Growth Color (Y/R)	Interpretation and Presumptive ID
	MSA	NA		

Lab Related Questions

1 You are studying a bacterial sample and you see that it failed to grow on MSA, but grew abundantly on nutrient agar. Which of the following is a reasonable conclusion about the bacterium?

a. It is not a *Staphylococcus* species.
b. It is *Escherichia coli*.
c. It is salt tolerant.
d. It is unable to ferment mannitol.
e. Its growth was inhibited by phenol red.

2 Choose the true statements about MSA (select all that apply).

a. It is an enriched media.
b. It is a selective media.
c. It is a differential media.
d. It only supports the growth of microbes that can use mannitol as a carbon source.
e. It supports the growth of pathogens.

3 If a bacterium grew on MSA and there is a _____ colored halo in the media, then it means _____ was fermented. In contrast, lack of a yellow halo around the bacterial growth means _____.

4 The role of phenol red in MSA is to:

a. Detect pH changes in the media.
b. Stabilize the media's pH.
c. Serve as a nutrient for the bacterial culture.
d. Prevent contamination.
e. Select for Gram-positive bacterial growth.

5 You are studying a bacterial sample and you see that it grew on MSA, but not on nutrient agar. What is the best conclusion?

a. The bacterium was an old stock sample and was unhealthy, which is why it didn't grow.
b. The bacterium is a fastidious microbe in terms of its nutrient requirements.
c. The bacterium requires an acidic pH to grow.
d. The bacterium is most likely a pathogen.
e. An error was made while inoculating the nutrient agar plate.

6 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The only carbon source in MSA is mannitol.

_____ MSA is a defined media.

_____ MSA selects for growth based on bacterial salt tolerance.

_____ *Staphylococcus* species are not the only bacteria that can grow on MSA.

7 Choose the true statement(s) about *Staphylococcus* species (select all that apply).

a. They exist as normal human microbiota of the skin.
b. They include pathogens.
c. They are salt tolerant.
d. They include opportunistic pathogens.
e. They all ferment mannitol.

8 Which of the following is a misconception about MSA?

a. It selects for salt tolerant bacterial growth.
b. It selects for bacterial growth based on Gram property.
c. It is a complex media.
d. It will not support the growth of pathogens.
e. *Staphylococcus* species grow well on it.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 22
DATA SHEET
(continued)

Critical Thinking

A technician prepares several batches of MSA. Before a batch is cleared for use in laboratory applications, it must pass quality control (QC) analysis. For QC analysis, an MSA plate from each batch is inoculated with three different bacterial species. Organisms A and B should both grow on MSA; Organism C should not grow on MSA. Organism A can also ferment mannitol, but Organism B cannot. As a positive control, the same process (side-by-side inoculation of Organisms A, B, and C) is used to inoculate a nutrient agar (NA) plate. As a negative control, an uninoculated control is also included for each test run. The data for several separate MSA batches are presented in the following tables.

QC Testing Batch 1	Organism A	Organism B	Organism C	Uninoculated Plate
MSA	Abundant Growth Yellow Halo	Abundant Growth No Yellow Halo	Abundant Growth No Yellow Halo	No Growth
Nutrient Agar	Abundant Growth	Abundant Growth	Abundant Growth	No Growth

QC Testing Batch 2	Organism A	Organism B	Organism C	Uninoculated Plate
MSA	Abundant Growth Yellow Halo	Abundant Growth No Yellow Halo	No Growth	No Growth
Nutrient Agar	Abundant Growth	Abundant Growth	Abundant Growth	No Growth

QC Testing Batch 3	Organism A	Organism B	Organism C	Uninoculated Plate
MSA	Abundant Growth No Yellow Halo	No Growth	No Growth	Some Growth
Nutrient Agar	Abundant Growth	Abundant Growth	Abundant Growth	No Growth

Based on the data provided, answer the following questions:

1 Applying what you have learned about MSA, what criteria must be met for a batch to pass QC?

2 Which batch (or batches), if any, would you pass for use in the laboratory? Explain your reasoning.

3 The technician learned that one of the batches was prepared with a below normal amount of NaCl. Which batch would that most likely be? How do you know?

EXERCISE 23

MacConkey (MAC) Agar

After doing this exercise, you should be able to:

1. Explain why MAC agar is a selective media.
2. Describe how MAC agar differentiates bacterial species from one another based on lactose fermentation.
3. Discuss the formulation of MAC agar and state specifically which ingredients allow it to be selective versus differential.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how different bacteria grow on MAC agar.

Background

The family *Enterobacteriaceae* includes a diverse collection of Gram-negative, facultatively anaerobic, rod-shaped bacteria that do not form endospores. Members of this family are widespread through nature, with some of them existing as normal microbial residents of the intestinal tract in humans and other animals. These gut-associated prokaryotes are often called **enteric bacteria.** Among the family *Enterobacteriaceae*, we find the **coliforms.** Coliforms have the same general features of their *Enterobacteriaceae* cousins, but the distinct feature of coliforms is they readily ferment lactose to form acids. For more information about fermentation, refer to Appendix A (Fig. A.6 and Table A.6).

There are two main categories of coliforms. First, there are general **environmental coliforms**, which are abundant in soil and surface water, and most are not concerning from a clinical standpoint. Second, there are **fecal coliforms,** which as their name suggests are present in feces from humans and other warm-blooded animals. Fecal coliforms can include true pathogens, opportunistic pathogens, commensal organisms (organisms that do not hurt the host, but do not necessarily confer a benefit), and mutualistic microbes (organisms that confer a benefit on their host).

Isolating fecal coliform bacteria is useful because they are common indicators of fecal contamination in food and water. While most fecal coliforms are *not* pathogens, detecting them above a specific threshold in a sample suggests potential gut-associated pathogens may also be present. Because *Escherichia coli* is one of the most abundant fecal coliforms, it is considered the hallmark bacterium for fecal contamination. **MacConkey (MAC) agar** is one example of an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample.

Selective and Differential Aspects of MAC Agar

As discussed, coliforms are Gram negative and ferment lactose. Thus, to detect coliforms, MAC agar is formulated to select for Gram-negative bacteria and then differentiate this broad grouping of microbes based on their ability to ferment lactose. The bile salts and crystal violet in the media inhibit the growth of Gram-positive bacteria while allowing most Gram-negative bacteria to grow. In addition, lactose is present in the media along with a pH indicator called neutral red dye. This pH indicator is red at a pH of 6.8 and below, and yellow above pH 6.8. Therefore, organisms that ferment lactose to acidic end products lower the pH (Fig. 23.1) and their colonies turn a pink to red color. Sometimes a pink/red bile precipitate will also form in the agar due to the lowered pH. Lactose non-fermenters retain their normal color (which often is colorless) and some may impart a slightly yellow/tan tinge to the media (Fig. 23.2).

It's worth noting that since MAC agar was first developed in 1900, it has undergone a variety of formulations. The recipe we use here is formulated with a concentration of sodium chloride and bile salts that reduces swarming by bacteria like *Proteus* species (see Fig. 6.16, p. 96) that interfere with interpretation of results and isolation of other species. Another MAC agar recipe leaves out crystal violet and only uses bile salts as the ingredient that confers selectivity. This modified recipe allows certain Gram-positive bacteria such as *Enterococcus* and some species of *Staphylococcus* to grow. Because these bacteria ferment lactose, they appear pink on MAC agar.

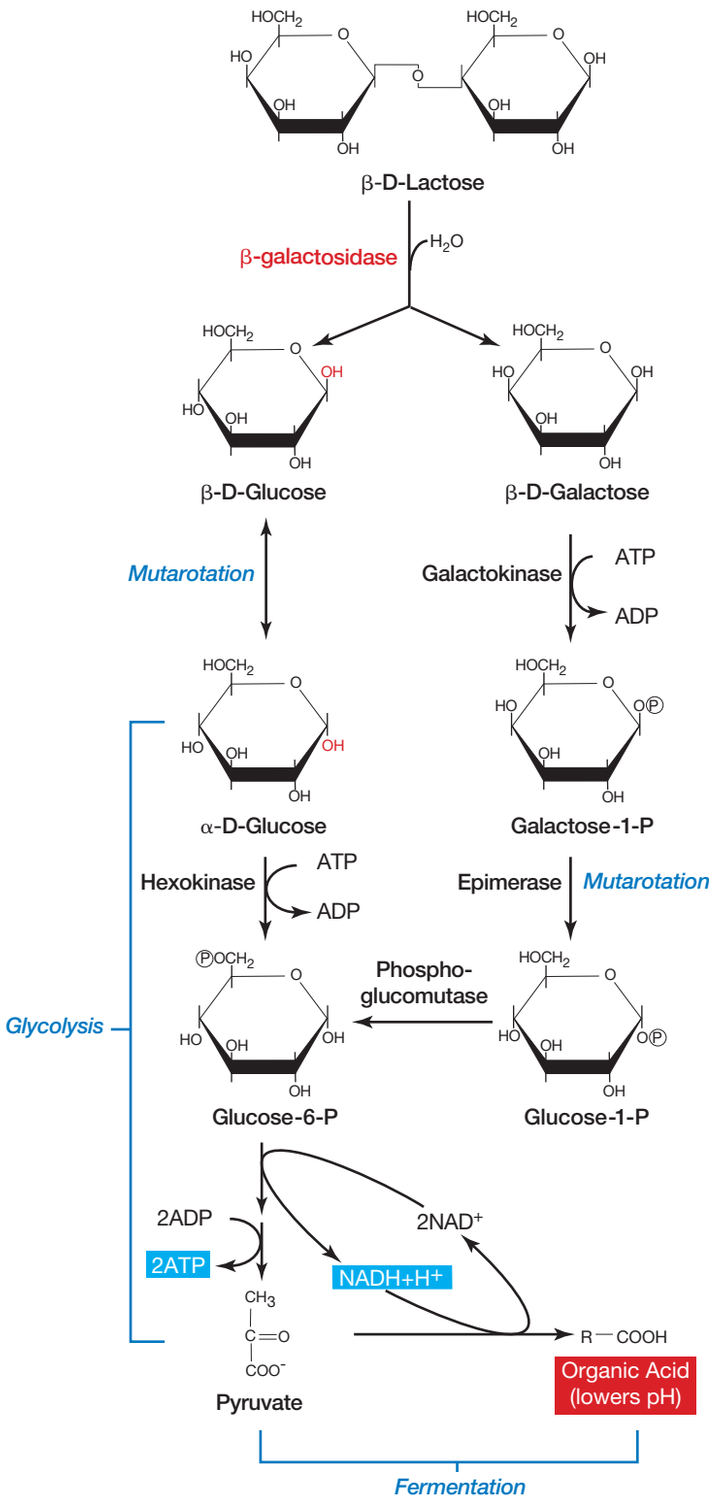


FIGURE **23.1** **Lactose Fermentation with Acid End Products** • A key end product of lactose fermentation is an organic acid (red box in the figure). That acid is detected by the neutral red pH indicator in MAC agar. For simplicity, only the relevant parts of glycolysis are shown. See Appendix A, Figure A.2 for details.

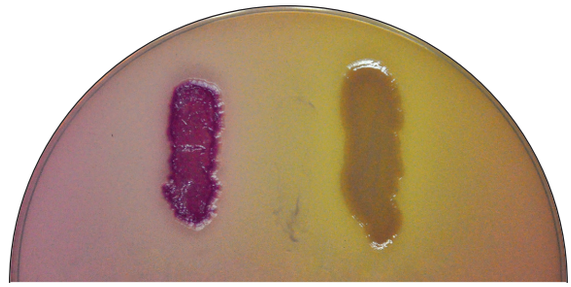


FIGURE **23.2** **MacConkey (MAC) Agar** • This MAC agar plate was inoculated with a Gram-positive coccus (center) and two members of *Enterobacteriaceae* (left and right). As expected, only the Gram-negative bacteria grew. The bacterium on the left (a coliform) fermented lactose and appears pinkish. The bacterium on the right (not a coliform), did not ferment lactose. It appears close to its natural color and has yellowed the medium.

Clinical Applications

Safety testing foods (e.g., milk, shellfish, and meats), drinking water, and recreational water (e.g., swimming pools and lakes) often involves checking for the presence of coliforms. This is because coliform bacteria are indicator organisms whose presence above a given threshold level suggest that certain enteric pathogens could be present. One example of an enteric pathogen that has caused numerous outbreaks by contaminating our food supply (e.g., beef products, various lettuces, and many other foods over the years) is *Escherichia coli* strain O157:H7. This strain is found in the gastrointestinal tract of cattle. In addition, the family *Enterobacteriaceae* includes a number of pathogens such as *Salmonella* serotype Typhi and *Salmonella* serotype Paratyphi, which causes typhoid fever and paratyphoid fever, respectively, as well as non-typhoidal *Salmonella* serotypes including Typhimurium and Enteritidis.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ MacConkey (MAC) agar plate (1)
- ❑ Nutrient agar (NA) plate (1)
- ❑ Inoculating loop
- ❑ Fresh broth cultures of these recommended bacteria:
  - ❑ *Escherichia coli*
  - ❑ *Providencia stuartii*
  - ❑ *Staphylococcus epidermidis*

Medium Recipes

MacConkey (MAC) Agar

- Pancreatic digest of gelatin 17.0 g
- Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$) 1.5 g
- Peptic digest of animal tissue 1.5 g
- Lactose ($C_{12}H_{22}O_{11}$) 10.0 g
- Bile salts 1.5 g
- Sodium chloride (NaCl) 5.0 g
- Neutral red 0.03 g
- Crystal violet 0.001 g
- Agar 13.5 g
- Distilled or deionized water 1.0 L

Adjust to pH 6.9–7.3 at 25°C

Nutrient Agar (NA)

- Beef extract 3.0 g
- Peptone 5.0 g
- Agar 15.0 g
- Distilled or deionized water 1.0 L

Adjust to pH 6.6–7.0 at 25°C



PROCEDURE

Today you will test how three organisms grow on nutrient agar versus on MacConkey (MAC) agar, a media that has selective and differential properties. MAC agar would normally be streaked for isolation, but in this lab, you will use a simple zigzag inoculation of pure cultures for the sole purpose of seeing what the different results look like.

1. **Obtain and Label Your Media:** Obtain your two plates of media (one MAC agar plate and one NA plate). Using a permanent marker, divide the bottom of each plate into three sectors. Label the base of each plate with the organisms' names (one organism in each plate section), your name, and the date. Use the same positions for each specimen on each plate.
2. **Inoculate Your Plates:** Gently mix each stock culture, being careful to not splash the culture into the tube's cap. Use an inoculating loop to perform a zigzag inoculation of each bacterium into their designated section on the plates. Inoculate three bacteria onto the MAC agar plate and the same three bacteria onto the NA plate. You will need to flame sterilize the loop between each organism so you do not cross-contaminate the samples.
3. **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.
4. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Examine and compare the plates for color and quality of growth. Note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **23.1** MacConkey Agar Results and Interpretations

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism is inhibited by crystal violet and/or bile	Gram-positive bacterium
Good growth (G)	Organism is not inhibited by crystal violet and/or bile	Gram-negative bacterium
Pink to red growth with or without bile precipitate (R)	Organism produces acid as a byproduct of lactose fermentation	Possible coliform bacterium
Growth is "colorless" (not red or pink) (C)	Organism does not ferment lactose	Unlikely to be a coliform bacterium

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 23
DATA SHEET

MacConkey (MAC) Agar

During the Lab

1 Record your results in the table provided, and use Table 23.1 to help you shape your interpretations.

Organism	Growth (N/P/G)		MAC Growth Color (R/C)	Interpretation and Presumptive ID
	MAC	NA		

Lab Related Questions

1 You are studying a bacterial sample and you see that it grew on MAC agar, but not on nutrient agar. What is the best conclusion?

a. The sample is a Gram-positive bacterium.
b. The sample is a Gram-negative bacterium.
c. The bacterium is most likely a pathogen.
d. There was an error made during the inoculation process.
e. The bacterium requires a neutral pH to grow.

2 Choose the true statements about MAC agar (select all that apply).

a. It is a defined media.
b. It is a selective media.
c. It supports the growth of pathogens.
d. It is a differential media.
e. Its recipe can be modified so that certain Gram-positive bacteria can grow on it.

3 The role of neutral red in MAC agar is to:

a. Serve as a nutrient for the bacterial culture.
b. Detect pH changes in the media.
c. Stabilize the media's pH.
d. Select for Gram-negative bacterial growth.
e. Select for Gram-positive bacterial growth.

4 If a bacterium grew on MAC agar and the bacteria takes on a _____ color, then it means _____ was fermented. In contrast, if the bacterial growth lacks coloration, it means _____.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ MAC agar is used for water safety testing.

_____ Only pathogens grow on MAC agar.

_____ MAC agar selects for Gram-positive bacteria.

_____ Coliforms ferment lactose.

6 Choose the true statement(s) about *Enterobacteriaceae* (select all that apply).

a. It is a taxonomic family.
b. They include harmless bacteria.
c. They include Gram-positive bacteria.
d. They include fecal coliforms.
e. They include coccus-shaped bacteria.

7 Which of the following render MAC agar selective? (Select all that apply.)

a. Sodium chloride (NaCl)
b. Bile salts
c. Neutral red
d. Crystal violet
e. Lactose

8 Select the true statements about coliforms (select all that apply).

a. They include normal gut microbiota.
b. They include spore-forming bacteria.
c. They are Gram-negative bacteria.
d. They are only present if pathogens are present.
e. They include *Escherichia coli*.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 23
DATA SHEET
(continued)

Critical Thinking

1 Why do bile salts make an ideal ingredient in MAC agar to grow fecal coliforms over other bacteria?

2 Occasionally MAC agar is prepared as a broth medium, but such a format would not be useful for food safety or water quality testing. Explain why.

— Lab Notes —

EXERCISE 24

Eosin Methylene Blue (EMB) Agar

After doing this exercise, you should be able to:

1. Explain why EMB agar is a selective media.
2. Describe how EMB agar differentiates bacterial species from one another based on lactose fermentation.
3. Discuss the formulation of EMB agar and state specifically which ingredients allow it to be selective versus differential.
4. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess how different bacteria grow on EMB agar.

Background

The media we explore in this lab is very similar to MacConkey (MAC) agar reviewed in Exercise 23. Like MAC agar, **eosin methylene blue (EMB) agar** is an enriched selective and differential growth media used for detecting members of the family *Enterobacteriaceae*. Recall that fecal coliforms are members of *Enterobacteriacea*. Because *Escherichia coli* is one of the most abundant fecal coliforms, it is considered the hallmark bacterium for fecal contamination of food and water.

Selective and Differential Aspects of EMB Agar

The gelatin in the media provides a rich nitrogen and organic carbon source. Lactose in the medium help us differentiate lactose fermenting coliforms, such as *Escherichia coli* and *Enterobacter aerogenes*, from pathogens such as *Proteus*, *Shigella*, and *Salmonella*, which do not ferment lactose. (See Fig. 23.1, p. 238, for an introduction to lactose fermentation.) The dyes eosin Y and methylene blue render the media selective and allow us to differentiate lactose fermenters from non-fermenters. Because the dyes inhibit the growth of most Gram-positive organisms (some *Enterococcus* and *Staphylococcus* species are exceptions), this media is selective for Gram-negative bacteria.

The capacity to differentiate between lactose fermenters and non-fermenters is due to how the dyes interact with the acidic end products that lactose fermenters produce. Based on the degree of lactose fermentation that occurs, these dyes will turn the bacterial growth a color ranging from a blackish/metallic-green to a pink/purple. A dark metallic-green growth is typical of vigorous lactose fermenters, like *Escherichia coli* (Fig. 24.1). Other, less vigorous lactose fermenters, such as *Enterobacter* or *Klebsiella* species, produce colonies that can range from pink to dark purple on the medium. Lactose non-fermenters typically retain their normal color or take on the coloration of the medium (Fig. 24.1).



FIGURE **24.1** **Eosin Methylene Blue (EMB) Agar** • This EMB agar plate was inoculated with a Gram-positive coccus (center) and two members of the *Enterobacteriaceae*, a coliform at the top and a noncoliform at the bottom. The Gram-positive coccus was inhibited by eosin and methylene blue dyes, but still grew enough to form a thin film on the agar. Vigorous lactose fermentation by the coliform gave its growth a shiny green appearance. The noncoliform bacterium did not ferment lactose, and therefore exhibits its regular coloration.

Clinical Applications

EMB agar is used to isolate fecal coliforms in food and dairy products as well as in environmental (especially water) samples. As mentioned in Exercise 23, this is because fecal coliform bacteria are indicator organisms whose presence above a given threshold level suggest that enteric pathogens such as certain *Escherichia coli* strains, *Salmonella*, *Shigella*, *Proteus*, *Klebsiella*, and *Yersinia*, may be present.

While pathogens within the *Enterobacteriaceae* family are important to consider in our food and water supply, these bacteria also cause a significant number of **nosocomial** or **health care-acquired infections** (**HAIs**) (infections that develop while a patient is receiving care in a health-care setting) and certain **community acquired infections** (infections that develop outside of a health-care setting). Compounding the importance of detecting

these bacteria in clinical samples is the fact that some *Enterobacteriaceae* family members are increasingly resistant to carbapenems along with many other antibiotics. These highly resistant bacteria are called **CRE strains**, which stands for carbapenem-resistant *Enterobacteriaceae*.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Eosin methylene blue (EMB) agar plate (1)
- ❑ Nutrient agar (NA) plate (1)
- ❑ Inoculating loop
- ❑ Fresh broth cultures of these recommended bacteria:
  - ☐ *Enterobacter aerogenes*
  - ☐ *Escherichia coli*
  - ☐ *Providencia stuartii*
  - ☐ *Staphylococcus epidermidis*

Medium Recipes

Eosin Methylene Blue (EMB) Agar (Levine)

Pancreatic digest of gelatin	10.0 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Dipotassium phosphate (K_2HPO_4)	2.0 g
Methylene blue	0.065 g
Eosin Y	0.4 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.9–7.3 at 25°C

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

Today you will test how four organisms grow on nutrient agar versus on EMB agar, a media that has selective and differential properties. EMB agar would normally be streaked for isolation, but in this lab, you will use a simple zigzag inoculation of pure cultures for the sole purpose of seeing what the different results look like.

1. **Obtain and Label Your Media:** Obtain your two plates of media (one EMB agar plate and one NA plate). Using a permanent marker, divide the bottom of each plate into four sectors. Label the base of each plate with the organisms' names (one organism in each plate section), your name, and the date. Use the same positions for each specimen on each plate.
2. **Inoculate Your Plates:** Gently mix each stock culture, being careful to not splash the culture into the tube's cap. Use an inoculating loop to perform a zigzag inoculation of each bacterium into their designated section on the plates. Inoculate four bacteria onto the EMB agar plate and the same four bacteria onto the NA plate. You will need to flame sterilize the loop between each organism so you do not cross-contaminate the samples.
3. **Incubate the Samples:** Incubate all the samples at 35 ± 2°C for 48 hours.
4. **Clean up:** Save or dispose of the original cultures as your instructor directs.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Examine and compare the plates for color and quality of growth. Note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **24.1** Eosin Methylene Blue Agar Results and Interpretations

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism is inhibited by eosin and/or methylene blue	Most likely a Gram-positive bacterium
Good growth (G)	Organism is not inhibited by eosin and/or methylene blue	Most likely a Gram-negative bacterium
Growth is pink and mucoid (Pi)	Organism produces acid as a byproduct of lactose fermentation	Possible coliform bacterium
Growth is "dark" (purple to black, with or without green metallic sheen) (D)	Organism produces acid as a byproduct of lactose fermentation	Probable coliform bacterium
Growth is "colorless" (not purple, green, red, or pink) (C)	Organism does not ferment lactose	Unlikely to be a coliform bacterium

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 24

DATA SHEET

Eosin Methylene Blue (EMB) Agar

During the Lab

1 Record your results in the table provided, and use Table 24.1 to help you shape your interpretations.

Organism	Growth (N/P/G)		EMB Growth Color (Pi/D/C)	Interpretation and Presumptive ID
	EMB	NA		

Lab Related Questions

1 You are studying a bacterial sample and you see that it grew on NA but not on EMB agar. What is the best conclusion?

a. The bacterium is likely Gram positive.
b. The bacterium does not use lactose as a carbon source.
c. The bacterium is a pathogen.
d. The bacterium was from an older culture, so it did not grow as expected.
e. The dye eosin was left out of the EMB agar when it was prepared.

2 Choose the true statements about EMB agar (select all that apply).

a. This is a defined medium.
b. This is a selective medium.
c. This is an enriched medium.
d. This is a differential medium.
e. This medium detects a bacterium's ability to ferment glucose.

3 What is the role of methylene blue in EMB agar? (Select all that apply.)

a. It allows bacteria to ferment lactose.
b. It serves as a nutrient source in EMB agar.
c. It acidifies the pH of the medium when lactose is fermented.
d. It is important for differentiating lactose fermenters from non-fermenters.
e. It has a role in inhibiting Gram-positive bacteria from growing.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Bacteria that ferment lactose will always grow on EMB agar.

_____ Gram-positive bacteria appear green when grown on EMB agar.

_____ EMB agar selects for Gram-negative bacteria.

_____ Coliforms do not typically grow on EMB agar.

5 Choose the true statements about the lactose in EMB agar (select all that apply).

a. Lactose fermenters may appear pink.
b. Lactose fermenters may appear dark green/blackish.
c. Most coliforms can ferment lactose.
d. EMB agar only allows us to assess lactose fermentation ability for Gram-positive bacteria.
e. Bacteria will only grow on EMB agar if they can use lactose as a carbon source.

6 What is the role of eosin Y in EMB agar? (Select all that apply.)

a. It selects for the growth of Gram-positive bacteria.
b. Bacteria that grow on EMB agar use eosin Y as a carbon source.
c. It stabilizes the media's pH.
d. It helps us visually differentiate lactose fermenters from non-fermenters.
e. It inhibits the growth of most Gram-positive bacteria.

7 Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? (Select all that apply.)

a. NA medium is selective for Gram-positive bacteria, while EMB agar is selective for Gram-negative bacteria.
b. NA medium is nutrient deficient and will not grow fastidious bacteria, while EMB agar is enriched and therefore will support the growth of fastidious bacteria.
c. We can expect anything that grows well on NA to also grow on EMB agar.
d. We can expect bacteria that grow well on EMB agar will also grow on NA.
e. Barring experimental process errors, a lack of growth on NA should be accompanied by a lack of growth on EMB agar.

8 If a bacterium grew on EMB agar and the bacteria takes on a dark-green color, then it means _____.

In contrast, if the bacterial growth lacks coloration, it means _____. A pink bacterial growth coloration suggests _____.

9 *Enterobacteriaceae* family members are important to detect because (select all that apply):

a. They can contaminate foods.
b. They can cause HAIs.
c. They can cause community-acquired infections.
d. They indicate fecal contamination of water.
e. They are all pathogens.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 24
DATA SHEET
(continued)

Critical Thinking

1 Would removing eosin Y and/or methylene blue from EMB agar alter the medium's sensitivity or specificity? Explain your answer. (See pp. 9–10, "A Word About Experimental Design," for assistance with these terms.)

2 Suppose a mistake was made while preparing a batch of EMB agar and the starting pH is 7.6 instead of 6.9–7.3. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pp. 9–10, "A Word About Experimental Design," for assistance with these terms.)

— Lab Notes —

EXERCISE 25

Blood Agar

After doing this exercise, you should be able to:

1. Describe the formulation of blood agar.
2. Explain how blood agar differentiates bacterial species from one another.
3. Discuss the forms of hemolysis.
4. State how blood agar can be clinically useful.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the hemolytic properties of select bacteria by growing them on blood agar.

CAUTION!

The U.S. Centers for Disease Control and Prevention cautions, "Either nonpathogenic or attenuated bacterial strains should be used when possible, especially in teaching laboratories." This lab exercise can either be performed with samples collected via throat swab or you may use known bacterial samples. Both protocols are described herein. Before deciding to use throat cultures as the inoculum source, faculty should check institutional policies regarding cultivating unknown bacteria from human subjects. Anytime such sampling is performed, BSL-2 agents that are *not* attenuated could be isolated. Faculty are strongly cautioned to review their institution's policies on students handling such samples.

Background

Blood agar is an enriched medium that includes 5% blood (usually sheep blood) in a tryptic soy agar base. Its rich composition allows for the growth of fastidious organisms, while also allowing for the differentiation of bacteria—especially certain Gram-positive cocci such as *Streptococcus*, *Staphylococcus*, and *Enterococcus* species—based on the degree to which they conduct **hemolysis** (break down RBCs).

The three major types of hemolysis are: **α-hemolysis** (alpha hemolysis), **β-hemolysis** (beta hemolysis), and **γ-hemolysis** (gamma hemolysis). Although all three of these include the word hemolysis, only one—β-hemolysis—completely breaks down RBCs and hemoglobin as indicated by a clearing of the medium around bacterial colonies (Fig. 25.1). In contrast, α-hemolysis, which is often described as a partial breakdown of RBCs, involves the oxidation of hemoglobin inside RBCs to methemoglobin.[1] This produces an olive-greenish discoloration of the blood agar around the colonies in reflected light (Fig. 25.2). Finally, in γ-hemolysis the RBCs are not broken down at all, nor is hemoglobin affected, so there is no change to the medium (Fig. 25.3).

But why would certain bacteria even conduct hemolysis? Well, iron is usually an essential nutrient for bacterial growth; however, it is not typically circulating freely in the human body. Instead it is bound in proteins like hemoglobin and other iron-containing proteins. Therefore, pathogens that conduct β-hemolysis may have a survival advantage due to their ability to completely break down RBCs and hemoglobin to obtain iron. While most pathogenic *Streptococcus* species happen to be α- or β-hemolytic, you should not assume that a bacterium must be α- or β-hemolytic to be pathogenic. Hemolytic activity is just one of many virulence factors that bacteria may have and there are plenty of non-hemolytic bacteria in nature that cause disease.



FIGURE **25.1** **β-Hemolysis** • *Streptococcus pyogenes* demonstrates β-hemolysis. The clearing around the growth is a result of complete lysis of red blood cells in the medium. For this photograph, the plate was illuminated with transmitted light.

[1] Hemoglobin is the oxygen-carrying molecule in RBCs. It contains an iron atom (Fe), which can lose electrons (undergo an oxidation to become Fe^{3+}) or gain electrons (undergo a reduction to become Fe^{2+}). Hemoglobin's iron atom must be in the Fe^{2+} state to bind oxygen. In methemoglobin, which cannot bind oxygen, the iron exists in the Fe^{3+} state.

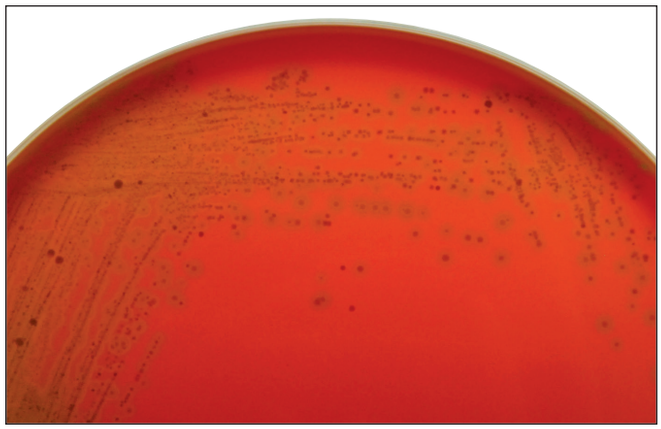


FIGURE **25.2** **α-Hemolysis** • This is a streak plate of *Streptococcus pneumoniae* demonstrating α-hemolysis. The greenish zone around the colonies results from incomplete lysis of red blood cells in the medium. For this photograph, the plate was illuminated with transmitted light, so the coloration appears more yellow-green than olive-green.

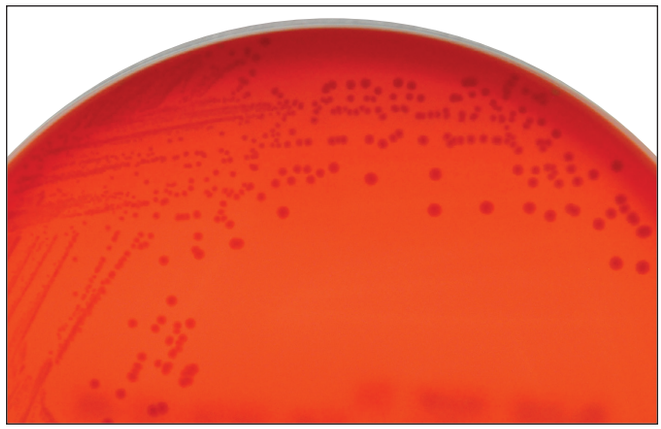


FIGURE **25.3** **γ-Hemolysis** • This streak plate of *Staphylococcus epidermidis* on a sheep blood agar shows that red blood cells in the medium are not lysed.

Hemolytic Bacteria

Many bacterial pathogens make **hemolysins**, which are specialized proteins that can affect RBCs, hemoglobin, and other cells such as white blood cells.

Here we focus on the hemolysins that certain members of the genus *Streptococcus* make, **streptolysins**. They come in two forms: type O and type S. **Streptolysin O** is oxygen labile (unstable in the presence of oxygen) and therefore works best under anaerobic conditions. **Streptolysin S** is oxygen stable, but also works best under anaerobic conditions. Therefore, it is useful to the **streak-stab technique** to detect streptolysin activity for bacteria inoculated onto blood agar. In this technique, a blood agar plate is streaked on the agar surface and then the agar is stabbed with the loop at the end of the streak pattern. The stab encourages streptolysin activity because of the reduced oxygen concentration of the subsurface environment. The result may be α-hemolysis on the surface, but β-hemolysis in the stab (Fig. 25.4).

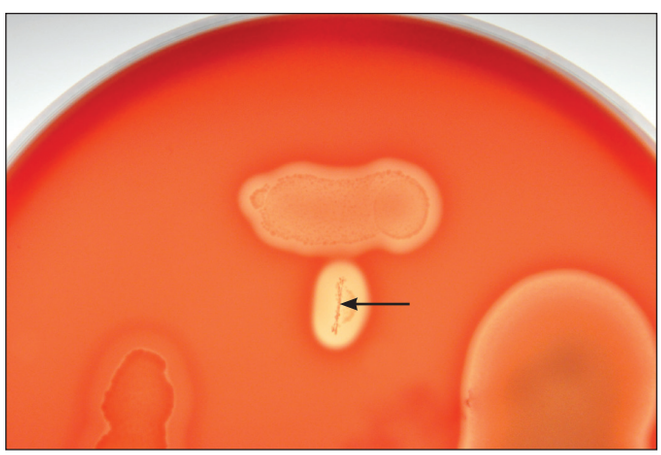


FIGURE **25.4** **Aerobic Versus Anaerobic Hemolysis** • An unidentified throat culture isolate demonstrates α-hemolysis when growing on the surface, but β-hemolysis beneath the surface surrounding the stab (**arrow**). This results from production of an oxygen-labile hemolysin.

Clinical Applications

Blood agar is commonly used to differentiate pathogenic *Streptococcus* species from non-pathogenic species of this diverse genus of Gram-positive cocci. In humans, streptococcal pathogens primarily infect the respiratory tract, skin, and bloodstream. One of the most common streptococcal pathogens identified in clinical samples is *S. pyogenes*. This bacterium is typically β-hemolytic and causes a wide range of infections to include streptococcal pharyngitis (strep throat), scarlet fever, streptococcal toxic shock syndrome, necrotizing fasciitis (flesh-eating bacteria), superficial skin infections (impetigo), and deeper skin infections (cellulitis). Blood agar remains an important means of detecting *S. pyogenes* in clinical samples, to include definitively diagnosing streptococcal pharyngitis. Other noteworthy streptococcal pathogens include *S. pneumonia* (bacterial pneumonia), and *S. mutans* (dental caries).

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sheep blood agar plate (1) (commercially available, TSA containing 5% sheep blood)
- ❑ Inoculating loop
- ❑ If a sample will not be collected by throat swab, then provide fresh broth cultures of these recommended bacteria:
  - ☐ *Streptococcus pyogenes* (BSL-2)
  - ☐ *Escherichia coli* (BSL-1 strains are standard)
  - ☐ *Streptococcus mutans* (BSL-1 strains are standard)

Medium Recipes

5% Sheep Blood Agar (TSA + 5% Sheep Blood)

Infusion from beef heart (solids)	2.0 g
Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	13.0 g
Sodium chloride (NaCl)	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
Defibrinated sheep blood	50.0 mL
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

PROCEDURE

Faculty may approach this lab differently depending on institutional safety regulations and the availability of BSL-2 graded lab facilities. As such, you will either inoculate stock cultures onto a blood agar plate to observe the various forms of hemolysis, or you may perform a throat swab and culture bacteria from that specimen. Follow whichever procedure your instructor decides to use. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

OPTION 1: Throat Culture Procedure

1. **Obtain and Label Your Media:** Obtain a blood agar plate. Use a permanent marker to mark the bottom of the plastic plate so it is divided into four equal quadrants. Then label the plate with your name, the date, and "throat culture."
2. **Perform a Throat Swab:** Have your lab partner (also appropriately protected) obtain a sterile cotton-tipped swab and follow the procedure in Appendix B, page 475, to obtain the specimen from your throat.
3. **Use the Swab to Inoculate the First Quadrant of the Blood Agar:** For this procedure, you will inoculate the agar using a zigzag inoculation combined with streak plating (see Ex. 4, p. 73, to review the process). Immediately inoculate the collected specimen into the first quadrant of the blood agar plate. Once you've inoculated the first plate quadrant, dispose of the swab into an approved biological waste container.
4. **Use an Inoculating Loop to Inoculate the Remaining Quadrants of the Blood Agar:** Use your inoculating loop to inoculate the remaining three plate sections. (Again, see Ex. 4, p. 69–71, to review the process.)
5. **Incubate the Samples:** Because of the potential for cultivating a pathogen from a throat swab specimen, tape down the plate lid once you have finished inoculating the plate. This will reduce the chance of the plate being accidentally opened. Invert the plate and incubate your sample at 35 ± 2°C for 24 hours; remove the plate to the refrigerator if you cannot examine it at 24 hours.
6. **Clean up:** Disinfect your work surface and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples). Do not open your plate until your instructor has seen it and given permission to do so.
2. **Observe Your Plates:** Holding your plate up to a light or using transmitted light from a light box/colony counter may help you better detect color changes and clearing around the colonies. Compare your observations to the information in Table 25.1 and note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

OPTION 2: Stock Culture Procedure

1. **Obtain and Label Your Media:** Obtain a blood agar plate. Use a permanent marker to mark the bottom of the plastic plate so it is divided into three equal sections. Label one section as *S. pyogenes*, another as *E. coli*, and the third as *S. mutans*.
2. **Inoculate the Blood Agar with the Streak-Stab Technique:** Use an inoculating loop to perform a zigzag inoculation of each sample into its designated plate section. At the tail end of the zigzag surface

inoculation, use the loop to make a small stab/incision into the agar as shown in Figure 25.4. This will allow you to see the difference in hemolytic action for each bacterium under reduced oxygen conditions. ***Note:*** Do not interrupt your streaked surface with this stab inoculation; instead stab at the tail end of the streak pattern.

3. **Incubate the Samples:** Invert the plate and incubate your sample at 35 ± 2°C for 24 hours; remove the plate to the refrigerator if you cannot examine it at 24 hours.
4. **Clean up:** Disinfect your work surface and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Plates:** Holding your plate up to a light or using transmitted light from a light box/colony counter may help you better detect color changes and clearing around the colonies. Compare your observations to the information in Table 25.1 and note your observations and results on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

TABLE **25.1** Blood Agar Test Results and Interpretations

Result	Interpretation	Symbol
Clearing around growth	Organism hemolyzes RBCs completely	β-hemolysis
Greening around growth	Organism partially hemolyzes RBCs/hemoglobin oxidized to methemoglobin	α-hemolysis
No change in the medium	Organism does not hemolyze RBCs	No hemolysis (also called γ-hemolysis)

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 25
DATA SHEET

Blood Agar

During the Lab

OPTION 1: Throat Culture Procedure

Observe your plate and find four different colonies growing on the blood agar surface (try to find a diversity of hemolysis reactions). Based on your observations, complete the following table. Refer to Table 25.1 for assistance with interpreting hemolytic property, and Figure 6.6 on page 91 when reporting colony morphology information.

	Colony Morphology and Agar Appearance	Hemolysis Result (α, β, γ)	Interpretation
Colony 1			
Colony 2			
Colony 3			
Colony 4			

OPTION 2: Stock Culture Procedure

Observe each of the three cultured bacteria and complete the following table. Refer to Table 25.1 for assistance with interpreting hemolytic property.

Organism	Agar Appearance Surface Inoculation	Agar Appearance Stab Inoculation	Surface Inoculation Hemolysis Result (α, β, γ)	Stab Inoculation Hemolysis Result (α, β, γ)	Interpretation

Lab Related Questions

1 Select the false statement(s) about blood agar (select all that apply).

a. It is a selective medium.
b. It is an enriched medium.
c. It is a differential medium.
d. It is useful for growing fastidious bacteria.
e. It inhibits the growth of Gram-negative bacteria.

2 Which of the following is not caused by *Streptococcus pyogenes*?

a. Streptococcal toxic shock syndrome
b. Necrotizing fasciitis
c. Dental caries
d. Streptococcal pharyngitis
e. Impetigo

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Methemoglobin is an oxidized form of hemoglobin.

_____ *Streptococcus* species are differentiated from one another on blood agar based on Gram property.

_____ Blood agar is usually prepared with bovine (cow) blood.

_____ Only pathogens grow on blood agar.

4 Choose the mismatched pair(s) (select all that apply).

a. *Streptococcus*; Gram-negative
b. *S. pyogenes*; β-hemolytic
c. Streptolysin O; oxygen labile
d. Streptolysin S; oxygen labile
e. γ-hemolysis; lack of hemolysis

5 Which of the following is not a reason for stabbing blood agar? (Select all that apply.)

a. It helps to detect streptolysin O activity.
b. It helps to detect streptolysin S activity.
c. It helps to isolate bacterial species from one another.
d. It tells us if we have a mixed culture.
e. It allows *Streptococcus* species to grow.

6 A bacterium with a _____ coloration around colonies is said to conduct _____ hemolysis, which is described as a partial lysis of the RBCs in the medium. In contrast, _____ generates a clearing around the bacteria due to _____.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 25
DATA SHEET
(continued)

7 Select the false statement(s) about hemolysis (select all that apply).

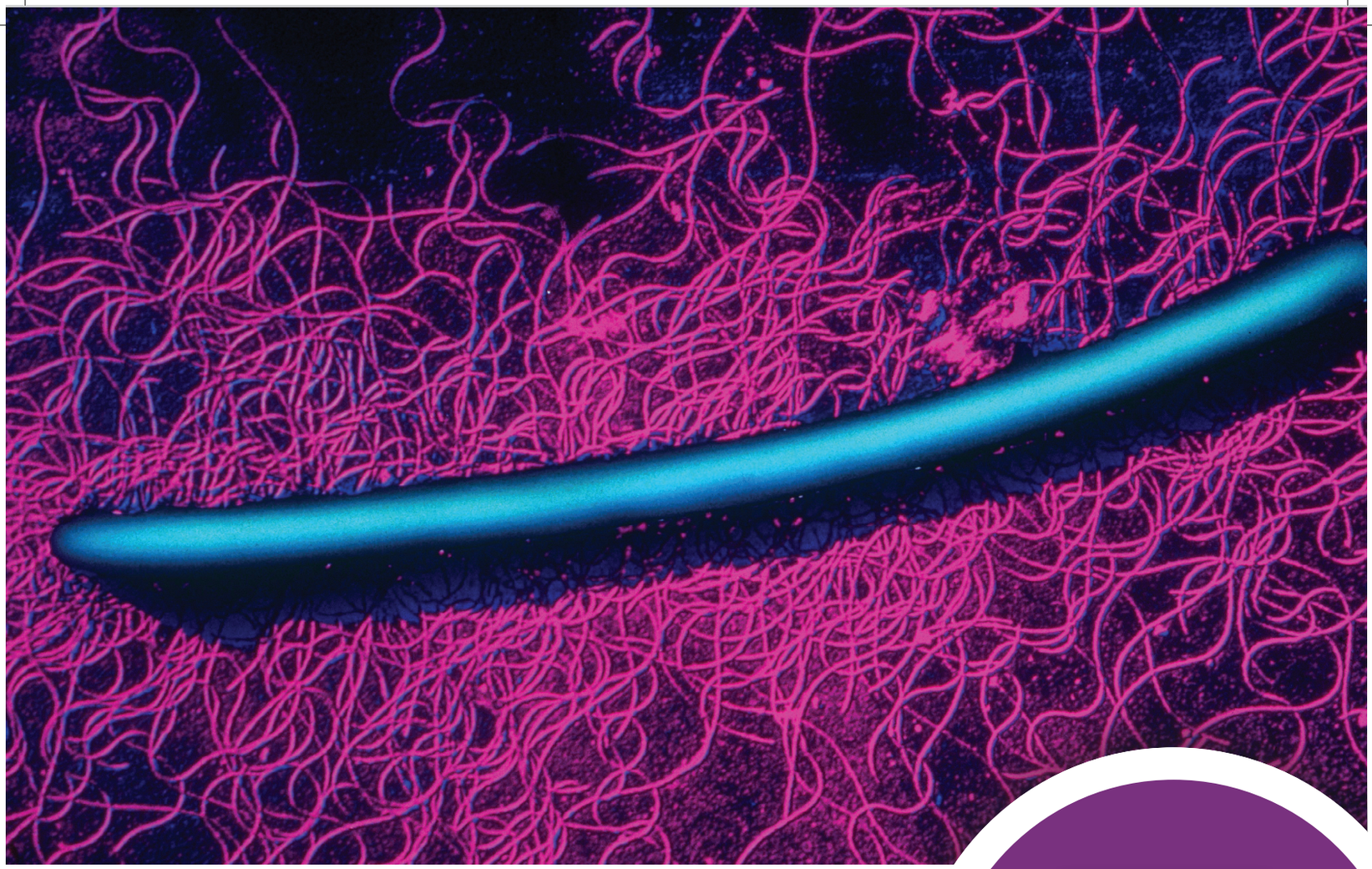
a. γ-hemolysis is a virulence factor.
b. All pathogens conduct α- or β-hemolysis.
c. Hemolysis only occurs under anaerobic conditions.
d. α-hemolysis must occur before β-hemolysis.
e. *S. pyogenes* performs β-hemolysis.

Critical Thinking

1 You are studying a newly emerging pathogen and determine the organism exhibits β-hemolysis when growing on the surface of blood agar, but exhibits γ-hemolysis when inoculated via a "stab" into blood agar. Based only on this observation, what may you conclude about this bacterium's unique hemolysin(s)?

2 Although streptolysins tend to be most active under decreased oxygen levels, throat culture samples are not analyzed using a stab inoculation. Explain why this is logical.

— Lab Notes —



SECTION

6

Biochemical Tests for Identifying Bacterial Isolates

In This Section

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EXERCISE 39 Gelatin Hydrolysis (Gelatinase Test) 357

— Lab Notes —

EXERCISE 26

Oxidation-Fermentation (O-F) Test

After doing this exercise, you should be able to:

1. State what O-F media can reveal about a bacterial species.
2. Discuss the possible interpretations for various O-F test results.
3. Describe the formulation of oxidation-fermentation (O-F) media.
4. Explain how O-F media is used in a clinical microbiology laboratory.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the oxidative-fermentative metabolic features of select bacteria by growing them on O-F media.

Background

The **Oxidation-Fermentation (O-F) test** (Hugh and Leifson's O-F media) differentiates bacteria—especially certain Gram-negative bacteria—based on how they metabolize carbohydrates. Specifically, this test reveals if a bacterial species uses aerobic oxidative respiration and/or fermentation to breakdown a given sugar.

Oxidative Respiration Versus Fermentation

Oxidative respiration involves stripping electrons off nutrients (via glycolysis, an intermediate step, and the Krebs cycle) and shuttling those harvested electrons through an electron transport chain to a final electron acceptor to generate ATP. In **aerobic oxidative respiration**, oxygen serves as the final electron acceptor in the electron transport chain and is therefore an absolute requirement for this pathway. In addition to making ATP, aerobic oxidative respiration also produces carbon dioxide (CO_2) and water (H_2O).

The O-F test is formulated to detect the mildly acidic end products that oxidative respiration generates. In addition, this test detects if a bacterium ferments a specific sugar to form acidic end products. Unlike aerobic oxidative respiration, **fermentation** does not use an electron transport chain and does not require oxygen to make ATP. Bacteria are metabolically versatile, so some species perform oxidative respiration and fermentation, choosing which is best based on the availability of oxygen.

How the O-F Test Works

Hugh and Leifson's O-F media contains a single sugar—usually glucose (dextrose)—as the carbohydrate source. In addition, the media has a high sugar-to-peptone ratio, which renders it especially useful for detecting the weakly acidic end products made during oxidative respiration. This is because the alkaline (basic) products produced when bacteria digest protein will not be present in quantities that readily neutralize weak acids that oxidative metabolism produces. Bromothymol blue dye serves as the pH indicator in the media. It appears yellow at a mildly acidic pH of 6.0, green at a near neutral pH of 7.1, and blue at the slightly alkaline pH of 7.6.

Before inoculation, the tubes of O-F media are placed in a boiling-water bath to drive off dissolved oxygen that may be in the media. Then, once the tubes of media have cooled, a bacterial sample is inoculated into two tubes; one tube will be set up to maintain anaerobic conditions, while the other will promote aerobic conditions. For the anaerobic tube, immediately following inoculation, the media's surface is sealed off with a layer of mineral oil. This prevents oxygen diffusion into the media and thereby prevents aerobic oxidative metabolism while allowing fermentation (Fig. 26.1). For the second tube, the media's surface is not sealed off with oil, allowing oxygen to diffuse through the semisolid media to support aerobic oxidative respiration (although fermentation is not prevented). Following incubation, the samples are observed to determine if there was a color change from green (the original media color) to yellow (Fig. 26.2).



FIGURE **26.1** **Adding the Sterile Mineral Oil Layer** • Tip the tube slightly to one side, and gently add sterile mineral oil to a depth of 3–4 mm. Be sure to use a sterile pipette for each tube.

Table 26.1 provides interpretations for the possible color outcomes. If the test organism ferments glucose, then *both* the sealed and unsealed media should turn yellow. If the organism performs *only* oxidative metabolism of glucose, then only the unsealed media turns yellow. Note that oxidative metabolism and fermentation both cause the unsealed media to turn yellow. Thus, while O-F media can reveal if an organism strictly uses oxidative metabolism to breakdown glucose, it does not reveal if an organism can metabolize glucose with oxidative *and* fermentation processes or strictly via fermentation. This is an important test limitation to recognize.

TABLE **26.1** **Oxidation-Fermentation (O-F) Test Results and Interpretations** ●

Sealed (anaerobic tube)	Unsealed (aerobic tube)	Interpretation	Symbol
Green or blue	Any amount of yellow	Oxidation only	O
Green or blue	Green or blue	No sugar metabolism; organism is nonsaccharolytic (cannot metabolize the sugar in the medium)	N
Yellow throughout	Yellow throughout	Oxidation and fermentation; or could be fermentation only	O-F or F[1]
Slightly yellow at the top	Slightly yellow at the top	Slow oxidation and slow fermentation; or could be slow fermentation only	O-F or F[1]

[1] The test does not differentiate between species that are strictly fermentative and species that conduct both fermentation *and* oxidative metabolism of the sugar. Therefore, when both tubes are yellow, the result is recorded as (O-F) or (F).

	Sealed Media Appearance	Unsealed Media Appearance	
Oxidative only	Green	Yellow	
Fermentation only	Yellow	Yellow	The test results for these two scenarios appear identical; thus, the O-F test does not allow us to differentiate between these two scenarios.
Oxidative and Fermentation	Yellow	Yellow	

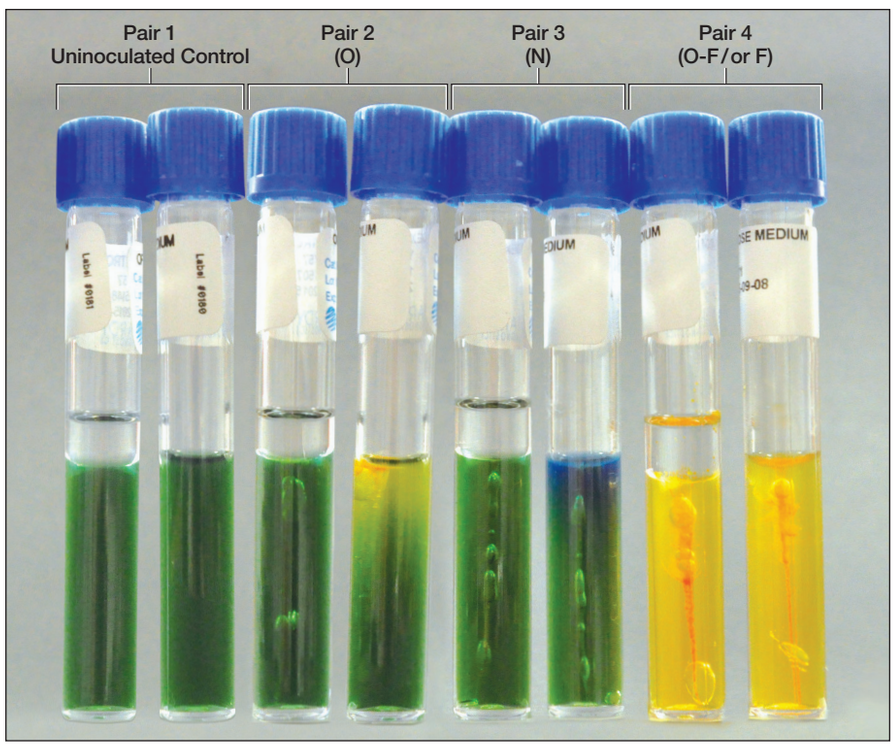


FIGURE **26.2** **Oxidation-Fermentation (O-F) Test Results** ● These tubes illustrate three possible results for the O-F test. **Pair 1** is an uninoculated control for color comparison. **Pair 2** was inoculated with a bacterium that only conducts oxidative metabolism (**O**) of glucose. **Pair 3** was inoculated with an organism incapable of utilizing glucose. Note the blue color in the upper portion of the unsealed media suggesting that the organism is both nonsaccharolytic (**N**) and strictly aerobic. **Pair 4** was inoculated with an organism capable of both oxidative and fermentative, or perhaps just fermentative, utilization of glucose (**O-F** or **F**). Recall the O-F test does not differentiate bacteria that use oxidative and fermentative processes from those that only use fermentative processes to metabolize the sugar in the media.

Clinical Applications

Determining a bacterium's identity is a key diagnostic tool. Many biochemical tests exist to help microbiologists accomplish this task. The O-F test is used to differentiate bacteria based on their ability to oxidize or ferment specific sugars. It allows presumptive separation of the fermentative *Enterobacteriaceae* (a large family that includes important pathogens such as *Escherichia coli* and *Salmonella*) from the strictly oxidative *Pseudomonas* (a genus that includes *P. aeruginosa*, which causes wound infections and swimmer's ear, otitis externa).

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ O-F glucose tubes (8)
- ❑ Sterile mineral oil
- ❑ Sterile transfer pipettes
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Escherichia coli*
  - ☐ *Kocuria rosea*

Medium Recipe

Hugh and Leifson's O-F Medium with Glucose

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	2.0 g
Sodium chloride (NaCl)	5.0 g
Dipotassium phosphate (K_2HPO_4)	0.3 g
Agar	2.5 g
Bromothymol blue	0.08 g
Glucose ($C_6H_{12}O_6$)	10.0 g
Distilled or deionized water	1.0 L

Adjust to pH = 6.6–7.0 at 25°C

PROCEDURE

Eight O-F media tubes will be used for this exercise, six for inoculation (two for each organism) and two for controls. One of each pair will receive a mineral oil overlay to maintain an anaerobic environment and force fermentation if the organism is capable of doing so. For best results, use a heavy inoculum and several stabs for each tube. Use only sterile pipettes to add the mineral oil. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain and Label Your Media:** Obtain eight O-F tubes. Label six (in three pairs) with the names of the organisms, your group name, and the date. Label the last pair "control."
2. **Inoculate the Media:** Using an inoculating needle, stab inoculate each pair (aside from the uninoculated control tubes) with the appropriate test organism to a depth of about 1 cm from the bottom of the agar. Most bacteria grow slowly in O-F media; therefore, to have observable results by your next class session, you will need to inoculate the tubes heavily. To introduce a heavy inoculum, simply repeat the inoculation process two additional times per tube so each tube is ultimately inoculated with three times the normal amount of inoculum.
3. **Add Mineral Oil:** Using a different sterile transfer pipette for each organism overlay one tube of each pair (including the controls) with 3–4 mm of sterile mineral oil (Fig. 26.1).
4. **Incubate the Samples:** Incubate all tubes at 35±2°C for 48 hours.
5. **Clean up:** Dispose of stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Samples:** Examine the tubes for color changes. Be sure to compare them to the controls before making a determination. Record your results in the table provided on the data sheet at the end of this lab.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 26

DATA SHEET

Oxidation-Fermentation (O-F) Test

During the Lab

1 Observe your tubes, and use Table 26.1 to help you complete the following table.

Organism	Color Results		Symbol	Interpretation
	Sealed	Unsealed		
Uninoculated controls				

Lab Related Questions

1 Select the true statement(s) about O-F media (select all that apply).

a. It is a differential medium.

b. It is a selective medium.

c. It contains methylene blue as a pH indicator.

d. The test is inoculated in pairs so that the media can be sealed off from atmospheric oxygen in one case, but not the other.

e. The media can differentiate between bacteria that use both oxidative and fermentation processes from those that only conduct fermentation.

2 Why is mineral oil added in the O-F test?

a. To serve as a nutrient source.

b. To keep potential pathogens from becoming airborne.

c. To remove oxygen from the media.

d. To help oxygen diffuse through the media.

e. To maintain an anaerobic environment.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ If a bacterium carries out oxidative metabolism, it cannot also carry out fermentation.

_____ O-F media is designed to detect the alkaline byproducts of fermentation.

_____ O-F media provides a single carbohydrate source.

_____ The O-F test is selective for bacteria that conduct fermentation versus aerobic oxidative respiration.

4 Facultative anaerobes, such as members of the family *Enterobacteriaceae*, are capable of both aerobic respiration and fermentation. What color results would you expect for such organisms when grown in O-F glucose media?

a. The sealed media should turn yellow and the unsealed media should remain green.
b. The sealed media should remain green and the unsealed media should turn yellow.
c. The sealed tube should turn yellow and the unsealed tube should turn blue.
d. Both the sealed and unsealed media should turn yellow.
e. Both the sealed and unsealed tubes should remain green.

5 Assume you were studying a newly discovered bacterium that is aerotolerant (can grow in the presence of oxygen) and performs anaerobic respiration where a certain amino acid that is common in proteins serves as the final electron acceptor. What observations would you expect for such a specimen?

a. The sealed media should turn yellow and the unsealed media should remain green.
b. The sealed media should remain green and the unsealed media should turn yellow.
c. The sealed tube should turn yellow and the unsealed tube should turn blue.
d. Both the sealed and unsealed media should turn yellow.
e. Both the sealed and unsealed tubes should remain green.

6 In the O-F test performed in this exercise, _____ served as the carbohydrate source while _____ served as a protein source.

Critical Thinking

1 Why is it necessary to use two controls (uninoculated control and a nonsaccharolytic sample) rather than just one? What are the specific purposes of each? Be thorough in your answer.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 26
DATA SHEET
(continued)

2 Suppose you perform this exercise and upon making your observations, you notice the unsealed, uninoculated control contained slight yellowing at the top. Suppose further that Pair 1 showed complete yellowing of both tubes and Pairs 2 and 3 showed slight yellowing of the unsealed tube. Assuming all other tubes were green, what conclusions could you safely make?

— Lab Notes —

EXERCISE 27

Phenol Red Fermentation Broth

After doing this exercise, you should be able to:

1. State what growing a bacterium in phenol red fermentation broth can reveal.
2. Discuss the possible interpretations for various phenol red fermentation broth results.
3. Describe how phenol red broth is formulated to provide metabolic information about a bacterial species.
4. Explain how phenol red fermentation broth can be useful in a clinical microbiology laboratory.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess the fermentative metabolic features of select bacteria by growing them in phenol red fermentation broth.

Background

Carbohydrate fermentation is a generalized biochemical process in which a carbohydrate, usually a sugar, is metabolized to make ATP without the use of oxidative phosphorylation. Recall that oxidative phosphorylation can be aerobic (the electron transport chain requires oxygen as a final electron acceptor) or anaerobic (the electron transport chain does not require oxygen as a final electron acceptor). Most microbes are metabolically flexible, so depending upon available nutrients and other environmental conditions they may conduct fermentation or perform oxidative phosphorylation (aerobic or anaerobic).

Monosaccharides and **disaccharides** are some of the most commonly fermented carbohydrates. Monosaccharides are the simplest carbohydrates, examples include the sugars glucose (dextrose), fructose (levulose), and galactose. Monosaccharides are sometimes called *simple sugars* because they do not need to be broken down into smaller carbohydrate units before entering metabolic pathways. In contrast, disaccharides are sugars made up of two monosaccharides. Disaccharides must be broken down (**hydrolyzed**) into their simple sugar components before entering metabolic pathways. Two common disaccharides are lactose and sucrose. The initial step in metabolically processing these sugars involves hydrolyzing them into their monosaccharide components: lactose is hydrolyzed into glucose and galactose whereas sucrose is split to release glucose and fructose. Thus, when an organism is described as a "lactose fermenter," it means the organism splits the disaccharide lactose into the monosaccharides glucose and galactose and then ferments those monosaccharides.

In microbiology we frequently use the term **fermenter**; unless expressly stated otherwise, this term should be assumed to include the initial hydrolysis of a carbohydrate into monosaccharide subunits followed by fermentation pathways that further metabolize the simple sugars. ***Note:*** Unlike aerobic oxidative phosphorylation, fermentation does not require oxygen to occur. However, this point should not lead you to assume that bacteria only conduct fermentation under anaerobic conditions. In this laboratory exercise your samples are grown in aerobic conditions.

In most cases, carbohydrate fermentation begins with simple sugars entering glycolysis, which makes pyruvate as a product. (Instead of glycolysis, some organisms use alternative pathways, such as the Entner-Doudoroff pathway [Fig. A.3, p. 470], to make pyruvate.) In fermentation, the resulting pyruvic acid is further metabolized to make a variety of organic acids, alcohols, and hydrogen or carbon dioxide gas. Fermentation reactions are diverse and the exact end products made depend on what enzymes the organism has available to perform a subset of reactions. Refer to Fig. 27.1 and Fig. A.6 for more information about fermentation pathways.

How Phenol Red Broth Works

Phenol red broth is a differential medium that can reveal if a bacterium ferments a specific carbohydrate. In addition to a protein source (pancreatic digest of casein), the media contains one of several carbohydrates (e.g., glucose, lactose, sucrose), the pH indicator phenol red, and a Durham tube to detect gas formation.

After inoculation and incubation, the phenol red broth is observed for two things:

Color Change

The media's color after incubation indicates if the sugar in the media was fermented. This is due to the media's pH indicator, phenol red, which appears yellow below pH 6.8, pink/magenta above pH 7.4, and red in between. Carbohydrate fermentation often produces acidic end products that lower the media's pH below the neutral range thereby turning the medium yellow (Fig. 27.2). If the organism did not ferment the sugar and instead digested protein, then the media may appear pink due

to the alkaline (basic) end products associated with protein metabolism. You can expect red media for uninoculated samples and for samples containing bacteria that either made neutral end products or failed to react at all.

Gas Production

Some organisms produce gas (often carbon dioxide or hydrogen gas) as a fermentation byproduct. If this occurs, the gas becomes trapped in the inverted Durham tube

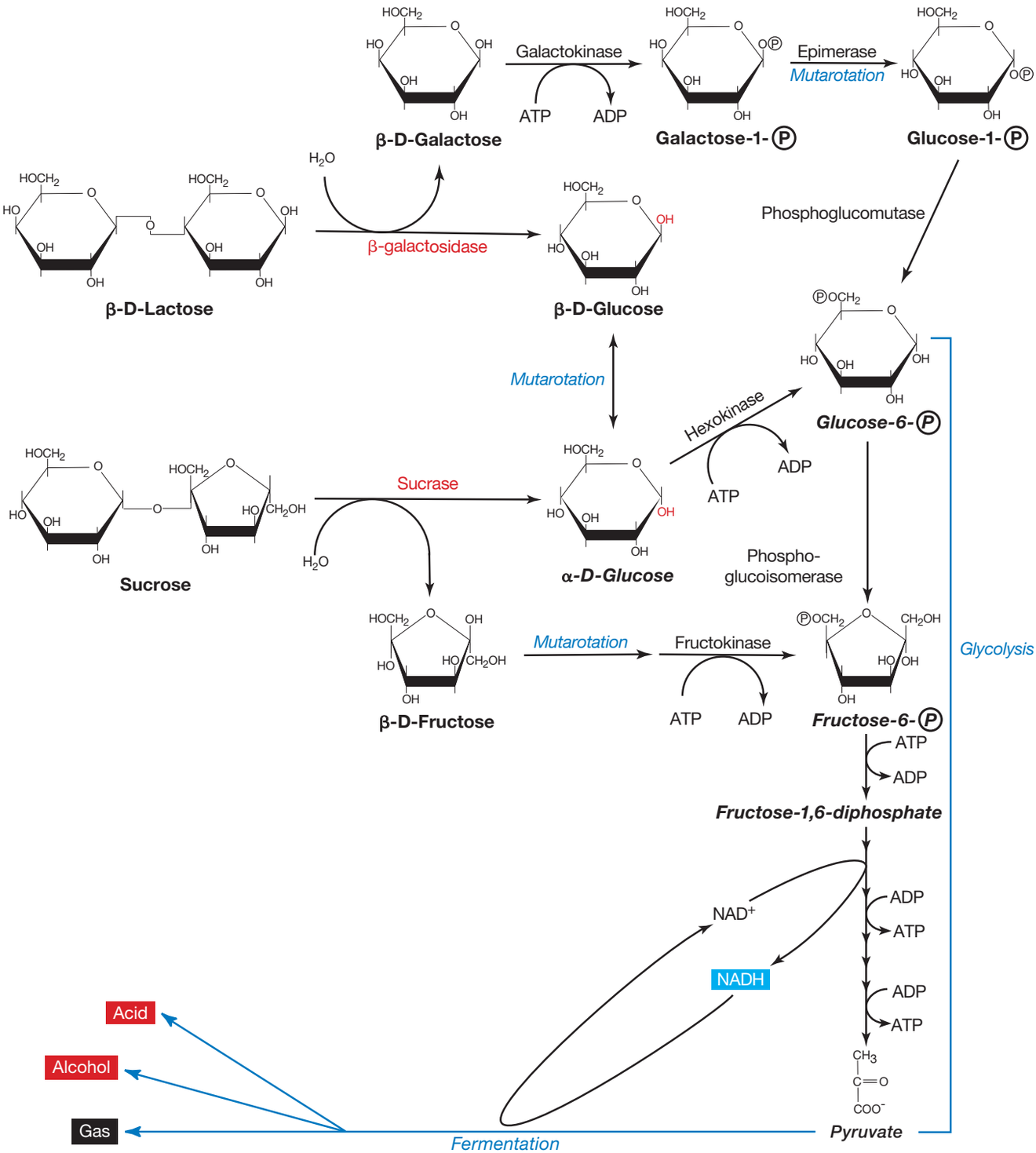


FIGURE **27.1** **Fermentation of Glucose and the Disaccharides Lactose and Sucrose** • Enzymes hydrolyze the disaccharides lactose and sucrose to release monosaccharides. These monosaccharides are then funneled into glycolysis as the initial stage in fermentation. The enzyme β-galactosidase hydrolyzes lactose into glucose and galactose whereas sucrase hydrolyzes sucrose to release glucose and fructose.

and a bubble is evident. Note that multiple tiny, pinpoint-sized bubbles in a Durham tube are typically artifacts of media preparation and/or due to samples being mixed. If gas is truly made, the bubble is comparatively large and easy to see.

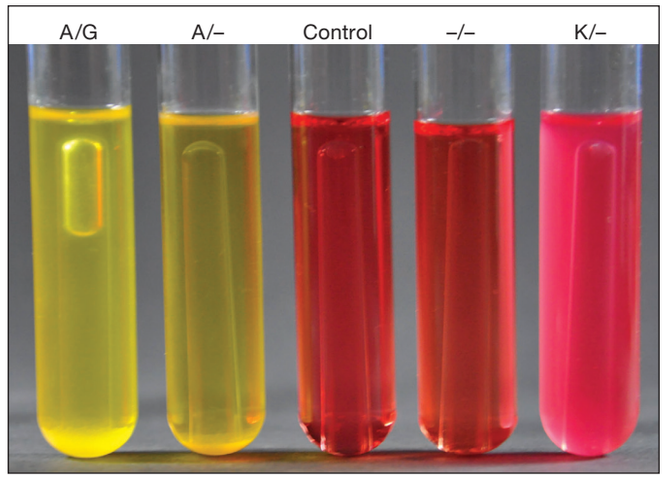


FIGURE **27.2** **Phenol Red Glucose Fermentation Broth Results** • These five phenol red glucose broths demonstrate various results. From left to right: **A/G** = fermentation with acid and gas production; **A/–** = fermentation with acid formation but without gas production; **Control** = uninoculated tube; **–/–** = no reaction (no fermentation reaction and no peptone breakdown)/lack of gas formation; **K/–** = no fermentation, instead proteins were metabolized to form basic end products/no gas produced.

TABLE **27.1** **Phenol Red Fermentation Broth Test Results and Interpretations** •

Result (list broth color first, then note if a bubble is present or absent)	Interpretation	Symbol
Yellow broth; bubble in Durham tube	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. (***Tip:*** Per Fig. 27.2, if lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase.)	A/G
Yellow broth; no bubble in Durham tube	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. (***Tip:*** Per Fig. 27.2, if lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase.)	A/–
Red broth; no bubble in Durham tube	The carbohydrate (insert sugar's name) was not fermented and gas was not produced.	–/–
Pink broth; no bubble in Durham tube	Degradation of peptone occurred to form alkaline end products, but the carbohydrate (insert sugar's name) was not fermented; gas was not produced.	K/–
Orange broth; no bubble in Durham tube	This is often noted as a negative test result for fermentation. However, it does suggest acidic products are present, albeit at very low levels since the media's pH remained above 6.8. Follow your instructor's directions on how they want you to note this result. They may want you to incubate the sample longer and/or note the result as inconclusive.	

Symbol Key:

A = Acidic end products (broth appears yellow due to fermentation reaction)
K = Alkaline end products (broth appears pink due to peptone breakdown; no fermentation of the given carbohydrate)
G = Gas bubble present in Durham tube
–/– = No reaction (no fermentation reaction and no peptone breakdown/lack of gas formation)

Clinical Applications

A variety of biochemical test procedures help clinical microbiologists identify pathogenic microbes in a patient sample (e.g., urine sample, throat swab, blood sample, etc.), phenol red medium is one such biochemical test. It helps us determine the fermentation profile for a specimen and is most commonly used to differentiate members of *Enterobacteriaceae* from other Gram-negative rods. It also allows us to distinguish between Gram-positive fermenters, such as *Streptococcus* and *Lactobacillus* species. Some *Streptococcus* species, such as *S. mutans* are prolific fermenters that make acidic end products that cause dental caries (cavities). The vaginal microbiome includes *Lactobacillus* species, which ferment glycogen (a polymer of glucose) and thereby establish an acidic pH in this tissue. The acidic vaginal pH inhibits the growth of a variety of potential pathogens to include the yeast *Candida albicans*, which causes vulvovaginal candidiasis (yeast infections).

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Phenol red glucose broths with Durham tubes (4)[1]
- ❑ Phenol red base broths (media without a carbohydrate added) with Durham tubes (4, optional)
- ❑ Phenol red broth containing other carbohydrates (e.g., lactose or sucrose) with Durham tubes (optional)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Alcaligenes faecalis* (subspecies *faecalis*) (recommend a BSL-1 strain ATCC 15554)
  - ☐ *Escherichia coli*
  - ☐ *Providencia stuartii*
  - ☐ *Streptococcus gallolyticus*; recommended if using additional phenol red broths (optional)

Medium Recipe

Phenol Red (Carbohydrate) Broth[2]

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	10.0 g
Sodium chloride (NaCl)	5.0 g
Carbohydrate (one only: glucose, lactose, sucrose, etc.)	5.0 g
Phenol red	0.018 g
Distilled or deionized water	1.0 L
Adjust to pH = 7.1–7.5 at 25°C	

[1] This lab exercise can also be performed using purple broths, which differ only slightly in ingredient composition, but use bromocresol purple as the pH indicator. Bromocresol purple turns yellow at pH 5.2 and is purple above pH 6.8.

[2] This formulation without carbohydrate is phenol red base broth.

PROCEDURE

Today you will inoculate phenol red glucose broths with three organisms to determine their fermentation characteristics. Your instructor may direct you to use phenol red broths containing other carbohydrates (e.g., lactose, sucrose, etc.) in addition to, or in place of, phenol red glucose broth. As a reminder, include an uninoculated control of each medium for color comparison. After your samples have incubated, use Table 27.1 as a guide for interpreting and recording your results.

1. **Obtain and Label Your Media:** Obtain four phenol red glucose broth tubes. Label each with your name, the medium name, and the date. Label the first three tubes with the names of the bacteria you will inoculate into each tube and label the fourth tube "uninoculated control." If your instructor wants you to use phenol red broth with other carbohydrates, you'll label those tubes in the same manner.
2. **Inoculate the Media:** Use an inoculating loop to inoculate the broth in each of your three experimental tubes with a test organism. Leave the fourth tube uninoculated.
3. **Incubate the Samples:** Incubate your samples at 35±2°C for 48 hours.
4. **Clean up:** Dispose of stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Observe Your Samples:** Using the uninoculated control as a color comparison and Table 27.1 as a guide, examine your samples and enter the results on the data sheet at the end of this lab exercise. When indicating the various reactions, use the standard symbols as shown, with the acid reading first, followed by a slash and then the gas reading.
3. **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 27

DATA SHEET

Phenol Red Fermentation Broth

During the Lab

1 Observe your tubes, and use Table 27.1 to write up your results in the following table.

	Phenol Red Results (list color first, then gas information)				
	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
Uninoculated control					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):
Organism Name	**Base Broth (optional)**	**Glucose**	**Lactose (optional)**	**Sucrose (optional)**	**Interpretation**
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Organism Name	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Organism Name	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Organism Name	Base Broth (optional)	Glucose	Lactose (optional)	Sucrose (optional)	Interpretation
					Base broth (optional):
					Glucose:
					Lactose (optional):
					Sucrose (optional):

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 27
DATA SHEET
(continued)

Lab Related Questions

1 Choose the false statement(s) about the phenol red test (select all that apply).

- **a.** It can detect if an organism ferments a specific sugar.
- **b.** It is a differential medium.
- **c.** It is a selective medium.
- **d.** It is inoculated with a mixed culture.
- **e.** It may contain multiple sugars in the same sample tube.

2 The purpose of the Durham tube in the media is:

- **a.** To stabilize the media's pH.
- **b.** To capture gas made as a fermentation byproduct.
- **c.** To remove oxygen from the media.
- **d.** To help oxygen diffuse through the media.
- **e.** To detect the media's pH.

3 Choose the true statement(s) about fermentation (select all that apply).

- **a.** If a fermentation reaction occurs, the phenol red media should turn yellow.
- **b.** All fermentation reactions produce gas as a byproduct.
- **c.** Fermentation reactions do not require oxygen.
- **d.** Glycolysis is the first stage of fermentation.
- **e.** Fermentation can only occur under anaerobic conditions.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Monosaccharides must be converted to disaccharides before being metabolized by fermentation pathways.

_____ Before it can be fermented, lactose must be split into simple sugars by the enzyme glucosidase.

_____ Sucrose is a disaccharide made up of fructose and glucose.

_____ A bacterium may conduct oxidative phosphorylation as well as fermentation.

5 The enzyme _____ is required to split sucrose into monosaccharides before fermentation can occur.

6 Assume you conduct a phenol red glucose test on an unknown bacterium and after incubation you obtain an A/– result. What can you reasonably conclude about the bacterium? (Select all that apply.)

- **a.** The bacterium ferments the sugar glucose.
- **b.** The bacterium makes alkaline metabolic byproducts.
- **c.** The bacterium made acidic end products.
- **d.** The bacterium does not make gas as a fermentation byproduct.
- **e.** There was an absence of gas as well as an absence of a color change.

Critical Thinking

1 Should you anticipate a bubble in the Durham tube if the media is red? Explain your reasoning.

2 Suppose you perform this exercise and upon making your observation, you notice the media *outside* the Durham tube is red, while the media *inside* the Durham tube is yellow. What conclusions could you make? Be sure to explain your reasoning.

EXERCISE 28

Methyl Red and Voges-Proskauer (MR-VP) Tests

After doing this exercise, you should be able to:

1. Describe the formulation of MR-VP media.
2. State how the MR and VP tests are each performed and interpreted.
3. Explain what the MR and VP tests each reveal about a bacteria species.
4. State why MR-VP tests are clinically useful.
5. Apply aseptic bacterial transfer and culture technique to set up an experiment that will assess if bacteria perform mixed acid fermentation or butanediol fermentation.

Background

Recall that fermentation reactions are metabolic processes that harvest energy from a nutrient source (often a sugar) without the use of an electron transport chain (without oxidative phosphorylation). While fermentation is an anaerobic process and therefore does not require oxygen to occur, that does not mean fermentation exclusively occurs when oxygen is absent; many microbes conduct fermentation when oxygen is present. Think of it like this: a flashlight may be especially useful during a power outage, but a power outage is not necessary for you to use a flashlight.

Fermentation reactions are extremely diverse, so much so that even when the same sugar is fermented (e.g., glucose), different end products can be made depending upon the pathway the bacterium uses. Consequently, determining the nature of a fermentation reaction's end products can help us differentiate bacterial species from one another. The **methyl red and Voges-Proskauer Tests** (**MR-VP**) help us differentiate bacterial species based on the fermentation pathway used to metabolize glucose.

Mixed Acid and Butanediol Fermentation Pathways

Fermentation pathways are diverse, but the MR-VP test differentiates between only two fermentation processes: mixed acid fermentation and butanediol fermentation (also called 2,3-butanediol fermentation).

In **mixed acid fermentation**, the pyruvate made in glycolysis is metabolized to form a variety of end products such as lactate, formate, acetate, succinate, ethanol, carbon dioxide (CO_2), and hydrogen gas (H_2) (Fig. 28.1). Among these end products, those with the suffix "ate" are acids—formate, acetate, succinate—that can stably lower (acidify) the MR-VP media's pH.

In **butanediol fermentation**, pyruvate is metabolized to form many of the same products seen for mixed acid fermentation, but in far smaller quantities. Instead, most of the end products are 2,3-butanediol and ethanol, which are neutral (Fig. 28.2). Because butanediol fermentation mainly generates neutral end products, the MR-VP media's pH will be closer to neutral than if mixed acid fermentation occurred (Fig. 28.3).

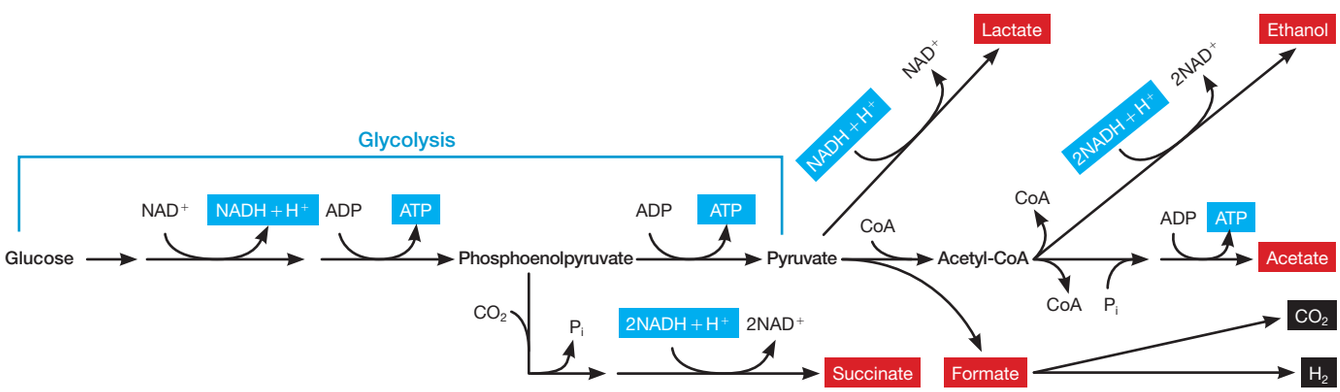


FIGURE **28.1** **Mixed Acid Fermentation Pathway of *E. coli*** • *E. coli* is a representative methyl red positive organism and is recommended as a positive control for the test. Its mixed acid fermentation products (in order of abundance) are: lactate, carbon dioxide (CO_2), hydrogen gas (H_2), ethanol, acetate, succinate, and formate. Most of the formate is converted to H_2 and CO_2 gases. With the exception of succinate, all the end products are derived from pyruvate; succinate is produced from phosphoenolpyruvate. *Salmonella* and *Shigella* also are methyl red positive, though specific end-product profiles can differ.

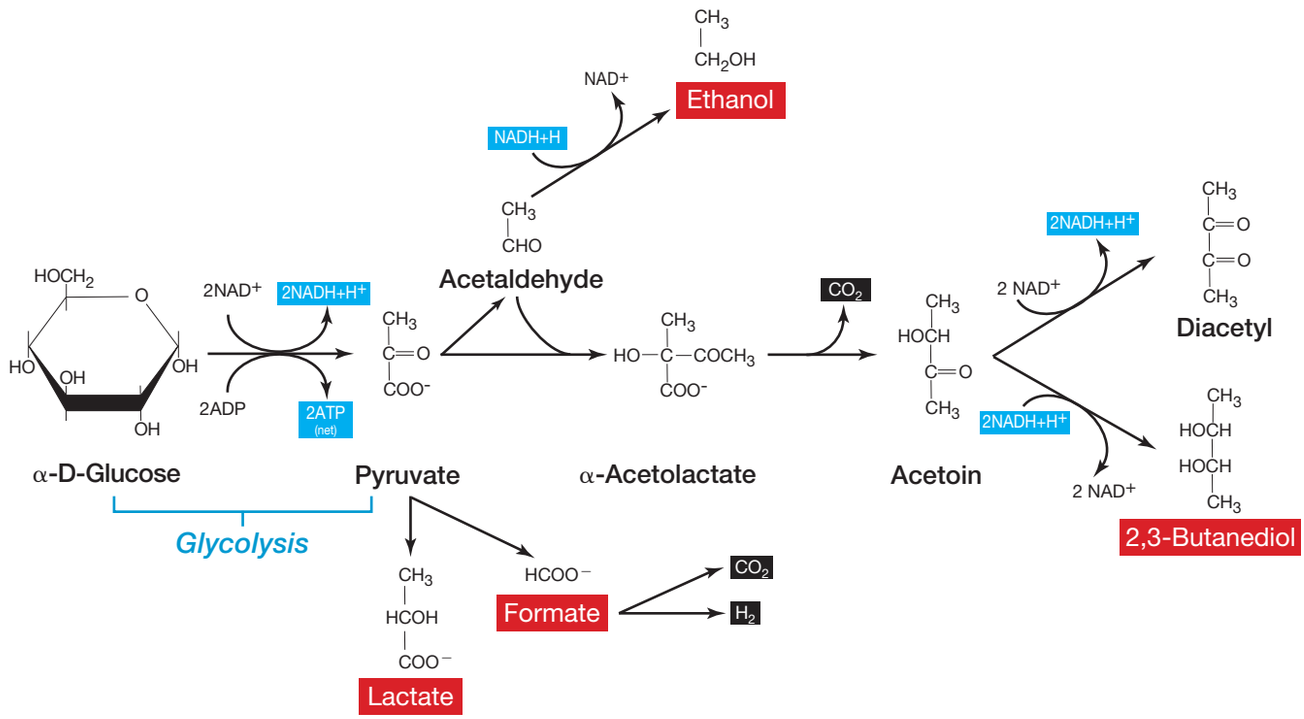


FIGURE **28.2** **Butanediol Fermentation Pathway** • Butanediol fermentation primarily produces the neutral molecules 2,3-butanediol and ethanol.

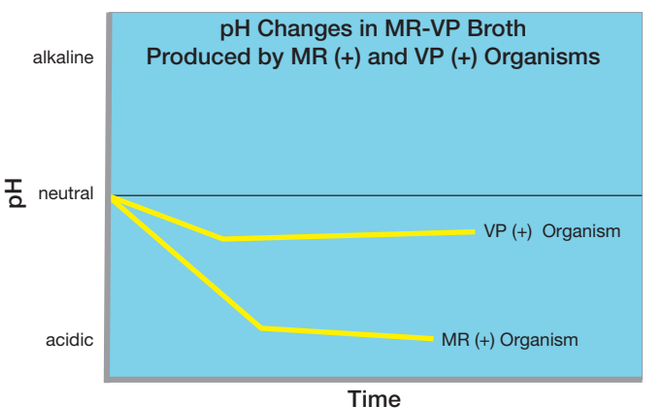


FIGURE **28.3** **Mixed Acid Versus Butanediol Fermentation and the Effects on MR-VP Media pH** • The MR-VP test differentiates between organisms that perform mixed acid fermentation and those that conduct butanediol fermentation. Mixed acid fermentation lowers the test broth's pH due to the abundance of acidic end products that are made. In contrast, organisms that perform butanediol fermentation make primarily neutral end products.

MR-VP Media Formulation

MR-VP broth is a defined media containing only peptone, glucose, and a phosphate buffer. The peptone provides protein, which is important as a nitrogen source. Glucose serves as the fermentable carbohydrate. The potassium phosphate buffer stabilizes the media's pH enough to guard against a significant pH decrease unless a substantive amount of acidic end products is made.

Interpreting MR-VP Results

For this test we inoculate only one tube of media, but after incubation we transfer 1 mL of the culture to a clean test tube to serve as the MR portion of the test and another 1 mL of culture is transferred to a tube and analyzed for the VP part of the test (Fig. 28.4).

Methyl Red (MR) Test

The MR portion of the test detects if mixed acid fermentation occurred. To assess if acidic end products are present or not, the pH indicator methyl red is added to 1 mL of the culture following incubation. Methyl red appears red at pH 4.4 and yellow at pH 6.2. Between these two pH values, it is various shades of orange. The development of a red color is the only true indication of a positive result (Fig. 28.5). As indicated in Table 28.1, a positive test result (red) means the bacterium performs mixed acid fermentation and converted glucose to acidic end products. A negative test result indicates that acidic end products were *not* detected, the test bacterium did *not* perform mixed acid fermentation.

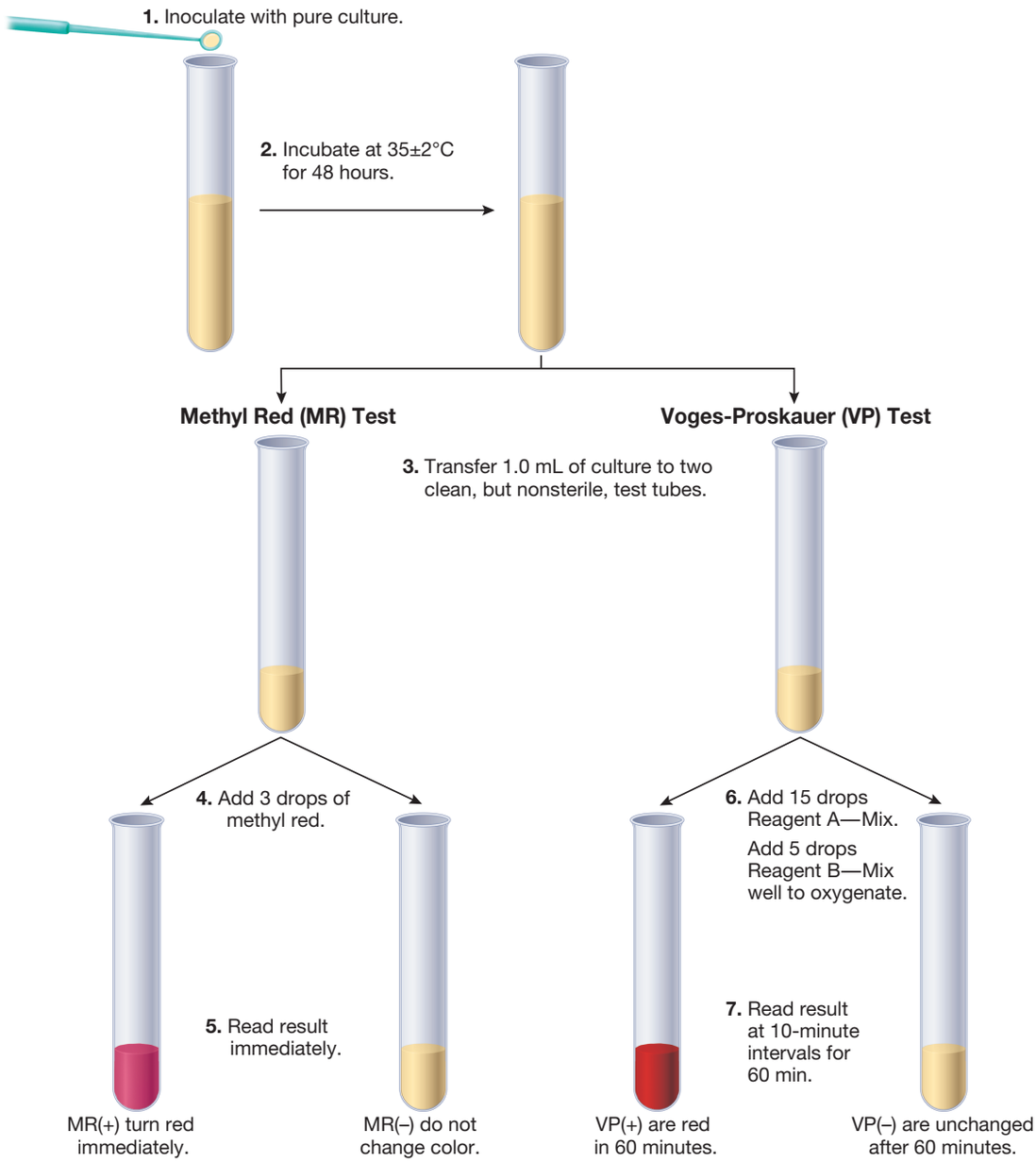


FIGURE **28.4** **Procedural Diagram for the Methyl Red and Voges-Proskauer Tests** ●

TABLE **28.1** **Methyl Red Test Results and Interpretations** ●

Result	Interpretation	Symbol
Red	Organism performs a mixed acid fermentation	+
No color change	Organism does not perform a mixed acid fermentation (acidic end products were not made in detectable amounts)	–

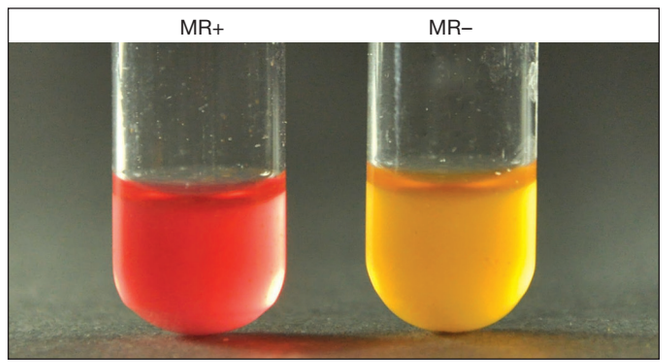


FIGURE **28.5** **Methyl Red Test Results** ● A positive MR result appears red and means the tested bacterium performs mixed acid fermentation. A negative MR result is yellow and indicates the tested bacterium does not conduct mixed acid fermentation.

Voges-Proskauer (VP) Test

The Voges-Proskauer test reveals if a bacterium metabolized glucose via the butanediol fermentation pathway. As previously reviewed, butanediol fermentation mainly produces neutral end products (ethanol and 2,3-butanediol). Therefore, unlike the MR portion of the test, we can't simply check for an acidic pH to determine if the bacterium performed butanediol fermentation. Instead, we add reagents to the sample to determine if **acetoin**, an intermediate made in the butanediol fermentation pathway, is present.

First, VP Reagent A—a 5% α-naphthol (alpha-naphthol) solution—is added to the sample. This reagent acts as a color intensifier that allows for enhanced test sensitivity. After mixing well to oxygenate the media, VP Reagent B—a 40% potassium hydroxide (KOH) solution—is added. Once the KOH is added to raise the sample's pH, the tube is again vigorously mixed to enhance the sample's oxygenation. The increased sample oxygenation combined with the alkaline pH promotes the oxidation of acetoin to form **diacetyl** (Fig. 28.6). The resulting diacetyl then reacts with **guanidine-containing compounds** present in the media's peptone, to form a red color (Fig. 28.7).

As reviewed in Table 28.2, a positive VP result indicates the bacterium made acetoin; it metabolizes glucose via the butanediol fermentation pathway. Absence of a color change (or development of copper color) after adding the reagents indicates a negative test result, meaning the bacterium does *not* metabolize glucose via the butanediol fermentation pathway. The copper color is a result of interactions between the reagents and should not be confused with the true red color of a positive result. It's helpful to include positive and negative control for color comparison for this test.

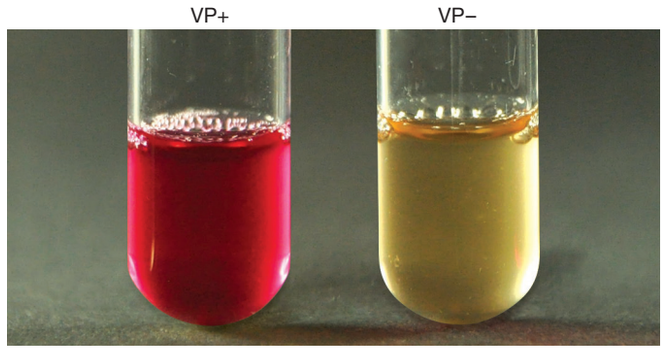


FIGURE **28.7** **Voges-Proskauer Test Results** • A positive VP test appears red and indicates acetoin was detected, thus butanediol fermentation occurred. A negative VP test appears yellow and indicates acetoin is not present, thus butanediol fermentation did not occur.

TABLE **28.2** **Voges-Proskauer Test Results and Interpretations** •

Result	Interpretation	Symbol
Red	Organism performs a 2,3-butanediol fermentation (acetoin was produced)	+
No color change	Organism does not perform a 2,3-butanediol fermentation (acetoin was not produced or it is not detectable)	–

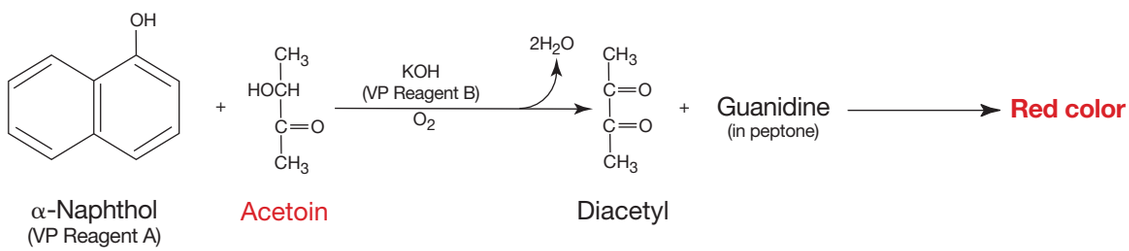


FIGURE **28.6** **Voges-Proskauer Test Indicator Reaction** • Adding potassium hydroxide (KOH) raises the sample's pH and promotes the oxidation of acetoin to form diacetyl. In this reaction, 5% α-naphthol enhances the development of the red color seen in a positive test result.

Clinical Applications

The methyl red and Voges-Proskauer tests are components of the *IMViC* battery of tests (*I*ndole, *M*ethyl red, *V*oges-Proskauer, and Citrate) used to distinguish between members of the family *Enterobacteriaceae* and differentiate them from other Gram-negative rods. Certain members of this family are clinically important because they cause diverse pathologies to include diarrheal illnesses, septicemia, pneumonia, meningitis, and urinary tract infections. Pathogens such as *Escherichia*, *Shigella*, and *Salmonella* are MR-positive and VP-negative. In contrast, certain other members of *Enterobacteriaceae*, such as *Enterobacter aerogenes* and *Klebsiella pneumoniae,* are MR-negative and VP-positive. Refer to Figure A.6 Sampling of Fermentation Pathways on page 473 to compare mixed acid and butanediol fermentation pathways to other fermentation pathways.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Methyl red and Voges-Proskauer (MR-VP) broths (3)
- ❑ Methyl red reagent
- ❑ VP Reagents A and B (formulations provided below)
- ❑ Nonsterile 12 mm × 75 mm test tubes (6)
- ❑ Nonsterile 1 mL pipettes (3)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Enterobacter aerogenes*

Medium and Reagent Recipes

MR-VP Broth

Buffered peptone	7.0 g
Dipotassium phosphate (K_2HPO_4)	5.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	5.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.7–7.1 at 25°C

Methyl Red Reagent

Methyl red dye	0.1 g
Ethanol	300.0 mL
Distilled water to bring volume to	500.0 mL

VP Reagent A (5% α-naphthol solution)

α-naphthol	5.0 g
Absolute alcohol to bring volume to	100.0 mL

VP Reagent B (40% KOH)

Potassium hydroxide (KOH)	40.0 g
Distilled water to bring volume to	100.0 mL

PROCEDURE

Today you will perform methyl red and Voges-Proskauer tests on *Escherichia coli* and *Enterobacter aerogenes*. After a 48-hour incubation period, you will observe the results of a mixed acid fermentation and a 2,3-butanediol fermentation. The exercise involves transfer of a portion of broth containing living organisms and the addition of measured reagents to complete the observable reactions. Please use care in your transfers and in your measurements. Also, wear a lab coat, gloves, and chemical eye protection when performing this laboratory exercise. For help with the procedure, refer to Figure 28.4. You'll also find Tables 28.1 and Table 28.2 helpful.

1. **Obtain and Label Your Media:** Obtain three tubes of MR-VP media. Label two of them with the names of the organisms, your name, and the date. The third tube, in addition to being marked with your name and the date, should be labeled "control."
2. **Inoculate the Media:** Use an inoculating loop to inoculate your two test broths with the test cultures. Do not inoculate the control.
3. **Incubate the Samples:** Incubate your samples at 35±2°C for 48 hours.
4. **Clean up:** Dispose of stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Mix and Transfer Your Samples**: Mix each broth well without splashing into the cap. Transfer 1.0 mL from each tube into two nonsterile tubes (Fig. 28.4). One tube will be designated for the MR test; the other will be for the VP test. Therefore, by the end of this step you should have one MR tube and one VP tube for each original sample (six tubes total if you started with three samples).

3 Develop Each Test and Make Observations:

Each MR test is processed as follows:

- Add 3 drops of methyl red reagent. Immediately observe the sample for the development of a red color.
- Using Table 28.1 as a guide, record your results in the table provided on the data sheet at the end of this exercise.

Each VP test is processed as follows:

- Add 15 drops (0.6 mL) of VP Reagent A. Mix well to oxygenate the medium, but be careful not to splash it.
- Add 5 drops (0.2 mL) of VP Reagent B. Carefully mix well again to oxygenate the medium.
- Place the tubes in a test-tube rack and observe for red color formation at regular intervals starting at 10 minutes for up to 1 hour. Mix again periodically to oxygenate the medium.
- Use Table 28.2 to help you interpret your results. Record your results in the table provided at the end of this exercise. (***Note:*** It is difficult to differentiate weak positive reactions from negative reactions. VP-negative reactions often produce a copper color. Weak VP-positive reactions produce a pink color. The more intense color change is usually observed at the media's surface.)

4 **Clean up:** Properly dispose of your samples as your instructor directs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 28

DATA SHEET

Methyl Red and Voges-Proskauer (MR-VP) Tests

During the Lab

1 Observe your tubes and use Tables 28.1 and 28.2 to write up your results in the following table.

Organism	MR Result	VP Result	Interpretation
Uninoculated control			

Lab Related Questions

1 Select the true statement(s) about an MR-negative bacterium (select all that apply).

a. It performed mixed acid fermentation.
b. It converted glucose to acidic products.
c. It may convert glucose to acetoin.
d. It produced methyl red.
e. It may perform 2,3-butanediol fermentation.

2 Select the false statement(s) about a VP-positive bacterium (select all that apply).

a. It performs 2,3-butanediol fermentation.
b. It performs mixed acid fermentation.
c. It produces acetoin.
d. It converts glucose to mostly acidic end products.
e. It cannot metabolize glucose.

3 Choose the true statement(s) about fermentation (select all that apply).

a. Fermentation requires oxygen to occur.
b. All fermentation reactions start with glucose.
c. Fermentation reactions can occur in the presence of oxygen.
d. Glycolysis is the first stage of fermentation.
e. Pathogens do not conduct fermentation.

4 In this procedure, the samples were not transferred into sterile tubes for developing the MR or VP test. Why weren't sterile tubes required?

_____

_____

5 Cross out the steps that do not apply to the VP test, and then number the remaining steps so they are in the proper order.

_____ Add methyl red.

_____ Immediately observe the sample for a color change.

_____ Add 1 mL of your sample to a clean tube.

_____ Inoculate MR-VP broth media.

_____ Add α-naphthol.

_____ Add potassium hydroxide.

_____ Record a yellow result as having a positive VP test result.

6 Cross out the steps that do not apply to the MR test, and then number the remaining steps so they are in the proper order.

_____ Inoculate MR-VP broth media.

_____ Add 1 mL of your sample to a clean tube.

_____ Add potassium hydroxide.

_____ Add α-naphthol.

_____ Incubate sample for an hour.

_____ Record a positive MR test result if a red color develops.

_____ Add methyl red.

7 Which of the following occurs in 2,3-butanediol fermentation? (Select all that apply.)

a. Acetoin is made as an intermediate.

b. Hydrogen sulfide (H_2S) is made.

c. Glucose is metabolized.

d. Lactate is made.

e. The media's pH becomes alkaline.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 28
DATA SHEET
(continued)

Critical Thinking

1 Why is the methyl red test read immediately after addition of methyl red reagent, whereas the Voges-Proskauer test is read up to 60 minutes after addition of Reagents A and B?

2 In the VP test, the most intense color change is often at the media's surface. Based on the reaction's chemistry, why does this make sense?

— Lab Notes —

EXERCISE 29

Catalase Test

After doing this exercise, you should be able to:

1. Describe how the catalase test is performed.
2. Explain what the catalase test reveals about a bacterial species.
3. State the applications of the catalase test.
4. Perform a catalase test and interpret the test results.

Background

Organisms that perform aerobic respiration tend to generate toxic oxygen intermediates in the process. Unless such intermediates are broken down, they will damage cellular components. Common examples of toxic oxygen intermediates are hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-). These agents damage cells by oxidizing proteins and other biomolecules, thereby rendering them nonfunctional.

As a protective mechanism, organisms that perform aerobic respiration tend to make enzymes that break down toxic oxygen intermediates. For example, the enzyme **superoxide dismutase** catalyzes the conversion of superoxide radicals, which are particularly damaging toxic oxygen intermediates, to the comparatively less damaging molecule, hydrogen peroxide (Fig. 29.1). **Catalase,** an enzyme in aerobes, microaerophiles, and facultative anaerobes, converts hydrogen peroxide to water and gaseous oxygen (Fig. 29.2). In large part (though exceptions exist), the ability to make these protective enzymes accounts for an organism's ability to live in aerobic environments (Table 29.1). Oxygen is toxic to obligate anaerobes because these microbes lack enzymes such as catalase and superoxide dismutase to effectively break down toxic oxygen intermediates that tend to evolve under aerobic conditions.

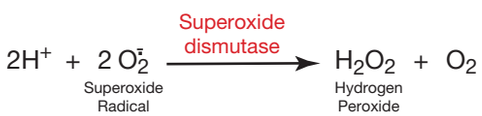


FIGURE **29.1** **Microbial Production of H_2O_2** • Superoxide dismutase produces hydrogen peroxide in the process of detoxifying superoxide radicals (O_2^-) made in aerobic cellular respiration.

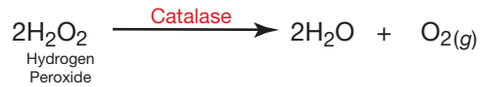


FIGURE **29.2** **Catalase Breaks Down H_2O_2** • Catalase converts hydrogen peroxide to water and oxygen gas.

TABLE **29.1** **Protective Enzymes in Various Aerotolerance Groups** •

Aerotolerance Group	Superoxide Dismutase	Catalase
Obligate aerobe	Present	Present
Facultative anaerobe	Present	Present
Microaerophile	Present	Present in small amounts
Aerotolerant anaerobe	Present	Absent (alternative mechanism)
Obligate anaerobe	Absent	Absent

Interpreting Catalase Test Results

By exposing bacteria to a 3% hydrogen peroxide solution (typical store-grade hydrogen peroxide), we can determine whether they make catalase or not. Upon adding hydrogen peroxide to a catalase-positive culture, oxygen gas bubbles immediately form. If bubbles do not form, the organism is catalase-negative (Table 29.2).

TABLE **29.2** **Catalase Test Results and Interpretations** •

Result	Interpretation	Symbol
Bubbles immediately develop (immediate effervescence)	Catalase is present; the organism is aerobic	+
No bubbles detected	Catalase is absent or not detectable	–

This test is often performed by adding hydrogen peroxide (Fig. 29.3) to a bacterial smear on a microscope slide. If using this "slide method," you should immediately cover the sample after adding hydrogen peroxide. This reduces workspace contamination from the aerosols a positive test may generate as bubbles develop (Fig. 29.4).

Another way to perform the catalase test is to add hydrogen peroxide directly to bacteria growing on an agar slant (Fig. 29.5) or on an agar plate. ***Note:*** Do not add hydrogen peroxide to cultures growing on blood agar because a false-positive result is likely to develop as catalase in the red blood cells in the agar breaks down the hydrogen peroxide. Lastly, upon adding hydrogen peroxide to the agar's surface (be it a slant or a plate culture), the culture will not be usable for other tests. As such, before performing the test in this way, ensure you do not need the culture for other applications.

A Note on Handling Hydrogen Peroxide

Hydrogen peroxide is a colorless liquid with disinfectant and bleaching properties. It is an irritant, so you should wear safety glasses when handling this substance. Also, this molecule is naturally unstable and decomposes slowly to form water and oxygen gas. Ultraviolet light and heat increase the rate of H_2O_2 decomposition, which is why we store the solution in a dark bottle in a cool place.

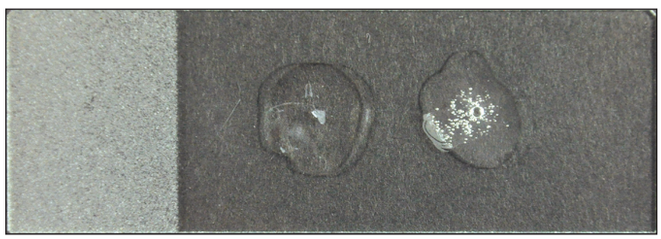


FIGURE **29.3** **Catalase Slide Test Results** • Visible bubbles upon adding hydrogen peroxide to a bacterial smear indicates a positive result in the catalase slide test. A catalase-negative organism is on the left. A catalase-positive organism is on the right. (For photography purposes, the slide was not covered, but you should cover the sample as shown in Fig. 29.4.)

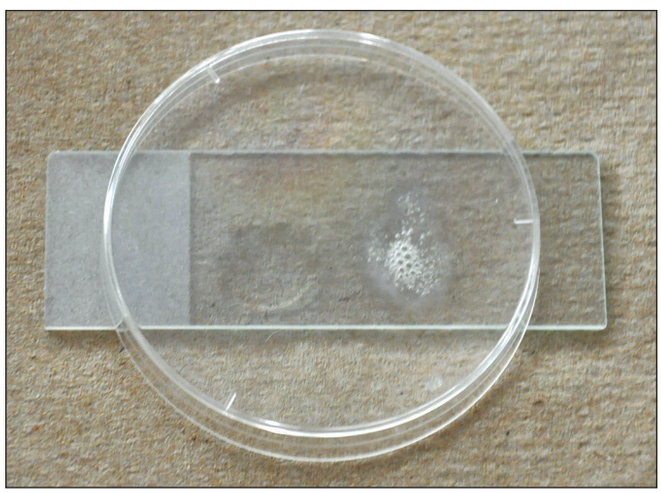


FIGURE **29.4** **Cover the Reaction** • Upon adding the hydrogen peroxide to the slide, cover it with a petri plate lid or base to prevent spattering from a positive result. When the reaction has subsided, the lid can be removed, disinfected, and saved for subsequent tests.

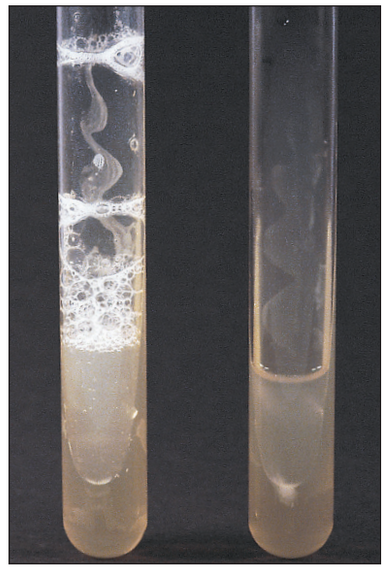


FIGURE **29.5** **Catalase Agar Slant Test Results** • The catalase test may also be performed by adding hydrogen peroxide to bacteria growing on an agar slant. A catalase-positive organism is on the left. A catalase-negative organism is on the right. In a similar fashion, you can also use a culture growing on an agar plate.



Clinical Applications

This test is useful for differentiating Gram-positive bacteria that make catalase from Gram-positive bacteria that do not. It's especially useful for the differentiation of catalase-positive *Staphylococcus* from catalase-negative *Streptococcus*. It can also differentiate catalase-positive *Micrococcus* species from the catalase-negative *Enterococcus*, and *Lactococcus* species. Variations on this test also help presumptively identify *Mycobacterium*, a genus that includes the causative agents of tuberculosis and leprosy.

Not only do catalase-positive bacteria typically thrive in an aerobic environment, but a high level of catalase is associated with pathogenicity (ability to cause disease). For example, some bacteria use catalase to thwart immune defenses by breaking down the hydrogen peroxide that certain phagocytic white blood cells (e.g., macrophages) use to kill bacteria.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ H_2O_2 (hydrogen peroxide, fresh 3% solution in amber dropper bottles)
- ❑ Microscope slides
- ❑ Petri plate base or lid
- ❑ Sterile wooden applicator or sterile cotton swab
- ❑ Nutrient agar (NA) slant (1, as an uninoculated control)
- ❑ Fresh (18- to 24-hour) agar slant cultures of these recommended organisms:
  - ❑ *Staphylococcus epidermidis*
  - ❑ *Lactococcus lactis*

PROCEDURE

For this exercise, you will perform the catalase test. Follow your instructor's directions regarding which version(s) of the test you will perform. They may have you perform the slide version and/or the slant version of the test. If you perform *both* versions, then you must perform the slide test first. This is because the slant version requires adding H_2O_2 directly to the slant, after which the culture is not usable. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

Slide Test

1. **Obtain Your Samples:** Obtain the test culture(s) from your instructor.
2. **Transfer Bacteria to a Slide:** Use a sterile wooden applicator or sterile cotton-tipped swab to smear a visible amount of bacteria onto a microscope slide. Properly dispose of the transfer tool. ***Note***: You must use fresh (18- to 24-hour) cultures for this test because older cultures may not have enough active catalase to produce visible bubbles.
3. **Add Hydrogen Peroxide to the Sample:** Aseptically place 1 or 2 drops of H_2O_2 directly onto the bacteria and immediately cover the sample with the base or lid of a petri plate (Fig. 29.4). This will contain any spattering due to bubbling that develops for a catalase-positive result. Observe the sample to see if bubbles develop. You can remove the cover if bubbling is absent or has subsided. ***Note***: When running this test on an unknown, you should simultaneously test a positive control to verify the quality of the peroxide.
4. **Record Your Data:** Record your results in the table at the end of the lab exercise.
5. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Agar Slant Test

1. **Obtain Your Samples:** Obtain the test culture(s) from your instructor.
2. **Add Hydrogen Peroxide to the Sample:** Before performing this test, confirm that you do not need the culture(s) for any other applications. Next, add approximately 1 mL of H_2O_2 to each of the three slants (two cultures and an uninoculated control) and replace the caps. Process the slants *one at a time*, observing and recording the results as you go. Refer to Table 29.2 to help you interpret the results.
3. **Record Your Data:** Record your results in the table at the end of the lab exercise.
4. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 29

DATA SHEET

Catalase Test

During the Lab

1 Observe your samples and use Table 29.2 to write up your results in the following table.

Organism	Bubbles? Y/N	+/–	Interpretation

Lab Related Questions

1 Oxygen is toxic to obligate anaerobes because these bacteria:

a. Make superoxide dismutase, which generates H_2O_2 from oxygen radicals.
b. Produce catalase, which produces H_2O_2 in aerobic environments.
c. Conduct anaerobic respiration, which produces oxygen radicals.
d. Cannot detoxify toxic oxygen intermediates.
e. Make catalase to breakdown H_2O_2.

2 Which of the following bacterial groups would you expect to have superoxide dismutase and catalase? (Select all that apply.)

a. Obligate aerobes
b. Obligate anaerobes
c. Facultative anaerobes
d. Aerotolerant anaerobe
e. Microaerophiles

3 Assume you grow a bacterium on blood agar and then perform the catalase test by adding H_2O_2 directly to the bacteria growing on the agar surface. Upon adding the peroxide, you see bubbles. Based on this, what could you conclude?

a. The organism is catalase positive.
b. The organism can break down H_2O_2.
c. The organism is an obligate aerobe.
d. The organism is an obligate anaerobe.
e. You cannot make a reliable conclusion as this may be a false-positive result.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Hydrogen peroxide is naturally stable.

_____ Certain phagocytic white blood cells use H_2O_2 as a defense mechanism against bacteria.

_____ Obligate aerobes cannot effectively detoxify toxic oxygen intermediates.

_____ Hydrogen peroxide degrades to water and hydrogen gas.

5 If an organism does not have catalase then:

a. It must be a prokaryotic cell.
b. It is less able to tolerate aerobic environments.
c. It cannot break down superoxide radicals.
d. It cannot metabolize nutrients.
e. It is not a pathogen.

Critical Thinking

1 In some cases the inclusion of an uninoculated control is recommended. In such a case, would the uninoculated control be a positive or a negative control? Explain your reasoning.

2 A negative catalase test is not indicative of a lack of aerobic respiration. Explain why.

EXERCISE 30

Oxidase Test

After doing this exercise, you should be able to:

1. Explain what the oxidase test reveals about a bacterial species.
2. State what cytochrome c oxidase is, where it's found, and what it does.
3. Describe how the oxidase test is performed.
4. State the applications of the oxidase test.
5. Perform an oxidase test and interpret the test results.

Background

The oxidase test reveals if a bacterium has **cytochrome c oxidase**, a participant in certain aerobic electron transport chains. Recall that electron transport chains facilitate the last stage of cellular respiration where oxidative phosphorylation generates ATP.

In ETCs, electrons pass from one membrane-associated electron carrier to the next until they reach a final electron acceptor—oxygen in aerobic respiration or something other than oxygen, such as nitrate or sulfate, in anaerobic respiration. The types of electron carriers that participate in ETCs are diverse (Fig. 30.1). In many cases the ETCs in prokaryotic cells resemble the ETCs in the mitochondria of eukaryotic cells. Furthermore, some organisms have more than one type of ETC and may alternate which ETCs they

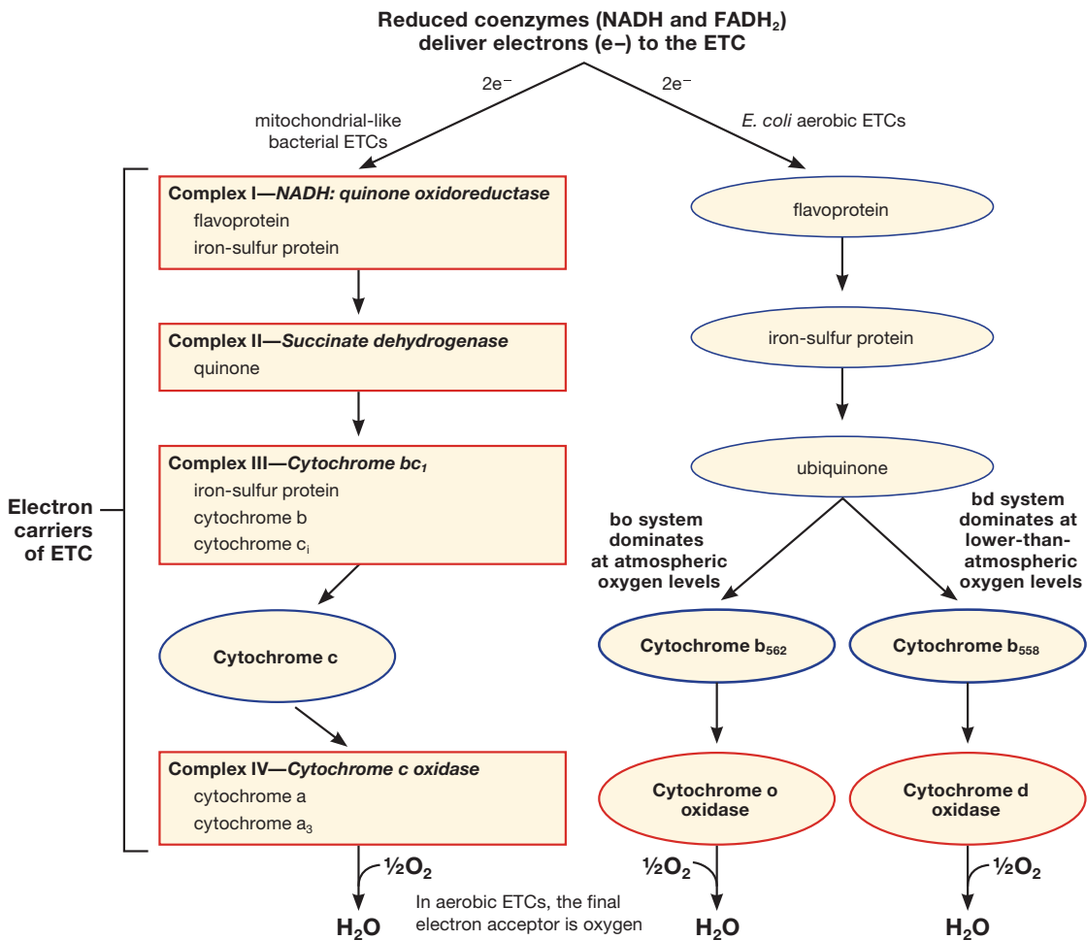


FIGURE **30.1** **Aerobic Electron Transport Chains (ETCs)** • (**Left**) Bacteria with chains resembling the mitochondrial ETC in eukaryotes contain cytochrome c oxidase (Complex IV), which transfers electrons from cytochrome c to oxygen. (**Middle** and **Right**) In the aerobic ETCs of *E. coli*, oxygen doesn't bind to the bo system (**Middle**) as well as it binds to the bd system (**Right**). Consequently, the bd system predominates under decreased oxygen levels. Therefore, the bd system allows *E. coli* to continue using aerobic respiration even when oxygen levels are low. Under low oxygen conditions, microbes without the bd system must either resort to less efficient oxygen independent ATP production pathways (anaerobic respiration or fermentation) or forgo ATP production and die.

use, depending on the availability of oxygen or other final electron acceptor(s). For example, *Escherichia coli* has two different types of aerobic ETCs as well as at least one type of anaerobic ETC that requires nitrate as the final electron acceptor.

While dissecting the diverse components of ETCs is an informative practice, for the purposes of this laboratory exercise we are primarily interested in only one electron carrier, cytochrome c oxidase, which is involved in some (though not all) aerobic respiratory chains. This carrier transfers electrons from cytochrome c to oxygen, the final electron acceptor in aerobic respiration.

How the Oxidase Test Works

The chemistry of ETCs is essentially a collection of redox (reduction-oxidation) reactions. As a reminder, in reduction reactions a molecule or atom gains one or more electrons; whereas, in an oxidation reaction, an atom or molecule loses one or more electrons. In nature, cytochrome c oxidase does exactly as its name implies, it oxidizes the electron carrier cytochrome c. The electron that cytochrome c oxidase obtains is then delivered to oxygen, thereby producing water (Fig. 30.2).

In the oxidase test, we establish a scenario in which cytochrome c oxidase obtains electrons from an added reagent instead of oxidizing its natural electron donor, cytochrome c. The reaction depends on adding a **chromogenic reducing agent** (color-generating agent) called **tetramethyl-*p*-phenylenediamine (TMPD)**, to serve as an electron donor. Upon oxidation, this chromogenic reducing agent develops a purple/dark-blue color. In the reaction, if cytochrome c oxidase is present, then it oxidizes TMPD thereby generating a purple/blue coloration (Fig. 30.3).

We can conclude that bacteria that have cytochrome c oxidase respire aerobically. However, a negative oxidase test result only means the bacterium lacks cytochrome c oxidase; it does not necessarily mean the bacteria cannot respire aerobically using some other ETC that doesn't require cytochrome c oxidase. For example, members of the *Enterobacteriaceae*, are capable of aerobic respiration, but they have a different terminal oxidase system and give a negative result for the oxidase test. There are several ways to perform the oxidase test. You may:

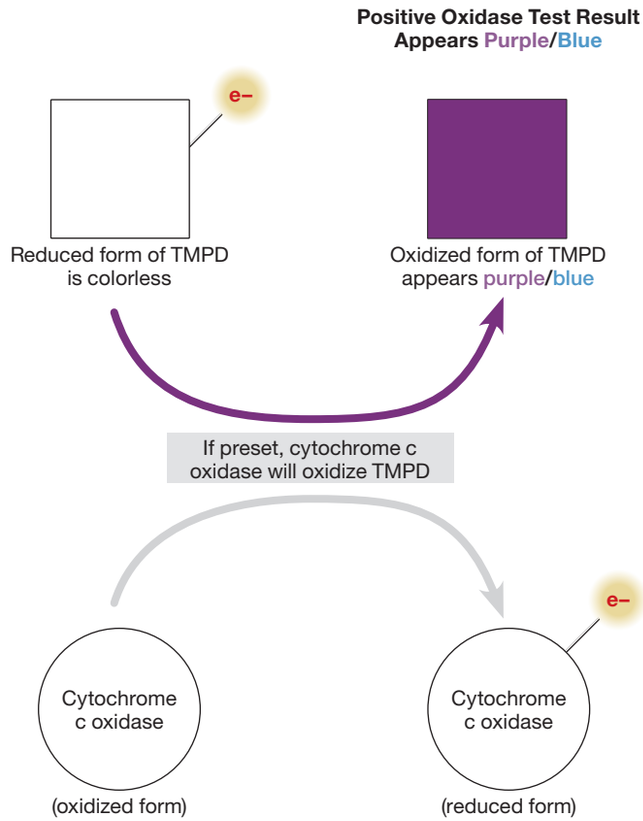


FIGURE **30.3** **Oxidation of Tetramethyl-*p*-phenylenediamine (TMPD) by Cytochrome c Oxidase** • If cytochrome c oxidase is present, it oxidizes TMPD. The oxidized form of TMPD appears purple/blue.

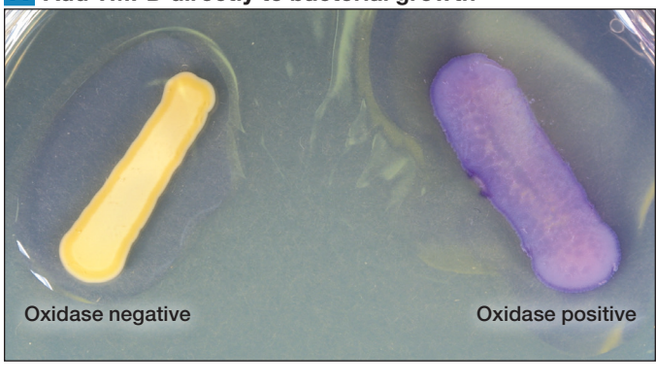
1. Add the TMPD reagent directly to bacterial growth on solid media (Fig. 30.4A); or
2. Smear the test bacterium onto filter paper, and then add TMPD to the smear (Fig. 30.4B); or
3. Smear the test bacterium onto paper infused with the TMPD reagent (Fig. 30.4C).

Regardless of the approach applied, if cytochrome c oxidase is present a color change to purple or blue occurs within seconds as TMPD is oxidized. Lack of a color change within the allotted time means that cytochrome c oxidase is not present and signifies a negative result (Table 30.1).

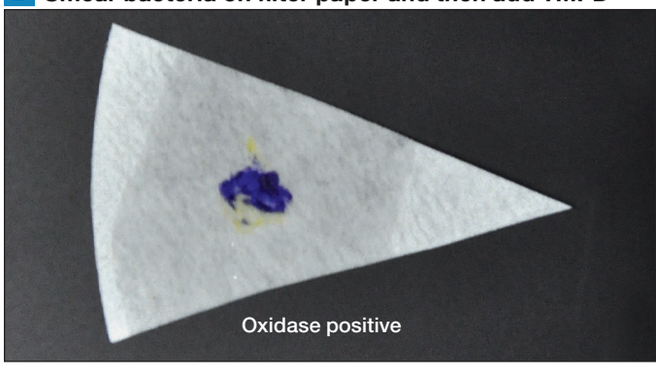
$$2\ \text{Cytochrome}\ c_{red} + 2H^+ + \tfrac{1}{2}O_2 \xrightarrow{\text{cytochrome c oxidase}} 2\ \text{Cytochrome}\ c_{ox} + H_2O$$

FIGURE **30.2** **Natural Oxidation of Cytochrome c** • Under natural conditions, cytochrome c oxidase removes electrons from cytochrome c (oxidizes cytochrome c) and transfers those electrons to O_2, producing water.

A Add TMPD directly to bacterial growth



B Smear bacteria on filter paper and then add TMPD



C Smear bacteria onto TMPD-infused paper

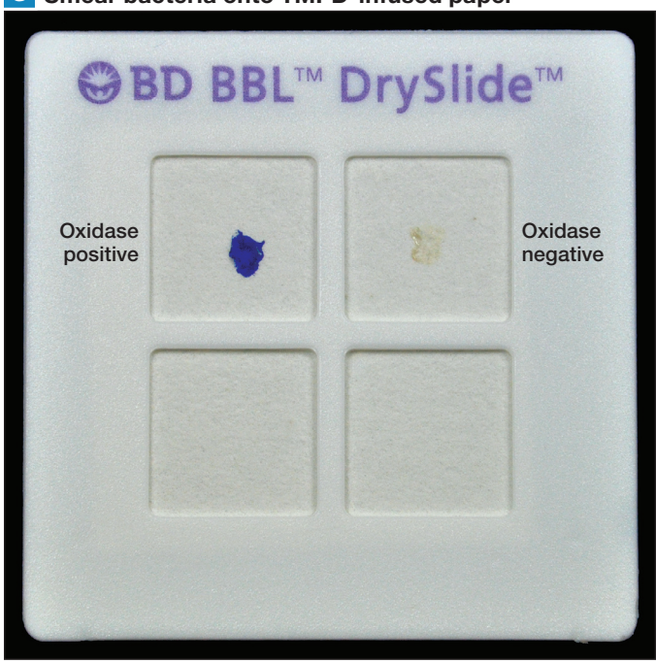


FIGURE **30.4** Three Ways to Perform the Oxidase Test

TABLE **30.1** Oxidase Test Results and Interpretations

Result	Interpretation	Symbol
Dark blue/purple within 20 seconds	Cytochrome c oxidase is present; the organism can perform aerobic respiration.	+
No color change to blue/purple within 20 seconds	Cytochrome c oxidase is not present or not detectable (***Note***: Do *not* assume the tested bacterium is anaerobic. The bacterium may have alternative aerobic ETCs that do not require cytochrome c oxidase.)	–

Avoiding Inaccurate Results

False-positive oxidase test results can develop if TMPD autoxidizes; that is, TMPD is oxidized independent of cytochrome c oxidase action. The most common teaching lab scenarios that trigger false-positive results are exposing the TMPD to a nickel-base alloy wire inoculating loop and/or excessive exposure of TMPD to atmospheric oxygen. To avoid these scenarios, only use a wooden applicator or cotton-tipped swab to apply the bacteria to the test and make your observations immediately upon adding the TMPD reagent.

False-negative oxidase test results can occur if cytochrome c oxidase activity is inhibited. This can occur if the cultures are older/not actively growing and/or if acids are present (pH below 5.1). As such, to avoid false-negative results, use fresh cultures that are 18–24 hours old and avoid using cultures grown under conditions that favor fermentation (e.g., don't use bacteria grown on/in media that detect fermentation).

6

Clinical Applications

Cytochrome c oxidase is an essential participant in aerobic respiration in animals. When one considers that cyanide, azide, and carbon monoxide are all deadly because they inhibit cytochrome c oxidase, it's easy to appreciate just how important this enzyme is. Because ETCs are often evolutionarily conserved, we can find this enzyme in certain aerobic bacteria.

In clinical laboratories, this test is useful for identifying a number of oxidase-positive pathogens of the *Neisseria*, *Moraxella*, *Campylobacter*, and *Pasteurella* genera. For example, the oxidase test can help differentiate *N. gonorrhoeae* (Gram-negative/oxidase-positive bacteria that cause the sexually transmitted infection gonorrhea) from *Chlamydia trachomatis*, a Gram-negative/oxidase-negative bacterium that causes chlamydia (a sexually transmitted infection with similar signs and symptoms as gonorrhea). The oxidase test is also useful in differentiating the oxidase-negative *Enterobacteriaceae* from the oxidase-positive *Pseudomonadaceae*.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile wooden applicator, sterile swab, or disposable plastic loop
- ❑ Small pieces of filter paper
- ❑ Sterile water
- ❑ Oxidase test reagent (TMPD solution)
- ❑ 24-hour agar slant cultures of these recommended organisms:
  - □ *Escherichia coli*
  - □ *Neisseria* species (e.g., *N. sicca* or *N. flava*)

Reagent Recipe

Oxidase Test Reagent

◗ Tetramethyl-*p*-phenylenediamine dihydrochloride	1.0 g
◗ Distilled or deionized water	100.0 mL

PROCEDURE

For this exercise, you will perform the oxidase test. Here we describe the oxidase test using filter paper and reagent. If you are using a commercial kit, follow its instructions. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Samples:** Obtain fresh test culture(s) from your instructor.
2. **Smear Bacteria onto Filter Paper:** Obtain a piece of filter paper and lay it on a paper towel. Then, use a sterile wooden applicator, sterile swab, or disposable loop to smear some of the culture onto the filter paper. Properly dispose of the applicator/loop.
3. **Add TMPD (Oxidase Reagent):** Add a few drops of TMPD (oxidase reagent) to the smear and observe for a color change within 20 seconds. ***Note***: If running an unknown sample, you should also test a known oxidase-positive organism.
4. **Repeat the Test for Other Samples:** Repeat Steps 2 and 3 for additional test organisms. The same filter paper can be used if there is room. When finished, properly dispose of the filter paper into a biowaste container.
5. **Record Your Data:** Record your results in the table at the end of the lab exercise.
6. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 30

DATA SHEET

Oxidase Test

During the Lab

1 Using Table 30.1 as a guide to help analyze your results, record them in the following table.

Organism	Color Result	+/–	Interpretation

Lab Related Questions

1 In nature, cytochrome c oxidase normally reduces:

a. Water.
b. Oxygen.
c. Cytochrome c.
d. TMPD.
e. Carbon monoxide.

2 Which of the following would not have cytochrome c oxidase? (Select all that apply.)

a. Obligate aerobes
b. Obligate anaerobes
c. Facultative anaerobes
d. Aerotolerant anaerobe
e. Human cells

3 In nature, cytochrome c oxidase normally oxidizes:

a. Water.
b. Oxygen.
c. Cytochrome c.
d. TMPD.
e. Carbon monoxide.

4 Mark each statezment as true or false **and** correct the false statements so they read as true.

_____ TMPD serves as an oxidizing agent in the oxidase test.

_____ Bacteria that lack cytochrome c oxidase may be anaerobic.

_____ Bacteria that perform aerobic respiration always have cytochrome c oxidase.

_____ If TMPD autoxidizes, then you may observe a false-negative result.

5 If an organism has cytochrome c oxidase then:

a. It must be a prokaryotic cell.
b. It can conduct aerobic respiration.
c. It will reduce TMPD.
d. It will die if oxygen is absent.
e. It is not a pathogen.

6 The oxidase test can differentiate (select all that apply):

a. *Neisseria* species from one another.
b. *Neisseria* species from *Moraxella* species.
c. *Neisseria gonorrhoeae* from *Enterobacteriaceae* members.
d. *Neisseria gonorrhoeae* from *Chlamydia trachomatis*.
e. Members of *Enterobacteriaceae* from *Chlamydia trachomatis*.

Critical Thinking

1 Assume you obtain the following data for a collection of oxidase tests performed on various 20-hour cultures of Organism A.

Growth Media	Oxidase Test Results		
	Observed at 10 Seconds	Observed at 30 Seconds	Observed at 1 Minute
Nutrient Agar (NA)	–	+	+
Tryptic Soy Agar (TSA)	+	+	+
Eosin Methylene Blue (EMB)	–	–	–

Based on the data, would you conclude Organism A is oxidase positive or oxidase negative? Explain your reasoning. Your explanation should include a discussion of why the data are conflicting despite performing the tests on the same organism.

2 You are performing an oxidase test and notice the positive control is not generating a blue color when exposed to TMPD. However, your test sample is clearly oxidizing TMPD. Assuming you performed the procedure correctly and used the same bottle of TMPD for both samples, what is the most likely explanation for the discrepancy in the test results?

EXERCISE 31

Nitrate Reduction Test

After doing this exercise, you should be able to:

1. Explain what the nitrate reduction test reveals about a bacterial species.
2. Describe how to perform and interpret the nitrate reduction test.
3. State the applications of the nitrate reduction test.
4. Perform a nitrate reduction test and interpret the test results.

Background

The nitrate test is a qualitative procedure that allows us to differentiate bacterial species from one another based on their ability to reduce nitrate (NO_3^-). Nitrate is one example of a final electron acceptor in anaerobic respiratory chains.

Nitrate reduction is complex; organisms may perform nitrate reduction with diverse outcomes (Fig. 31.1). For example, members of the *Enterobacteriaceae* use an enzyme called **nitrate reductase** to perform a single-step reaction in which a reduction reaction reduces nitrate to nitrite (NO_2^-). Other bacteria, called "denitrifiers," perform a multistep process called **dissimilatory nitrate reduction** (sometimes also called denitrification). In this process, nitrate is reduced to form molecular nitrogen (N_2) or nitrous oxide (N_2O), both of which are gases. Denitrifiers are ecologically important in the nitrogen cycle. Other organisms perform **assimilatory nitrification**, a process in which nitrate or nitrite is reduced to form ammonium (NH_4^+), which may then be used to build amino acids.

Interpreting the Nitrate Reduction Test

Nitrate broth is a complex (or enriched) medium of beef extract, peptone, and potassium nitrate (KNO_3). An inverted Durham tube in each broth serves to trap a portion of any produced gas. Following incubation, we analyze the test via a three-step process (Fig. 31.2).

First, check if there is a bubble in the Durham tube. If there is a bubble *and* you are certain the bacterium does *not* conduct fermentation, then you may safely conclude the tested organism reduces nitrate to gaseous end products such as molecular nitrogen (N_2) or nitrous oxide (N_2O) (Fig. 31.3A). (For more information on fermentation, see Ex. 27: Phenol Red Fermentation Broth.)

Because fermentation pathways can generate gas byproducts such as CO_2, a bubble in the Durham tube is not a specific indictor of nitrate reduction. As such, in most cases you should process each sample by adding a variety of reagents. First, nitrate Reagent A (sulfanilic acid) and nitrate Reagent B (α-naphthylamine) are added to the medium to test for nitrate reduction to nitrite. If present, nitrite will form nitrous acid (HNO_2) in the aqueous medium (Fig. 31.4). Nitrous acid reacts with the added reagents to produce a red, water-soluble compound (Fig. 31.3B). So, if you see a red color after adding Reagents A and B, then you may conclude the tested bacterium reduces nitrate to nitrite.

However, if you do *not* see a color change to red after adding Reagents A and B, then you must add nitrate Reagent C (zinc dust) to determine if the bacterium reduced nitrate beyond nitrite. Zinc dust catalyzes the reduction of any nitrate still in the medium to nitrite. The generated nitrite spontaneously becomes nitrous

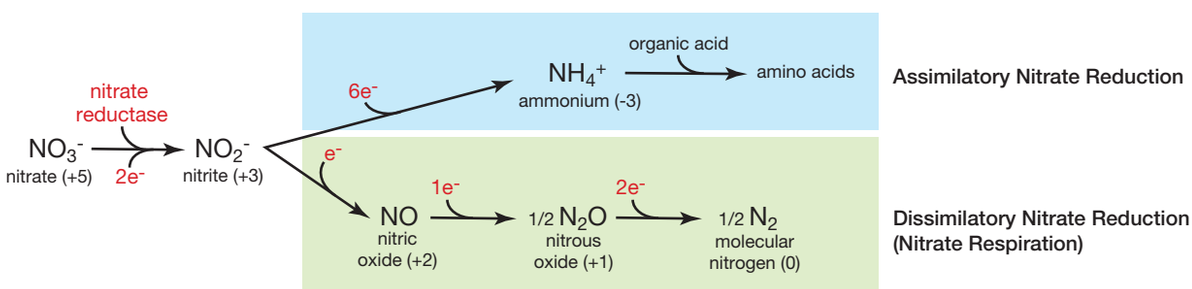


FIGURE **31.1** **Possible End Products of Nitrate Reduction** • In the presence of nitrate reductase, nitrate (NO_3^-) undergoes a single reduction to form nitrite (NO_2^-). Nitrite can then be further reduced via dissimilatory nitrate reduction or assimilatory nitrification. In each compound, nitrogen's oxidation state is shown in parentheses. Notice that as electrons are added, nitrogen's oxidation state decreases.

acid, which reacts with the already present Reagents A and B to turn the medium red. In this instance, the red color indicates the organism did *not* reduce nitrate (Fig. 31.3C). Therefore, lack of a color change after adding Reagent C indicates the organism reduced nitrate to NH_4^+, NO, N_2O, or some other nitrogenous compound other than nitrite.

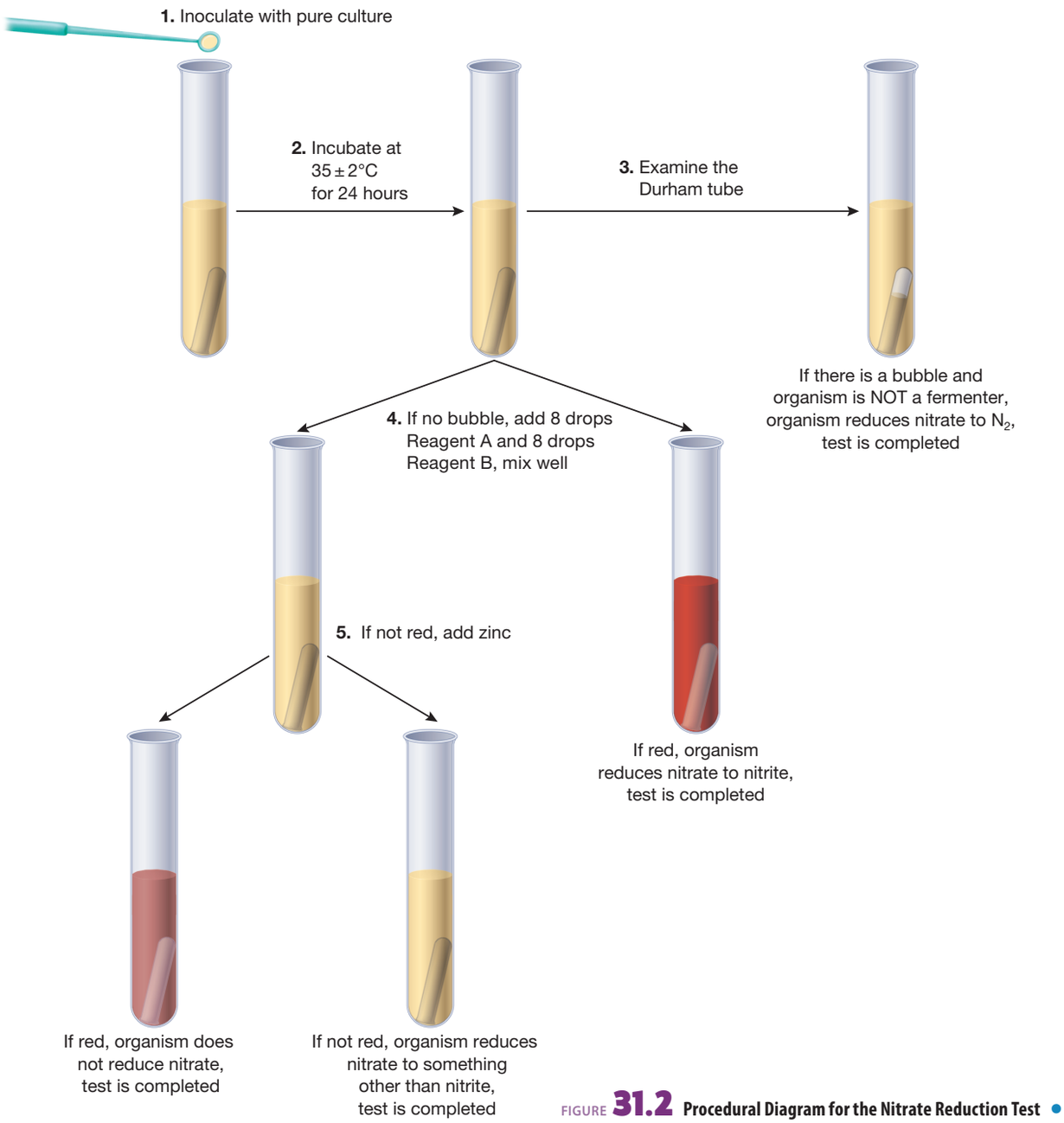


FIGURE **31.2** **Procedural Diagram for the Nitrate Reduction Test**

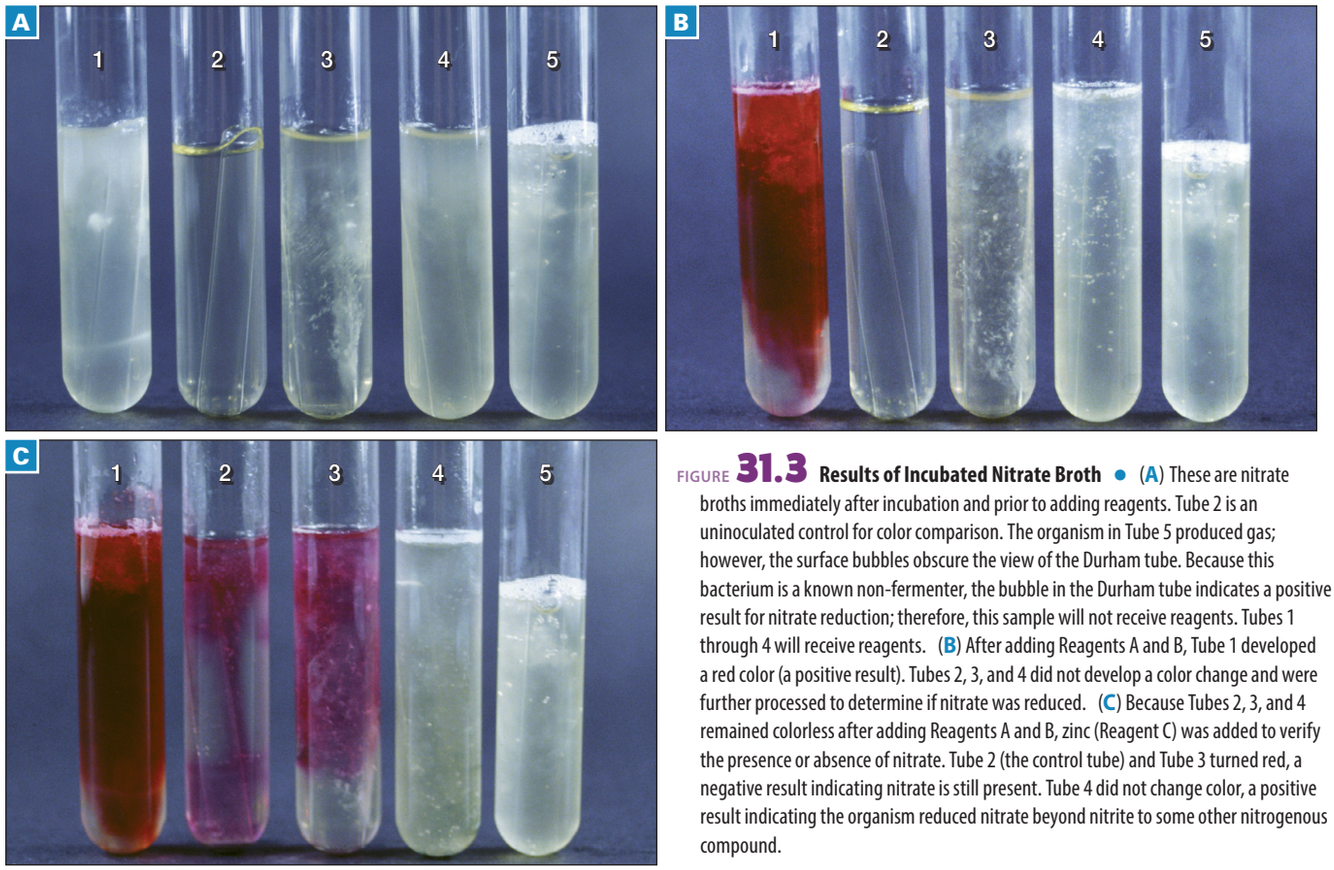


FIGURE **31.3** **Results of Incubated Nitrate Broth** • (**A**) These are nitrate broths immediately after incubation and prior to adding reagents. Tube 2 is an uninoculated control for color comparison. The organism in Tube 5 produced gas; however, the surface bubbles obscure the view of the Durham tube. Because this bacterium is a known non-fermenter, the bubble in the Durham tube indicates a positive result for nitrate reduction; therefore, this sample will not receive reagents. Tubes 1 through 4 will receive reagents. (**B**) After adding Reagents A and B, Tube 1 developed a red color (a positive result). Tubes 2, 3, and 4 did not develop a color change and were further processed to determine if nitrate was reduced. (**C**) Because Tubes 2, 3, and 4 remained colorless after adding Reagents A and B, zinc (Reagent C) was added to verify the presence or absence of nitrate. Tube 2 (the control tube) and Tube 3 turned red, a negative result indicating nitrate is still present. Tube 4 did not change color, a positive result indicating the organism reduced nitrate beyond nitrite to some other nitrogenous compound.

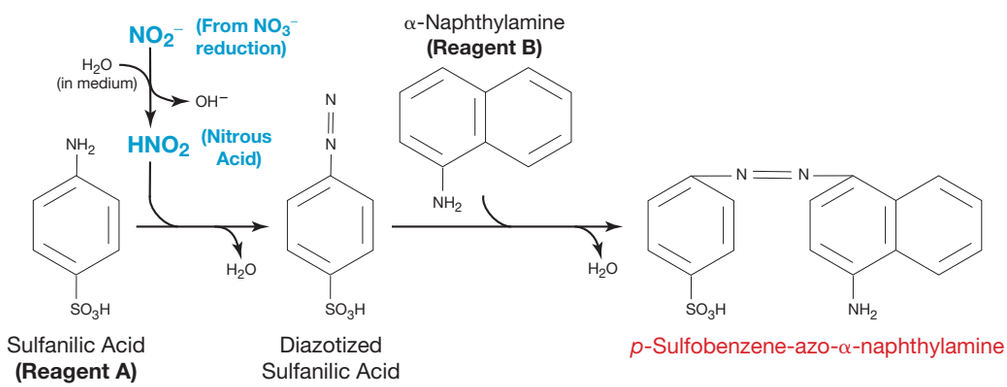


FIGURE **31.4** **Indicator Reaction for Nitrate Reduction to Nitrite** • If nitrate is reduced to nitrite, nitrous acid (HNO_2) will form in the medium. Nitrous acid then reacts with sulfanilic acid (Reagent A) to form diazotized sulfanilic acid, which reacts with the α-naphthylamine (Reagent B) to form *p*-sulfobenzene-azo-α-naphthylamine, which is red. Thus, a red color indicates the presence of nitrite and is considered a positive result for nitrate reduction to nitrite.

Clinical Applications

Virtually all members of *Enterobacteriaceae* perform a one-step reduction of nitrate to nitrite. The nitrate test differentiates them from Gram-negative rods that either do not reduce nitrate or reduce it beyond nitrite to N_2 or other compounds.

The nitrate reduction test is especially useful in clinical laboratories for differentiating *Neisseria gonorrhoeae* (negative for nitrate reduction) from *Kingella denitrificans* (positive for nitrate reduction), a Gram-negative bacterium routinely isolated on selective media for *N. gonorrhoeae*. This test is also useful for differentiating the causative agent of tuberculosis, *Mycobacterium tuberculosis* (positive for nitrate reduction), from similar *Mycobacterium* species that do not perform nitrate reduction.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Nitrate broths (4)
- ❑ Sterile wooden applicators or disposable loops
- ❑ Nitrate test Reagents A and B
- ❑ Zinc powder (Reagent C)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - □ *Alcaligenes faecalis*
  - □ *Escherichia coli*
  - □ *Pseudomonas aeruginosa* (BSL-2) (optional)

Medium and Reagent Recipes

Nitrate Broth

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate (KNO_3)	1.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.8–7.2 at 25°C

Reagent A

Sulfanilic acid	0.8 g
5N acetic acid (30%)	100.0 mL

Reagent B

N,*N*-Dimethyl-α-naphthylamine	0.6 g
5N acetic acid (30%)	100.0 mL

PROCEDURE

After incubating your inoculated nitrate broths, you should first inspect the Durham tubes in the media for bubbles, an indication of gas production. One or more of the organisms used in the exercise ferment sugars and therefore may produce a gas other than the molecular nitrogen (N_2) made in certain nitrate reduction pathways. To distinguish if the gas made is due to nitrate reduction versus fermentation, you *must* add reagents to all the sample tubes, those where you see a bubble and those where you do not see a bubble. Record any gas observed in the Durham tubes and see if there is a correlation between that and the color results after the reagents and zinc dust have been added. Follow the procedure carefully, and use Table 31.1 as a guide when interpreting the results. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** Use BSL-2 precautions with *Pseudomonas aeruginosa*, if used.

1. **Obtain Your Samples:** Obtain fresh test culture(s) from your instructor. Obtain three—or if *P. aeruginosa* is used, obtain four—nitrate broths. Label two (or three) of them with the names of the organisms, your group name, and the date. Label the third (or fourth) tube "control."
2. **Inoculate the Media:** Use an inoculating loop to inoculate two (or three) broths with the test organisms. Do not inoculate the control.
3. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 24 to 48 hours.
4. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Samples for Bubbles in the Durham Tube:** Following the procedural diagram in Figure 31.2, first examine each tube for evidence of gas production. Record your results in the table provided at the end of the exercise. Refer to Table 31.1 when making your interpretations. (Be methodical when recording your results. Red color can have opposite interpretations depending on where you are in the procedure.)

3 **Add Reagents A and B to Each Sample and Make Observations:** Continue using Figure 31.2 as a guide and proceed to the addition of Reagents A and B to *all* tubes. Add 8 drops (approximately 0.5 mL) of Reagent A and then 8 drops (approximately 0.5 mL) of Reagent B to each tube. Mix well, but carefully, and let the tubes stand undisturbed for 10 minutes. Using the control as a comparison, record your test results in the table provided in the data sheet. Set aside any test broths that are positive.

4 **Add Reagent C to Samples That Have Not Developed a Red Color:** Where appropriate, add a very small pinch of zinc dust (Reagent C). You can dip a wooden applicator into the zinc dust and transfer only the amount that clings to the wood. Let the tubes stand for 10 minutes. Record your results in the table provided in the data sheet at the end of this exercise.

5 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

TABLE **31.1** Nitrate Reduction Test Results and Interpretations

Result	Interpretation	Symbol
Gas in Durham tube (***Note:*** You can only rely on this measure if you are absolutely certain the tested bacterium does *not* conduct fermentation. If you do not know if the test bacterium performs fermentation, then you must add reagents to assess if nitrate was reduced.)	If you are certain the test bacterium does *not* perform fermentation, then you may conclude denitrification occurred ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$).	+
Red color (after addition of Reagents A and B)	Nitrate reduction to nitrite ($NO_3^- \rightarrow NO_2^-$); nitrate reductase is present	+
No color change (after the addition of Reagents A and B)	Incomplete test; requires the addition of zinc dust	
No color change (after addition of Reagent C, zinc dust)	Nitrate reduction to gaseous (NO, $\frac{1}{2}N_2O$, or $\frac{1}{2}N_2$) or reduction to nongaseous nitrogen compounds such as ammonium (NH_4^+)	+
Red color (after addition of Reagent C, zinc dust)	No nitrate reduction	–

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 31

DATA SHEET

Nitrate Reduction Test

During the Lab

1 Using Table 31.1 as a guide, enter your results and interpretations in the following table. If you have a positive for gas production, look up the fermentation characteristics of that organism in a standard reference, such as ***Bergey's Manual of Determinative Bacteriology*** and consider that information as you shape your interpretation.

Organism	Results			Interpretation (be as specific as possible with respect to end products if positive)
	Gas	Color		
	Y/N	After Reagents	After Zinc	
Uninoculated control				

Lab Related Questions

1 Choose the true statement(s) about nitrate (select all that apply).

a. It can be oxidized to nitrite.

b. It can be reduced to molecular nitrogen (N_2).

c. It can serve as a final electron acceptor in anaerobic respiration.

d. It is present in the media used for this exercise.

e. It can be reduced to ammonium.

2 The nitrate reduction test is performed using:

a. Differential media.

b. Selective media.

c. Enriched media.

d. Defined media.

e. Synthetic media.

3 Reagents A and B are added to:

a. Detect nitrate production.
b. Detect nitrite production.
c. Convert nitrite to nitrate.
d. Convert nitrate to nitrite.
e. Activate nitrate reductase.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Nitrate reductases convert nitrite to nitrate.

_____ Bacteria that are anaerobic may use nitrate as a final electron acceptor.

_____ Reagent C facilitates the detection of nitrate in the media.

_____ An organism that reduces nitrate to ammonium will turn red after adding Reagents A, B, and C.

5 Number the steps below so they are in the proper order for the nitrate reduction test.

_____ Development of a red color indicates a negative result.

_____ Add Reagent B.

_____ Look for a bubble in the Durham tube.

_____ Inoculate the media.

_____ Development of a red color indicates a positive result.

_____ Add Reagent C if no color developed.

_____ Incubate the media.

_____ Add Reagent A.

6 Assume you see gas in the Durham tube and after adding Reagents A and B, a red color develops. Based on these observations, what can you conclude? (Select all that apply.)

a. The bacterium is an obligate aerobe.
b. The bacterium makes nitrate reductase.
c. The gas observed may be due to a fermentation reaction.
d. The organism is *not Neisseria gonorrhoeae*.
e. You must add zinc to the media before making any conclusions.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 31
DATA SHEET
(continued)

Critical Thinking

1 Suppose you remove your test cultures from the incubator and notice that one of them—a known fermenter—has a gas bubble in the Durham tube. Knowing that fermenters frequently produce gas, you ignore the bubble and proceed to the next step. Adding reagents produces no change, and neither does adding zinc. Based on this information what can you conclude? Be sure to explain your reasoning.

2 Although strict anaerobes may reduce nitrate, the nitrate reduction test as we performed it in this exercise would not determine the nitrate reduction potential of a strict anaerobe. Explain why.

— Lab Notes —

EXERCISE 32

Citrate Utilization Test

After doing this exercise, you should be able to:

1. Explain what the citrate utilization test reveals about a bacterial species.
2. Describe how to perform and interpret the citrate utilization test.
3. State the applications of the citrate utilization test.
4. Perform a citrate utilization test and interpret the test results.

Background

In the following exercise you will perform an example of a utilization test using Simmons citrate medium. **Citrate** is a six-carbon weak organic acid. It's found throughout nature, such as in citrus fruits. It is also an intermediate in the Krebs cycle, and thereby associated with aerobic respiration. Despite citrate being an intermediate in aerobic respiration, the citrate utilization test does not reveal if an organism performs aerobic respiration. Rather, it reveals if an organism can ferment citrate and therefore rely on citrate as its sole carbon source.

While numerous bacterial species make citrate, not all that make it can use it as a sole carbon source. Organisms that can use citrate as their sole carbon source must import the molecule into the cell and then metabolize it. To achieve this, bacteria require **citrate permease** to import citrate from the extracellular environment into the cell. Then, **citrate lyase** (Fig. 32.1) converts the imported citrate into oxaloacetate and acetate. The oxaloacetate is then decarboxylated (CO_2 is removed) by **oxaloacetate decarboxylase** to form pyruvate. The resulting pyruvate may then be fermented to a variety of intermediates. The cell's environmental pH and the availability of certain enzymes influences which fermentation pathway(s) further metabolizes pyruvate. From a metabolic standpoint, it is worth pointing out that the bacteria that thrive on Simmons citrate agar tend to perform fermentation pathways that generate ATP and reducing power (i.e., NADPH or NADH).

Citrate Media Formulation

Simmons citrate agar is a defined solidified agar medium (usually poured in a slant format) where sodium citrate is the only carbon source in the medium and ammonium phosphate serves as an inorganic nitrogen source.

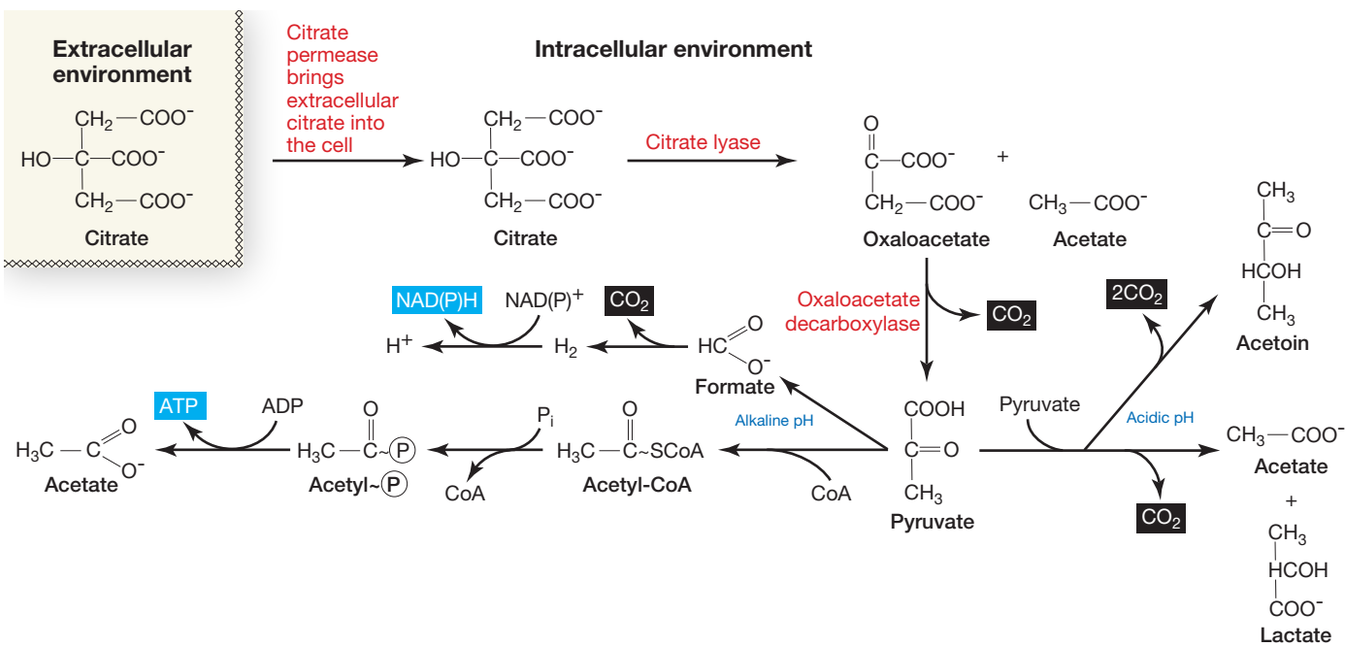


FIGURE **32.1** **Citrate Chemistry** • Citrate permease imports citrate into the cell. From there, citrate lyase converts citrate into oxaloacetate and acetate. Next, the enzyme oxaloacetate decarboxylase performs a decarboxylation reaction in which CO_2 is removed from oxaloacetate thereby forming pyruvate. The resulting pyruvate can be converted to a variety of products depending on the pH of the cell's environment and the enzymes the cell possesses. One option, the formate/acetyl-CoA pathway shown in detail to the left, produces the useful compounds ATP and NADH/NADPH.

Consequently, only bacteria that can survive with citrate as their sole carbon source and use ammonium phosphate as their nitrogen source will grow on this media.

As bacteria grow on the media and metabolize citrate, they also convert ammonium phosphate in the media to ammonia (NH_3) and ammonium hydroxide (NH_4OH). Because NH_3 and NH_4OH are bases, they will alkalinize the agar (raise the media's pH). The media includes a pH indicator dye, bromothymol blue, which allows us to determine if a pH shift to an alkaline state has occurred. The dye appears green at pH 6.9 (the media's original preparation pH) and blue at a pH level of pH 7.6 (the media's alkalinized pH if NH_3 and NH_4OH were made). Thus, conversion of the medium from its original green color to blue means the bacteria are actively growing and indicates a positive citrate test result (Fig. 32.2 and Table 32.1).

Occasionally bacteria will grow on a Simmons citrate slant *without* producing a color change in the media. In most cases, this is because of too short an incubation. In the absence of a color change, growth on the slant suffices as evidence of citrate utilization and thereby constitutes a positive test result. To avoid confusion between actual growth and a heavy inoculum, which may be misinterpreted as growth, citrate slants should be lightly inoculated. One way to decrease the amount of inoculum added to the agar is to use an inoculating needle rather than a loop to streak the agar slant surface. However, if this strategy is employed, you should be careful that you do not gouge the agar surface with the needle.

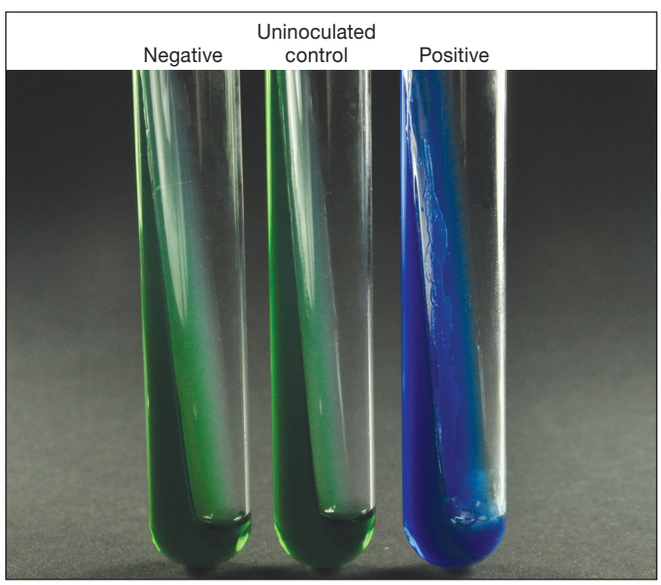


FIGURE **32.2** **Citrate Utilization Test Results** • After incubation, if the media appears green and there is no growth on the slant's surface, then the test result is negative; the bacterium cannot use citrate as its sole carbon source. In contrast, a blue media coloration and the presence of growth indicates a positive test result; the bacterium can use citrate as its sole carbon source.

Interpreting Citrate Utilization Test Results

TABLE **32.1** **Citrate Utilization Test Results and Interpretations** •

Result	Interpretation	Symbol
Media appears blue (even if only a portion of the media turned blue) *and* growth is evident	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source)	+
No color change, but growth is evident	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source)	+
No color change *and* no growth	Citrate is not utilized (organism lacks one or more of the enzymes needed to use citrate as its sole carbon source)	–

Clinical Applications

As previously discussed, identifying metabolic properties is an important means of determining a bacterial isolate's identity. The citrate utilization test reveals if a bacterium can use citrate as its sole carbon source. Citrate utilization is one part of a test series referred to as the IMViC (*I*ndole, *M*ethyl Red, *V*oges-Proskauer and Citrate tests) that helps differentiate members of the family *Enterobacteriaceae* from one another. In particular, the citrate test is useful for differentiating certain fecal coliforms from one another. For example, *E. coli* and *Shigella flexneri* do not typically grow on Simmons citrate agar while *Enterobacter aerogenes* does. Recall that a number of fecal coliforms cause gastrointestinal illness and are primarily transmitted via fecal-contaminated food and/or water.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Simmons citrate slants (3)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Enterobacter aerogenes*
  - ☐ *Escherichia coli*

Medium Recipe

Simmons Citrate Agar

Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium phosphate (K_2HPO_4)	1.0 g
Sodium chloride (NaCl)	5.0 g
Sodium citrate ($C_6H_5O_7Na_3$)	2.0 g
Magnesium sulfate ($MgSO_4$)	0.2 g
Agar	15.0 g
Bromothymol blue	0.08 g
Distilled or deionized water	1.0 L

Adjust to pH 6.7–7.1 at 25°C



PROCEDURE

You will be inoculating Simmons citrate slants with a citrate-positive and a citrate-negative organism. To avoid confusing growth on the slant with a heavy inoculum, be sure to lightly inoculate (your instructor may suggest using a needle instead of a loop to help you reduce how much bacteria you add). Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain Your Samples:** Obtain three Simmons citrate tubes. Label two with the names of the organisms, your group name, and the date. Label the third tube "control."
2. **Inoculate the Media:** Using an inoculating needle and *light inoculum*, streak the slants with the test organisms. Do not inoculate the control.
3. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 48 hours.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Observe each sample for color changes and/or growth on the media.
3. **Record Your Result:** Record your results in the table provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 32

DATA SHEET

Citrate Utilization Test

During the Lab

1 Using Table 32.1 as a guide, enter your results in the following table.

Organism	Color Result	(+ or –)	Interpretation
Control			

Lab Related Questions

1 Choose the true statement(s) about citrate (select all that apply).

a. It is an intermediate of the Krebs cycle.
b. It is a three-carbon compound.
c. It can be converted to oxaloacetate.
d. It raises the media's pH if present.
e. It can be used as a nitrogen source.

2 Which of the following is indicative of a positive test result for citrate utilization? (Select all that apply.)

a. The media appears green and growth is absent.
b. The media appears blue and growth is present.
c. The media appears green and growth is present.
d. The media appears blue and growth is absent.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Only bacteria that can use citrate as their sole carbon source will grow on Simmons citrate agar.

_____ *E. coli* will typically generate a positive test result when grown on Simmons citrate agar.

_____ This test can determine if a bacterium performs aerobic respiration.

_____ An organism that requires an organic nitrogen source will not grow on Simmons citrate agar.

4 The citrate test determines if a bacterium:

a. Metabolizes citrate.
b. Requires a carbon source.
c. Requires a nitrogen source.
d. Produces acidic metabolic byproducts.
e. Is a pathogen.

5 Which of the following are unlikely to grow on Simmons citrate agar? (Select all that apply.)

a. *E. coli*
b. *S. flexneri*
c. A bacterium that requires an inorganic nitrogen source
d. A bacterium that lacks citrate permease
e. *E. aerogenes*

6 Why may a heavy inoculum be problematic in this test?

a. The medium's pH can be falsely elevated if too many bacteria are growing on the agar.
b. You will not be able to achieve colony isolation.
c. You may introduce a contaminant into the agar.
d. You may mistake the inoculum for actual growth.
e. You may be unable to assess the media's color if the slant is overgrown.

7 Choose the statement(s) that accurately describe(s) Simmons citrate agar (select all that apply).

a. It is considered enriched media.
b. It is considered a defined media.
c. There is an organic source of carbon in the media.
d. Simmons citrate agar contains methylene blue as a pH indicator.
e. After incubation, you must add reagents to this media before interpreting test results.

Critical Thinking

1 It is a Friday afternoon and you head to the lab to inspect the Simmons citrate agar bacterial samples that you inoculated on Wednesday of the same week. The samples were all grown at 37°C. You do not observe any growth in the tube for your test sample nor for the negative control. However, the positive control grew just fine. You decide your tested bacteria cannot utilize citrate as a sole carbon source. Planning to dispose of the samples on Monday, you simply place the samples in a test-tube rack at room temperature in the biosafety hood where they will be safe until your return. When you return to the lab on Monday, you notice that your unknown sample has abundant growth and the media is completely blue. Your negative control sample still does not exhibit growth and remained green. Based on this, what can you conclude about your test specimen? Be sure to explain your reasoning and provide details from the scenario that support your conclusion.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 32
DATA SHEET
(continued)

2 You perform the citrate utilization test and after 48 hours of incubation you make the following observations. Should you trust your findings? Be sure to explain your reasoning.

Sample Description	Media Color after 48 Hours of Incubation	Growth after 48 Hours of Incubation
E. coli	Blue	Present
E. aerogenes	Blue	Present
Unknown sample 2	Blue	Present

— Lab Notes —

EXERCISE 33

Amino Acid Decarboxylation (Decarboxylase Tests)

After doing this exercise, you should be able to:

1. Explain what amino acid decarboxylation tests reveal about a bacterial species.
2. Describe how to perform and interpret amino acid decarboxylation tests.
3. State the applications of amino acid decarboxylation tests.
4. Perform amino acid decarboxylation tests and interpret the test results.

Background

Amino acids are the building blocks of protein, but when resources are limited, some bacteria use them as a carbon and energy source. Using amino acids for purposes other than making proteins generally involves removing one or both of their functional groups: the amine (NH_2) by a **deaminase** and/or the carboxylic acid (COOH) group using a **decarboxylase enzyme.**

In this exercise, you will examine the ability of bacteria to decarboxylate the amino acid lysine using the enzyme lysine decarboxylase. As optional procedures, you may also test for the presence of ornithine decarboxylase and arginine decarboxylase in the assigned bacteria. (***Note:*** Each decarboxylase is specific to its own amino acid substrate.) The indole test, discussed in Exercise 36, relies on a bacterium's ability to deaminate an amino acid.

Møller's Decarboxylase Medium Formulation

To prepare the media for the decarboxylase tests, a single amino acid is added to a foundational medium called Møller's decarboxylase base medium. The base medium contains beef extract and peptone as nitrogenous nutrients for bacteria; glucose serves as the fermentable carbohydrate; and pyridoxal is a necessary cofactor for decarboxylase enzymes to function. The type of amino acid added to the base medium depends on which decarboxylase you are aiming to detect. For example, if you want to determine if your test bacterium has the enzyme lysine decarboxylase, then the base media would be supplemented with the amino acid lysine. The dye bromocresol purple serves as the pH indicator in the media. It appears yellow below pH 5.2 and purple at pH 6.8 or higher.

Performing and Interpreting Amino Acid Decarboxylation Tests

After inoculation, an overlay of mineral oil is added to seal the medium from the air. This ensures the surface of the media's pH is not artificially raised (alkalized) through interactions with the ambient air and it helps to maintain a lower oxygen environment that favors glucose fermentation (Fig. 33.1). Recall that glucose fermentation tends to lead to an accumulation of acidic end products; all *Enterobacteriaceae* family members ferment glucose to acidic end products. A low pH combined with the presence of a specific amino acid induces **decarboxylase-positive** organisms to produce the enzyme that can **catabolize** (break down) the given amino acid. That is, the specific decarboxylase gene is "switched on."

Decarboxylation of the amino acid results in accumulation of amines, which are alkaline (Fig. 33.2). As amines accumulate, the resulting alkaline pH causes the pH indicator, bromocresol purple, to appear purple; this is a positive test result (Fig. 33.3, left). If the organism is a glucose fermenter but does not produce the appropriate

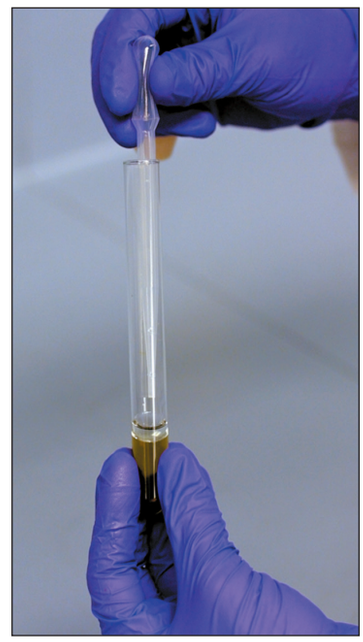


FIGURE **33.1 Adding Sterile Mineral Oil to the Tubes** • Gently let the oil slide down the side of the test tube to a depth of approximately 4 mm (not mL). It is important to keep the mineral oil sterile to prevent contamination.

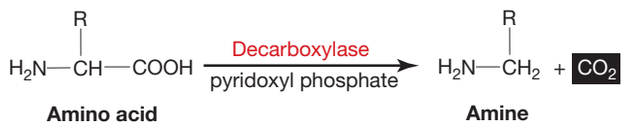


FIGURE **33.2 Amino Acid Decarboxylation** • Removal of an amino acid's carboxyl group results in the formation of an amine and carbon dioxide.

decarboxylase, the medium will appear yellow (Fig. 33.3, middle). If the organism does not ferment glucose, the medium does not exhibit a color change (Fig. 33.3, right).

Different amino acids can be added to the base broth. The three amino acids most frequently used in decarboxylase test media are lysine, ornithine, and arginine. Figures 33.4 through 33.6 show the chemistry associated with decarboxylating each of these amino acids. Although the chemistry slightly differs based on the amino acid catabolized, positive and negative test results are interpreted in the same manner as used for the lysine decarboxylase test (Table 33.1).

Lastly, it is worth noting that arginine decarboxylase medium can not only reveal if a bacterium makes arginine decarboxylase, but it can also detect the presence of a second pathway for arginine catabolism, the arginine dihydrolase system. In this system, arginine is converted to citrulline and then into ornithine, ATP, CO_2, and two NH_3 (ammonia) (Fig. 33.7). The generated ammonia raises the pH and turns the medium purple, just as would occur if arginine decarboxylase were present.

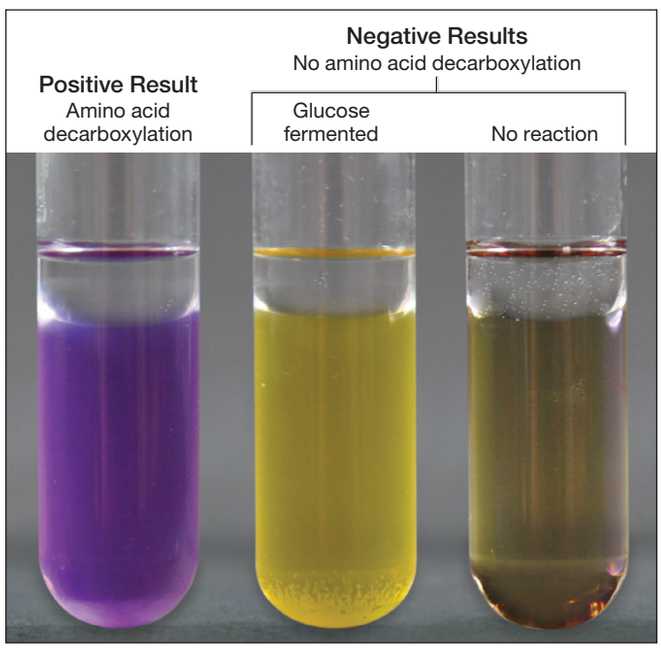


FIGURE **33.3** **Decarboxylation Test Results** • (**Left**) A lysine decarboxylase-positive result appears purple; (**Middle**) a lysine decarboxylase-negative result with glucose fermentation appears yellow; (**Right**) a decarboxylase-negative result without fermentation appears a murky yellow/tan color, the same color as your uninoculated control, which is not shown here. The color results shown here are representative of other decarboxylase media as well.

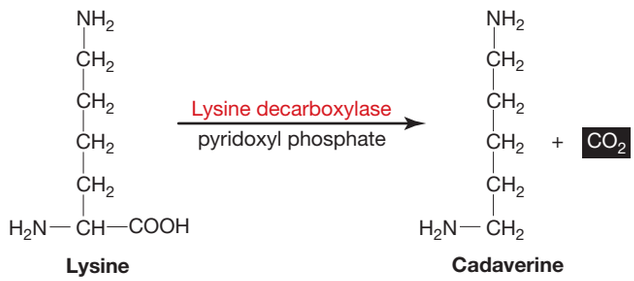


FIGURE **33.4** **Lysine Decarboxylation** • Decarboxylation of the amino acid lysine produces cadaverine and CO_2.

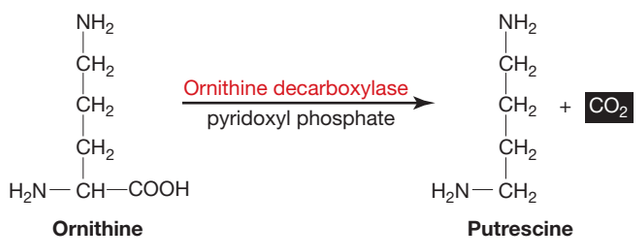


FIGURE **33.5** **Ornithine Decarboxylation** • Decarboxylation of the amino acid ornithine produces putrescine and CO_2.

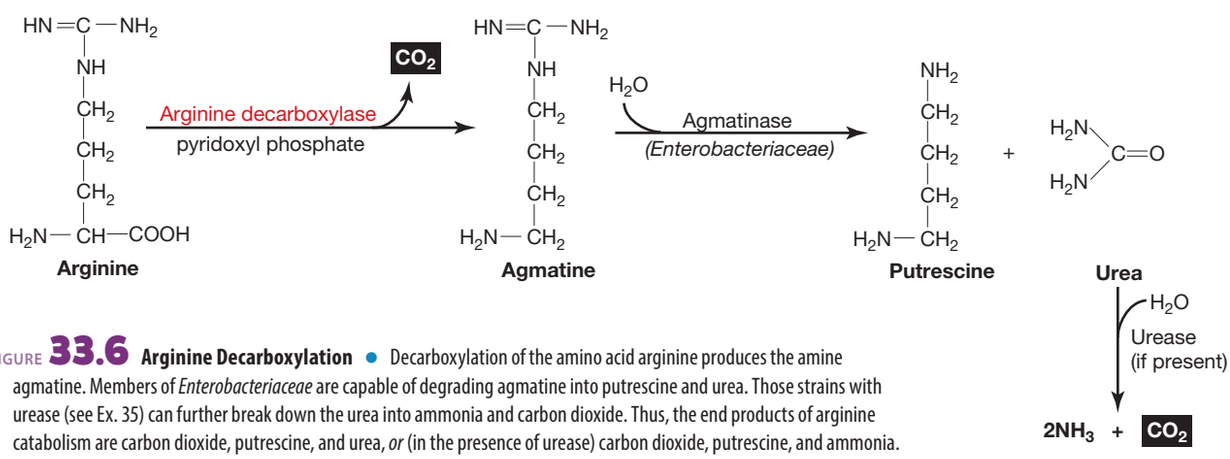


FIGURE **33.6** **Arginine Decarboxylation** • Decarboxylation of the amino acid arginine produces the amine agmatine. Members of *Enterobacteriaceae* are capable of degrading agmatine into putrescine and urea. Those strains with urease (see Ex. 35) can further break down the urea into ammonia and carbon dioxide. Thus, the end products of arginine catabolism are carbon dioxide, putrescine, and urea, *or* (in the presence of urease) carbon dioxide, putrescine, and ammonia.

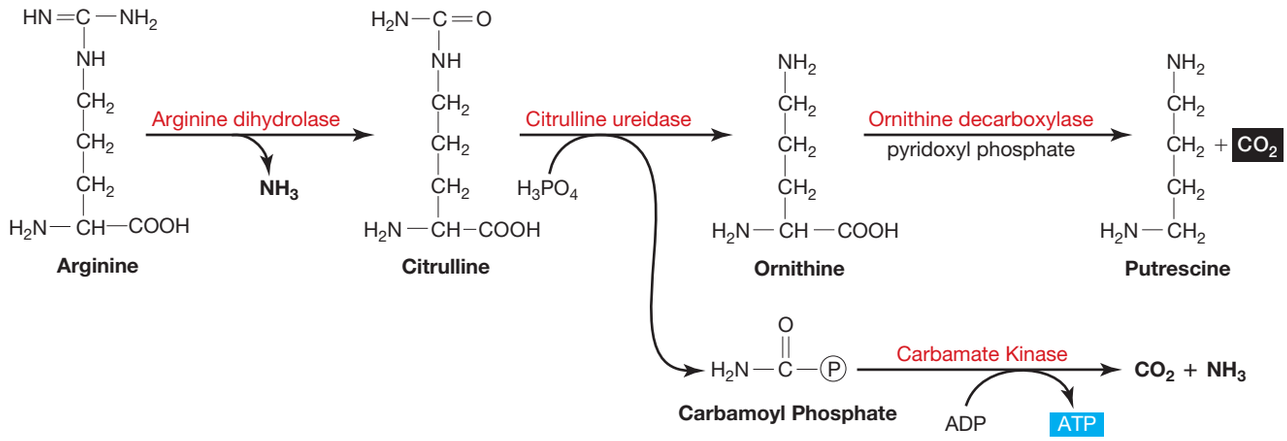


FIGURE **33.7** **Arginine Dihydrolase System** • In addition to decarboxylation, arginine may also be catabolized by a dihydrolase enzyme with the production of ornithine, NH_3, and CO_2. In ornithine decarboxylase-positive organisms, the ornithine is further degraded into putrescine and CO_2. The example shown here is a dihydrolase system of *Pseudomonas putida* in which ATP synthesis occurs. Regardless of the specifics, the arginine dihydrolase pathway is indistinguishable from the arginine decarboxylase pathway because both produce alkaline end products.

TABLE **33.1** **Decarboxylase Test Results and Interpretations** •

Result	Interpretation	Symbol
No color change	No decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable	–
Yellow	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable	–
Purple (may be slight)	Decarboxylation of the amino acid (insert amino acid name)*; organism produces the specific decarboxylase enzyme	+

* The amino acid included in the media can vary. Therefore, all interpretations must be described as they relate to the specific amino acid in the media. Example: If using lysine decarboxylase media, the amino acid in the media is lysine.

Clinical Applications

The decarboxylase tests are a form of utilization test that helps microbiologists identify bacterial species. Decarboxylase media can include any one of several amino acids, with the most frequently used amino acids being lysine, arginine, and ornithine. Typically, decarboxylase media are used to differentiate organisms in the family *Enterobacteriaceae* and to distinguish them from other Gram-negative rods.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Lysine decarboxylase broths[1] (3)
- ❑ Tubes (3) of the Møller's decarboxylase base media (media lacking the test amino acid)
- ❑ Sterile mineral oil in microtubes
- ❑ Sterile transfer pipettes (16)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Enterobacter aerogenes*
  - ☐ *Providencia stuartii*
  - ☐ *Enterobacter cloacae* (optional, see footnote)

[1] This exercise assumes that only two bacterial species will be tested and that only lysine decarboxylase media will be used. However, if desired, arginine and ornithine decarboxylase media may also be used in this exercise. If these other decarboxylase test media are included, it is recommended that you also include *Enterobacter cloacae* as one of the test organisms.

Medium Recipe

Lysine Decarboxylase Medium (Møller)[2]

- Peptone 5.0 g
- Beef extract 5.0 g
- Glucose (dextrose) ($C_6H_{12}O_6$) 0.5 g
- Bromocresol purple 0.01 g
- Cresol red 0.005 g
- Pyridoxal 0.005 g
- L-Lysine ($C_4H_7COOH(NH_2)_2$) 10.0 g
- Distilled or deionized water 1.0 L

Adjust to pH 5.8–6.2 at 25°C

[2] ***Note:*** The base medium used for your controls is the same as the lysine decarboxylase medium, except lysine is not added.

PROCEDURE

This exercise assumes you will test two different bacterial species for their ability to decarboxylate the amino acid lysine. Please note, your instructor may modify the number and/or type of bacteria tested. They may also alter which decarboxylases test(s) is/are performed. That said, positive and negative results—with and without acid production—look the same regardless of the amino acid in the decarboxylase medium. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

6

1. **Obtain Your Samples and Media:** Obtain the two bacterial stock cultures that you are going to test. You will also need three tubes of Møller's decarboxylase base medium (media lacking the test amino acid) and three tubes of lysine decarboxylase media (the media that contains the test amino acid). ***Note:*** Your instructor may modify the number of tubes and the type of decarboxylase media you use, so pay attention and adapt your process accordingly.
2. **Label the Media Tubes:**
   a. **Label the Three Tubes of Base Media that Lack Lysine:** One tube will serve as your uninoculated control; the other two tubes are to grow the two test bacteria. Label each tube with your name, the date, the sample description (i.e., the bacterium's name or uninoculated), and the media's name.
   b. **Label the Three Tubes of Lysine Decarboxylase Media:** One tube will serve as your uninoculated control; the other two tubes are to grow the two test bacteria. Label each tube with your name, the date, the sample description (i.e., the bacterium's name or uninoculated), and the media's name.
3. **Inoculate the Media:** Use an inoculating loop to inoculate a separate lysine decarboxylase broth for each test bacterium. Repeat the process for the base media. As a reminder, do not inoculate the tubes you labeled "uninoculated."
4. **Mineral Oil Overlay:** Aseptically add mineral oil to each tube (Fig. 33.1). Be careful not to introduce contaminants while adding the oil.
5. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 96 hours (4 days) up to a full week.
6. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Observe each sample for color changes. Refer to Figure 33.3 and Table 33.1 when interpreting your results. If the inoculated base medium without the added amino acid becomes alkaline during incubation, the test is considered invalid.
3. **Record Your Result:** Record your results in the table provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 33

DATA SHEET

Amino Acid Decarboxylation (Decarboxylase Tests)

During the Lab

1 Using Table 33.1 as a guide, enter your results in the following table.

Organism	Results				Interpretation
	Base		Lysine		
	Color	+/–	Color	+/–	
Control					

Lab Related Questions

1 Choose the true statement(s) (select all that apply).

a. If an organism has lysine decarboxylase, it can make lysine.
b. If an organism has lysine decarboxylase, it can catabolize lysine.
c. If an organism has lysine decarboxylase, it can deaminate lysine.
d. If an organism has lysine decarboxylase, it will produce putrescine.
e. If an organism has lysine decarboxylase, it will produce CO_2.

2 Which of the following is (are) indicative of a negative test result for amino acid decarboxylation? (Select all that apply.)

a. The media appears yellow.
b. The media appears purple.
c. The media did not manifest a color change.
d. The media appears yellow and cloudy.
e. The media appears blue.

3 Why is mineral oil added in the amino acid decarboxylase test? (Select all that apply.)

a. It protects the samples from contamination.
b. It serves as a carbon source for the bacteria.
c. It promotes glucose fermentation.
d. It acidifies the media.
e. It prevents artificial alkalization of the media.

4 You observe your inoculated sample at Day 3 and notice it is yellow. By Day 6, it is purple. What can you conclude about the organism? (Select all that apply.)

a. It ferments glucose.
b. It made lysine.
c. It cannot ferment glucose.
d. It catabolized lysine.
e. It cannot catabolize lysine.

5 A typical decarboxylase enzyme removes _____ from an amino acid to produce _____ and _____.

6 The pH of media in a positive amino acid decarboxylase test result is:

a. Purple.
b. Basic.
c. Neutral.
d. Acidic.
e. Yellow.

7 In this experiment, the Møller's decarboxylase base medium serves as a:

a. Positive control.
b. Negative control.
c. Practice sample.
d. Back-up sample.
e. Calibration sample.

Critical Thinking

1 How would you modify the test media to determine if a bacterium that ferments lactose can decarboxylate lysine?

2 Think about the effect of the mineral oil on the test results. Would omitting the sterile mineral oil affect either the specificity or the sensitivity of the test, or both? Explain.

EXERCISE 34

Starch Hydrolysis (Amylase Test)

After doing this exercise, you should be able to:

1. Explain what the amylase test reveals about a bacterial species.
2. Describe how to perform and interpret the starch hydrolysis test.
3. State the applications of the starch hydrolysis test.
4. Perform a starch hydrolysis test and interpret the test results.

Background

The starch hydrolysis test (or amylase test) reveals if a bacterium can break down the complex polysaccharide starch. **Starch** is essentially a string of α-glucose molecules bonded together. There are two general versions of this polymer, a linear form called **amylose** and a branched form called **amylopectin**. In most cases, starch exists as a mixture of these two forms, with amylopectin predominating.

Starch is too large to pass through the bacterial cell membrane to be metabolized. Therefore, it must first be broken down extracellularly. This is accomplished by three enzymes: **α-amylase**, **β-amylase**, and **α-1,6-glucosidase**. These enzymes are all secreted extracellularly and are therefore considered **exoenzymes**. Furthermore, because all three enzymes use water to split complex molecules, they are called **hydrolytic** enzymes. Although these enzymes have the aforementioned features in common, they have different roles in breaking down starch into smaller molecules.

The enzyme α-amylase breaks amylose into the monosaccharide α-glucose and the disaccharide α-maltose, whereas β-amylase hydrolyzes amylopectin to form β-maltose and **limit dextrin**, a small branched carbohydrate molecule. Because neither α nor β amylases can disassemble the glucose subunits around branch points of limit dextrin, **α-1,6-glucosidase** breaks down limit dextrin into short, linear oligosaccharides that α-amylase then hydrolyzes to α-glucose and α-maltose (Fig. 34.1).[1]

[1] The carbons of organic molecules are numbered. A "1,4 linkage" is a covalent bond between the first carbon of one glucose and the fourth carbon of another glucose. A "1,6 linkage" is a covalent bond between the first and sixth carbons of two glucose molecules. The "α" and "β" refer to whether the two glucose molecules have the same orientation (α) or are "flipped" 180° relative to one another (β).

Starch Medium Formulation and Test Result Interpretation

Starch agar is a plated medium of beef extract, soluble starch, and agar. When organisms that produce α-amylase, β-amylase, and oligo-1,6-glucosidase are grown on starch agar, they hydrolyze the starch in the area surrounding their growth. Because both starch and its sugar subunits are virtually invisible in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity of the bacterial growth. Iodine reacts with starch and produces a blue or dark-brown color. If the bacterium hydrolyzed starch, the added iodine will not encounter starch to react with and therefore you will observe a clear zone around the bacterial growth (Fig. 34.2). Table 34.1 summarizes interpretations for possible test results.

TABLE **34.1** Starch Hydrolysis Test Results and Interpretations

Result	Interpretation	Symbol
Clearing around growth	α-amylase, β-amylase, and/or α-1,6-glucosidase is present (or more simply, amylase is present)	+
No clearing around growth	α-amylase, β-amylase, and α-1,6-glucosidase are absent; (or more simply, amylase is absent or not detectable)	–

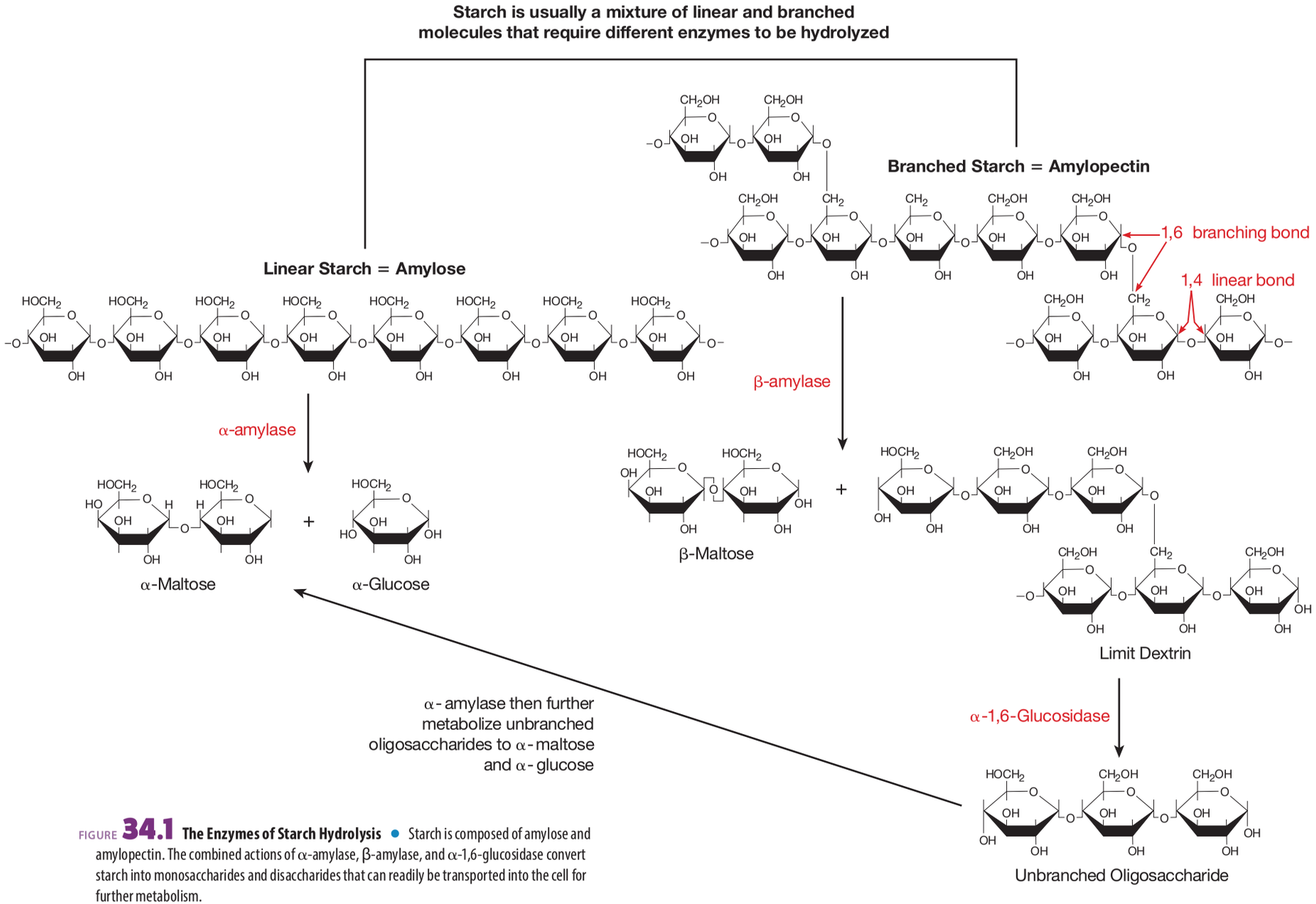


FIGURE **34.1** **The Enzymes of Starch Hydrolysis** • Starch is composed of amylose and amylopectin. The combined actions of α-amylase, β-amylase, and α-1,6-glucosidase convert starch into monosaccharides and disaccharides that can readily be transported into the cell for further metabolism.

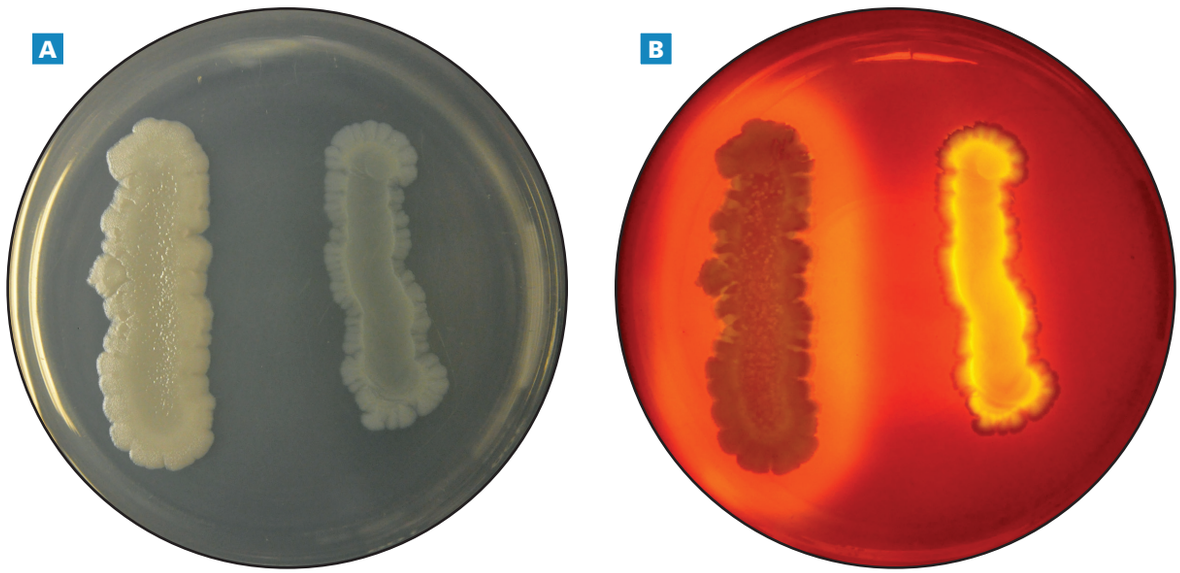


FIGURE **34.2** **Starch Hydrolysis Test Results** • (**A**) This is an image of a double inoculated starch agar plate before adding iodine. Notice the wavy margins of the bacterial growth. (**B**) This is the same plate pictured in Panel **A**, but with iodine added. The clearing in the medium around the organism on the left demonstrates a positive result for starch hydrolysis. The organism on the right, which lacks a clearing, is negative. Notice that the wavy margin of the negative organism's growth appears lighter and could be misinterpreted as a positive result. Drawing a line around the edges of growth prior to adding iodine can help avoid this sort of misinterpretation.

Clinical Applications

Starch agar was originally designed for cultivating *Neisseria*, a bacterial genus that includes *N. gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhea. However, this medium is no longer used for *Neisseria* isolation. Instead a modified formulation, which has pH indicators added, is useful for isolating and presumptively identifying *Gardnerella vaginalis*, bacteria associated with bacterial vaginosis. It also aids in differentiating members of the genera *Corynebacterium*, *Clostridium*, *Clostridioides*, *Bacillus*, *Bacteroides*, *Fusobacterium*, and *Enterococcus*, most of which include amylase-positive and amylase-negative species.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Starch agar (SA) plate (1)
- ❑ Gram's iodine (from your Gram stain kit)
- ❑ Fresh broth cultures of these recommended organisms:
  - ☐ *Bacillus subtilis*
  - ☐ *Escherichia coli*

Medium Recipe

Starch Agar (SA)

Beef extract	3.0 g
Soluble starch	10.0 g
Agar	12.0 g
Distilled or deionized water	1.0 L

Adjust to pH 7.3–7.7 at 25°C

6

PROCEDURE

You will inoculate a starch agar plate with two organisms. After incubation, you will add a few drops of iodine to determine whether the tested bacteria hydrolyzed starch. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1 **Obtain Your Samples and Media:** Obtain the two bacterial stock cultures that you are going to test. You will also need one plate of starch agar.

2. **Label the Starch Plate:** Using a marking pen, divide the starch agar plate into three equal sectors. Label one section for each test bacterium and the third section as an uninoculated control section. Be sure to mark on the bottom of the plate, not the lid. Also, write your name and the date on your plate.
3. **Inoculate the Media:** Use an inoculating loop to inoculate each test bacterium in a zigzag pattern in its designated section. Be careful to keep your inoculation well within each section's boundaries. Leave the control section uninoculated.
4. **Incubate Your Samples:** Incubate all tubes at 35±2°C for 24 hours. If incubated longer, you run the risk of one organism overgrowing the plate and obscuring the results of the other. If the next lab session is more than 24 hours after inoculation, then at the 24-hour mark, the plates can be transferred from the incubator to a refrigerator and maintained at 4°C until your next lab meeting.
5. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Indicate the Growths' Margins:** Remove the plate from the incubator, and then note the location and appearance of the growth. Use a marking pen to indicate the margins of the growth.
3. **Add Iodine:** Cover the growth and surrounding areas with Gram's iodine. Do not flood the plate, but do add enough iodine so that the agar is completely colorized. *Allow the iodine to soak in.*
4. **Examine Your Samples:** Examine the areas surrounding the growth for clearing. ***Note:*** Usually the growth on the agar prevents contact between the starch and the iodine so no color reaction takes place at that point. Beginning students sometimes look at this lack of color change and judge it incorrectly as a positive result. Therefore, when examining the agar for clearing, look for a halo *around* the growth, not at the growth itself.
5. **Record Your Result:** Refer to Figure 34.2 and Table 34.1 when interpreting your results. Record your results in the table provided in the data sheet at the end of this exercise.
6. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 34

DATA SHEET

Starch Hydrolysis (Amylase Test)

During the Lab

1 Using Table 34.1 and Figure 34.2 for guidance, enter your findings in the following table.

Organism	Result	(+/–)	Interpretation (include enzyme)
Control sector			

Lab Related Questions

1 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The media was originally designed for cultivating *Gardnerella species*.

_____ Test results are visible only after adding iodine.

_____ Iodine reacts with glucose in the media.

_____ The uninoculated region of the plate should appear clear at the end of the experiment.

2 For a bacterium to generate a positive test result in the starch hydrolysis test, which of the following must be present? (Select all that apply.)

a. α-amylase must be present.
b. β-amylase must be present.
c. α-1,4-glucosidase must be present.
d. β-1,6-glucosidase must be present.
e. α-1,6-glucosidase must be present.

3 What serves as the differentiation aspect in this test?

a. The presence or absence of growth on the media.
b. A change in media pH following starch hydrolysis.
c. A media color change during incubation.
d. A color change in the bacterial culture during incubation.
e. The color of the media in the presence of iodine.

4 Choose the true statement(s) about β-amylase (select all that apply).

a. It breaks α 1,6 linkages between glucose molecules.
b. It breaks α 1,4 linkages between glucose molecules.
c. It generates α-glucose.
d. It generates β-maltose.
e. It acts intracellularly.

5 Choose the true statement(s) about α-amylase (select all that apply).

a. It is an exoenzyme.
b. It is a hydrolytic enzyme.
c. It breaks α 1,6 linkages between glucose molecules.
d. It reacts with iodine.
e. It breaks α 1,4 linkages between glucose molecules.

6 How would observing the test results at 12 hours likely affect the experimental outcome? (Select all that apply.)

a. You would be less likely to observe a positive test result.
b. You would be less likely to observe a negative test result.
c. You would be more likely to observe a false-negative test result.
d. You would be more likely to observe a positive test result.
e. You would be more likely to obtain a false-positive result.

7 How would adding glucose to the medium affect the experimental outcome?

a. The bacteria would be more likely to grow, thereby generating a false-positive result.
b. Bacteria would metabolize glucose over starch, generating a false-negative test result.
c. The added glucose would bind to the iodine, generating a false-positive result.
d. The added glucose would inhibit growth, thereby generating a false-negative result.
e. Bacteria would ferment the glucose, thereby generating a false-positive result.

Critical Thinking

1 Suppose you could selectively prevent production of α-amylase or α-1,6-glucosidase in an organism that normally hydrolyzes starch. Which enzyme would the organism miss the most and why?

2 Design an experiment that would allow you to determine if an isolated bacterium has α-amylase and β-amylase, but lacks α-1,6-glucosidase. (***Hint***: You only need the starch hydrolysis test to do this, although it should be modified.)

EXERCISE 35

Urea Hydrolysis (Urease Test)

After doing this exercise, you should be able to:

1. Explain what the urea hydrolysis test reveals about a bacterial species.
2. Describe how to perform and interpret the urea hydrolysis test.
3. State the applications of the urea hydrolysis test.
4. Perform a urea hydrolysis test and interpret the test results.

Background

This test reveals if a bacterium produces the intracellular hydrolytic enzyme **urease**, which uses water to catabolize (break down) urea to form ammonia and carbon dioxide (Fig. 35.1). **Urea** is produced as a byproduct of amino acid catabolism and is the primary nitrogenous waste in the urine of many land animals (including mammals). Although urea is present at high levels in urine, it is not exclusively present in urine; it is also present in blood, sweat, and even in the stomach.

Because urease generates an alkaline byproduct (ammonia), the enzyme can serve as a virulence factor for some pathogens by counteracting acid in the microbe's environment. For example, *Helicobacter pylori* (the bacterium that causes stomach ulcers) uses urease to survive in the acidic stomach environment. *Streptococcus salivarius*, an oral commensal, also produces urease. Furthermore, many enteric bacteria will metabolize urea if it is present and another usable nitrogen source is absent.

Urea Medium Formulation and Test Result Interpretation

The original urease test used urea agar, which was formulated to differentiate enteric organisms capable of rapid urea hydrolysis (rapid urease-positive bacteria, such as *Proteus*, *Morganella morganii*, and some *Providencia stuartii* strains) from slower urease-positive and urease-negative bacteria. A pH indicator was used to track the rising pH due to accumulation of ammonia. Rapid urease-positive organisms produce a positive result within a day; slower urease-positive organisms may take several days to produce a readable result.

In this exercise, you will be using urea broth, which differs from urea agar in two important ways. First, the only nutrient source other than urea is a trace level (0.0001%) of yeast extract. Second, it contains buffers strong enough to inhibit alkalinization of the medium by all but the rapid urease-positive organisms mentioned previously. The pH indicator phenol red, which is yellow or orange below pH 8.4 and red or pink above, is included to expose any increase in pH. The development of a pink color in the medium in 24 hours or less indicates a rapid urease-positive organism. If the media appears orange or yellow, the test result is deemed negative (Fig. 35.2 and Table 35.1).

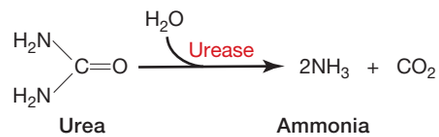


FIGURE **35.1** **Urea Hydrolysis** • Urea hydrolysis produces ammonia, which raises the pH in the medium and turns the pH indicator pink.

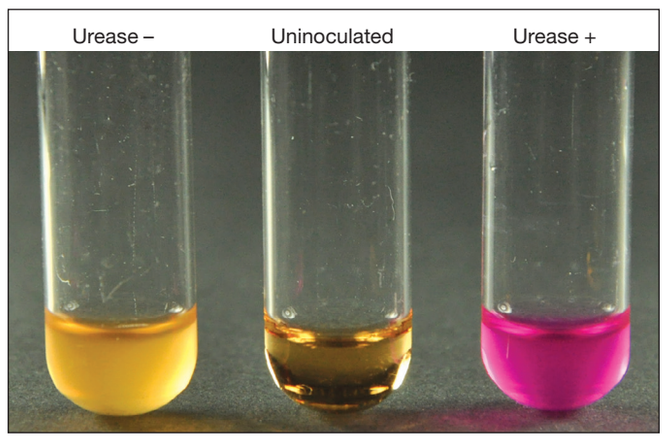


FIGURE **35.2** **Urea Hydrolysis Test Results** • These are urea broth tubes after a 24-hour incubation. Urease-positive samples will appear bright pink after incubation. Urease-negative samples remain yellow.

TABLE **35.1** **Urea Hydrolysis Test Results and Interpretations** •

Result	Interpretation	Symbol
Orange or yellow	No urea hydrolysis; urease is absent or not produced in enough quantity to produce results in 24 hours; or the organism cannot live in urease broth	–
Pink	Rapid urea hydrolysis; urease was produced	+

Clinical Applications

Various pathogenic bacteria make urease as a virulence factor to survive in host tissues; the presence of urease is therefore an important marker of a number of bacterial infections. For example, common urinary tract pathogens such as *Staphylococcus saprophyticus* and *Proteus* species are rapid hydrolyzers of urea; thus, the urease test readily differentiates these bacteria from urease-negative pathogens like *E. coli*, which are also a common cause of urinary tract infections. The urease test also helps microbiologists identify *H. pylori*, which is associated with gastric and duodenal ulcers, as well as stomach cancer.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Urea broths (3)
- ❑ Sterile wooden applicators or sterile disposable loops (3)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - □ *Escherichia coli*
  - □ *Proteus vulgaris* (BSL-2)

Medium Recipe

Rustigian and Stuart's Urea Broth

Yeast extract	0.1 g
Potassium phosphate, monobasic (KH_2PO_4)	9.1 g
Potassium phosphate, dibasic (K_2HPO_4)	9.5 g
Urea ($CO(NH_2)_2$)	20.0 g
Phenol red	0.01 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

6

PROCEDURE

You will perform the urease test with urea broths, which should not be read beyond 24 hours of incubation. If your labs are scheduled more than 24 hours apart, arrange to have someone place your broths in a refrigerator until you can examine them. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. *Caution!* **Follow BSL-2 precautions if using *P. vulgaris*.**

1. **Obtain Your Samples and Media:** Obtain the two bacterial stock cultures that you are going to test and three tubes of urease broth medium. Label two with the names of the organisms, your group name, and the date. Label the third tube "control."
2. **Inoculate the Media:** Heavily inoculate the two urea broths with the test organisms. Do not inoculate the control tube.
3. **Incubate Your Samples:** Incubate all tubes aerobically at 35±2°C for 24 hours.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Examine the samples for color changes.
3. **Record Your Result:** Refer to Table 35.1 and Figure 35.2 when interpreting your results. Record your results in the table provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 35
DATA SHEET

Urea Hydrolysis (Urease Test)

During the Lab

1 Using Table 35.1 and Figure 35.2 for guidance, enter your findings in the following table.

Organism	Color Result	(+/–)	Interpretation (include enzyme)
Control			

Lab Related Questions

1 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The media was originally designed as an agar plate.

_____ A positive test result indicates ammonium was made.

_____ A negative test result indicates urea was not hydrolyzed.

_____ Test results should be viewed no later than 48 hours after incubation.

2 _____ is the enzyme that hydrolyzed urea.

3 Choose the true statement(s) about urea (select all that apply).

a. Urea is a virulence factor.
b. Urea can serve as a nitrogen source.
c. Urea is present at high levels in urine.
d. Urea is only found in urine.
e. Urea is a byproduct of amino acid catabolism.

4 Choose the true statement(s) about urease (select all that apply).

a. Urease is a virulence factor.
b. Urease is only made by pathogens.
c. Urease is a hydrolytic enzyme.
d. Urease acts extracellularly.
e. Urease produces urine.

5 Choose the true statement about urea hydrolysis test media.

a. Only microbes that are urease positive can grow in the media.
b. The pH indicator methyl red is present in the media to detect pH changes.
c. The media is rich in nutrients.
d. The media can detect the production of urea.
e. The media can differentiate *Proteus* species from *E. coli*.

6 How would observing the test results at 48 hours likely affect the experimental outcome?

a. You would be less likely to observe a positive test result.
b. You would be less likely to observe a negative test result.
c. You would be more likely to observe a false-negative test result.
d. You would be more likely to observe a false-positive test result.
e. You would be more likely to observe contamination.

Critical Thinking

1 You are working in a clinical microbiology lab, and immediately after you open a patient's urine sample, your colleague, who is sitting near you, declares, "Wow, if that isn't a urease-positive sample, then I don't know what is!" How could your colleague possibly know anything about the probable urease test result when you haven't even performed a urease test?

2 Your professor informs you that the test media was prepared without the pH indicator, but that you should still be able to make test result conclusions using tools in the lab. How would you proceed?

EXERCISE 36

Assessing Sulfur Reduction, Indole Production, and Motility (SIM Media and General Motility Agar)

After doing this exercise, you should be able to:

1. Describe how to use media to test for a bacterium's sulfur reduction ability, indole production, and motility.
2. State the differences between SIM (sulfur reduction, indole production, motility) media and general motility agar.
3. Describe how to perform and interpret sulfur reduction, indole production, and motility tests.
4. Discuss why assessing sulfur reduction, indole production, and motility is useful.
5. Test bacteria for sulfur reduction ability, indole production ability, and motility, and interpret the test results.

Background

In this exercise, you will explore how to assess a bacterium's ability to reduce sulfur, make indole, and move. Learning if a bacterial isolate has these features assists with bacterial species identification. It's worth noting that we can detect any one of these three capabilities using a variety of tests; however, here we will focus on using SIM media (a three-in-one test that assesses sulfur reduction, indole production from tryptophan, and motility) and general motility agar. Before we dive into how we perform the SIM and general motility tests, let's first walk through each of these three features.

1. **Sulfur Reduction:** Bacteria accomplish sulfur reduction to H_2S in two different ways, depending on the enzymes present. Both mechanisms produce hydrogen sulfide (H_2S) gas, which we can detect using SIM (sulfur reduction, indole production, motility) media.[1]
   - The enzyme **cysteine desulfurase** catalyzes the hydrolysis of the sulfur-containing amino acid cysteine to pyruvate, ammonia (NH_3), and hydrogen sulfide (H_2S) during putrefaction (Fig. 36.1). The resulting pyruvate may enter fermentation or respiration pathways for energy extraction.
   - In a totally unrelated way, the enzyme **thiosulfate reductase** catalyzes the reduction of sulfur (in the form of sulfate) to H_2S at the end of an anaerobic respiratory electron transport chain (Fig. 36.2).
2. **Indole Production:** Bacteria that make the enzyme **tryptophanase** can hydrolyze the amino acid tryptophan to pyruvate, ammonia (by deamination), and indole (Fig. 36.3). The cell may then extract energy from pyruvate by funneling it into fermentation reactions or cellular respiration. SIM media allows us to assess bacteria for indole production.[2]

[1] The triple sugar iron (TSI) test also reveals information about a bacterium's ability to perform sulfur reduction. We discuss that test in Exercise 37.

[2] Theoretically, any media containing enough tryptophan can be used to detect indole production. SIM is one such media, but tryptone broth is another common media for this application.



FIGURE **36.2** **Sulfur Reduction: H_2S Production from Anaerobic Respiration (Thiosulfate Reductase Reaction)** • Anaerobic respiration with thiosulfate as the final electron acceptor produces hydrogen sulfide (H_2S).

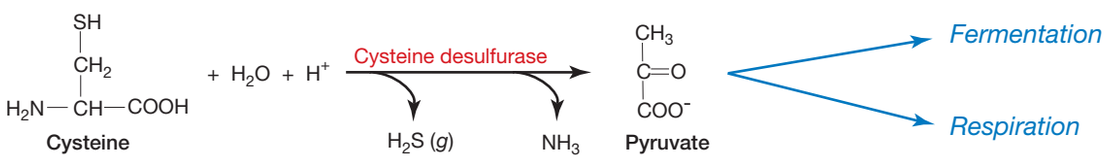


FIGURE **36.1** **Sulfur Reduction: H_2S Production from Cysteine Hydrolysis (Cysteine Desulferase Reaction)** • In putrefaction, cysteine desulfurase catalyzes the hydrolysis of the amino acid cysteine with the production of pyruvate, ammonia (NH_3), and hydrogen sulfide (H_2S).

3. **Motility**: Testing for motility will only reveal if a bacterium can move. This specific assessment does not reveal anything about the presence or absence of an enzyme or a biochemical product, and is therefore not technically considered a biochemical test. Some bacteria move using a single flagellum or multiple flagella; others use gliding or cilia. While we can note a bacterium's motility and occasionally its mechanism of motility by observing it under a microscope, here we review how to use SIM media and general motility agar to test if the bacterium can move. ***Note:*** Neither of these media reveals how the tested bacterium moves (e.g., flagella versus cilia).

Media Formulations and Test Result Interpretations

Let's now review how we can use media to assess sulfur reduction, indole production, and motility. Here we review SIM media *and* general motility agar. That said, your instructor may direct you to only use SIM media or they may include the general motility agar as an additional test to guard against inconclusive motility results that can occur when using SIM media. An inoculating needle is used to stab SIM and general motility agar.

Using SIM Media to Assess Sulfur Reduction

As previously reviewed, there are two main ways that bacteria reduce sulfur. In both cases, bacteria make hydrogen sulfide gas (H_2S). SIM media can detect H_2S production because the media contains iron in the form of ferrous ammonium sulfate. This ingredient combines with H_2S to form ferric sulfide (FeS), an easily visible black precipitate (Fig. 36.4). Therefore, any blackening of the SIM medium is an indication of sulfur reduction and a positive test. If there is no blackening in the SIM media, that indicates sulfur reduction did not occur, so the test result is negative (Fig. 36.5). Table 36.1 summarizes test results and interpretations for the "S" (sulfur reduction) part of SIM media.



FIGURE **36.4** **Indicator Reaction for H_2S Production in SIM Media** • Hydrogen sulfide (H_2S), a colorless gas, reacts with ferrous sulfate in SIM media to generate an easily detectable black precipitate.

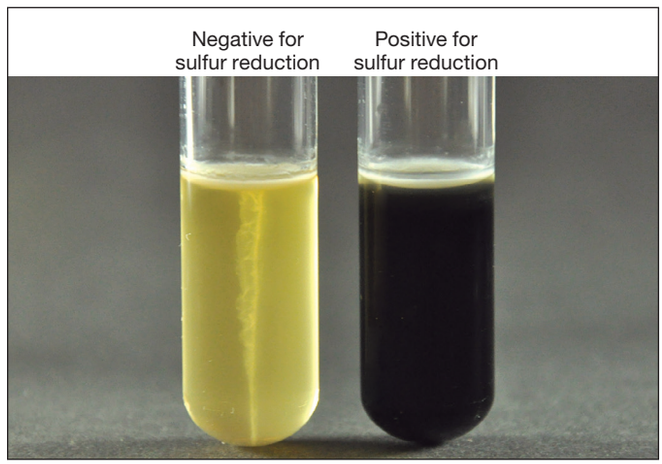


FIGURE **36.5** **Sulfur Reduction in SIM Media** • The lack of a black precipitate for the organism on the left indicates the bacterium did not make H_2S, this is a negative test result. The black precipitate, evident in the tube on the right, indicates the organism reduced sulfur and made H_2S, this is a positive test result.

TABLE **36.1** **Sulfur Reduction (H_2S Production) Test Results and Interpretations** •

Result	Interpretation	Symbol
No black in the medium	Sulfur is not reduced, or is not reduced in detectable amounts	–
Black in the medium	Sulfur reduction occurred (H_2S was made); organism either respires anaerobically with sulfate as the final electron acceptor (thiosulfate reductase) or hydrolyzes the amino acid cysteine (cysteine desulfurase)	+

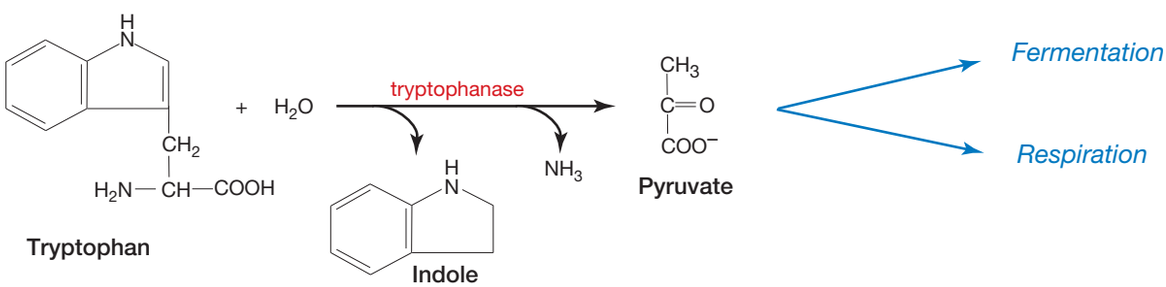


FIGURE **36.3** **Tryptophan Catabolism** • The enzyme tryptophanase breaks down the amino acid tryptophan.

Using SIM Media to Assess Indole Production

Tryptophan hydrolysis in SIM medium can be detected by the addition of Kovac's reagent after the incubation period. Kovac's reagent contains ***p*-dimethylaminobenzaldehyde** (DMABA) and HCl dissolved in amyl alcohol. When a few drops of Kovac's reagent are added to the tube, it forms a layer over the agar. DMABA reacts with any indole present and produces a quinoidal compound that turns the reagent layer red (Figs. 36.6 and 36.7). The formation of red color in the reagent layer indicates a positive reaction and the presence of tryptophanase. The test result is negative if a red color fails to develop after adding Kovac's reagent. Table 36.2 summarizes test results and interpretations for the "I" (indole production) part of SIM media.

TABLE **36.2** **Indole Production Test Results and Interpretations** •

Result	Interpretation	Symbol
No red layer evident after adding Kovac's reagent	Tryptophanase is absent and indole is not produced, or is produced but not in detectable amounts	–
Red layer evident after adding Kovac's reagent	Organism produces tryptophanase and hydrolyzes tryptophan into indole, NH_3, and pyruvate	+

Using SIM Media and General Motility Agar to Assess Motility

SIM agar is a semisolid medium that has a reduced agar concentration (0.35% vs. 1.5% in nutrient agar). Its semisolid formulation allows us to detect motility by looking to see if growth radiated away from the line of inoculation. Observing slightly fuzzy growth in *all* directions away from the line of inoculation is an indication of motility (Fig. 36.8). This should not be confused with the (seemingly) spreading growth produced by lateral movement of the inoculating needle when stabbing. Table 36.3 summarizes test results and interpretations for the "M" (motility) part of SIM media.

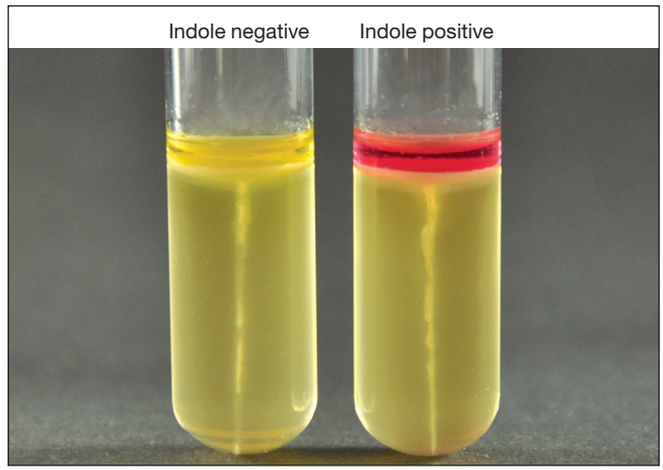


FIGURE **36.7** **Indole Production Test Results in SIM Media** • These SIM tubes were inoculated with an indole-negative organism on the left (no red layer evident after adding Kovac's reagent) and an indole-positive organism on the right (red layer evident upon adding Kovac's reagent). Notice how little Kovac's reagent is used.

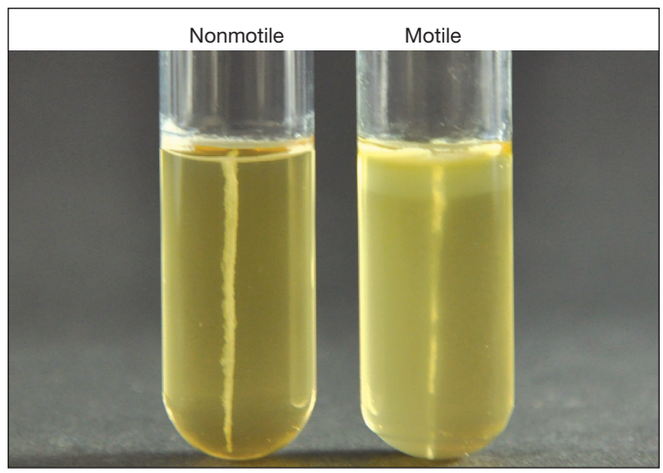


FIGURE **36.8** **Motility in SIM Medium** • Nonmotile bacteria will remain along the line of inoculation. In contrast, for motile bacteria, you should see a haziness in the agar radiating away from the line of inoculation. If results are not interpretable (vague or obscured by the black precipitate indicative of sulfur reduction), try general motility agar.

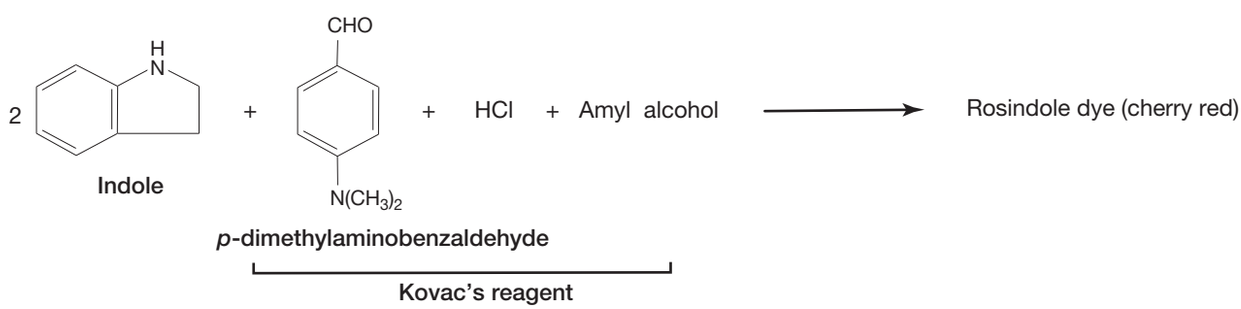


FIGURE **36.6** **Indicator Reaction for Indole Production in SIM Media** • Indole, if present, reacts with the DMABA in Kovac's reagent to produce a red color.

TABLE **36.3** Motility Test Results and Interpretations (SIM media)

Result	Interpretation	Symbol
No growth radiating away from the original inoculation/ stab line	Nonmotile	–
Growth radiating outward from inoculation/stab line	Motile	+
Blackened medium (H_2S produced)	Motility not determined with this media (use general motility agar to assess motility)	ND

TABLE **36.4** General Motility Agar Test Results and Interpretations

Result	Interpretation	Symbol
Red diffuse growth radiating outward from the stab line	Organism is motile	+
Red growth only along the stab line	Organism is nonmotile	–

General motility agar is another media that reveals information about motility. This agar is especially useful in cases where sulfur reduction obscures motility observations in SIM agar. Like SIM agar, general motility agar is a semisolid medium. Unlike SIM media, general motility agar only reveals information about a bacterium's motility.

As with SIM media, general motility agar is inoculated via a single stab with an inoculating needle. Following incubation, motile organisms are observed as a radiating growth that extends outward in all directions from the central stab line. A tetrazolium salt (TTC) is sometimes added to the medium to make interpretation easier. TTC is used by the bacteria as an electron acceptor. In its oxidized form, TTC is colorless and soluble; when reduced, it is red and insoluble (Fig. 36.9). As summarized in Table 36.4, a positive result for motility is indicated when the red (reduced) TTC is seen radiating outward from the central stab; a negative result shows red only along the stab line (Fig. 36.10).

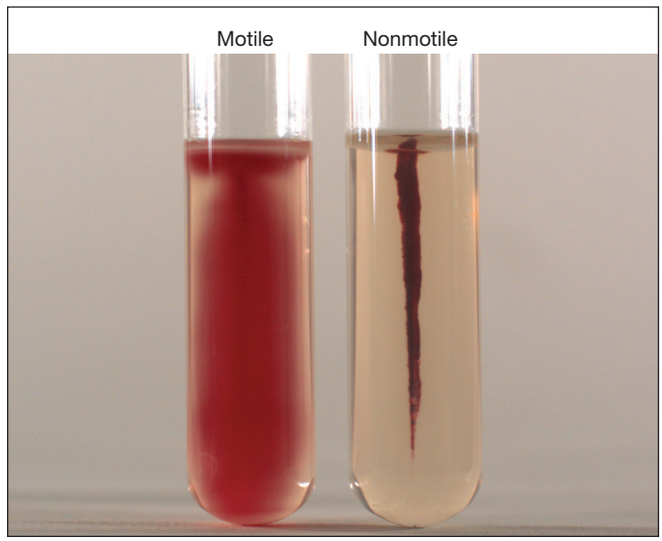
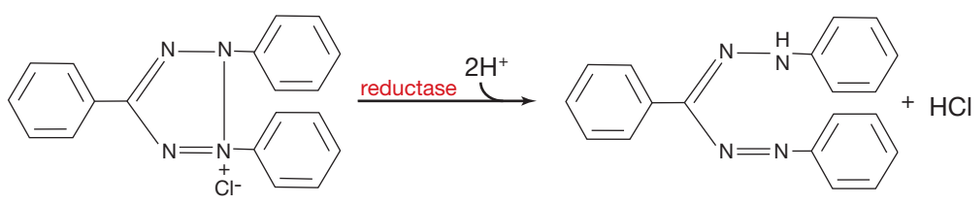


FIGURE **36.10** **Results in General Motility Agar with TTC** • A red color spreading away from the line of inoculation indicates the tested bacterium is motile. If the red color remains isolated to the line of inoculation, then the bacterium is nonmotile.



2,3,5-Triphenyltetrazolium chloride$_{oxidized}$ (TTC$_{ox}$)
colorless and soluble

Formazan$_{reduced}$
red color and insoluble

FIGURE **36.9** **General Motility Agar Indicator Reaction: Reduction of TTC** • When 2,3,5-triphenyltetrazolium chloride (TTC) is reduced, it is converted to formazan, a red insoluble compound. In general motility media, this red compound serves as an indicator of bacterial growth location.

Clinical Applications

SIM medium is used to differentiate a variety of pathogenic bacteria. For example, sulfur-reducing members of *Enterobacteriaceae*, especially members of the genera *Salmonella, Shigella,* and *Proteus* are readily differentiated from H_2S negative *Morganella morganii* and *Providencia rettgeri*.

SIM medium also detects bacteria that can produce indole using the enzyme tryptophanase. The indole test is one component of the IMViC battery of tests (*I*ndole, *M*ethyl red, *V*oges-Proskauer, and Citrate) used to differentiate indole-positive *E. coli* from indole-negative members of the *Enterobacter*, *Klebsiella*, *Hafnia*, and *Serratia* genera.

In addition to the first two functions, SIM can assess bacterial motility, an important differential characteristic of *Enterobacteriaceae*, which are usually motile. Recall that the family *Enterobacteriaceae* includes the genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Serratia*, and *Citrobacter* (all referred to as coliform bacilli) as well as *Proteus*. These genera encompass members of the normal microbiota of the intestinal tract, some of which act as true pathogens that cause a wide range of infections, while others are categorized as opportunistic pathogens. Aside from *Klebsiella* species, other *Enterobacteriaceae* coliforms are motile.

General motility agar is another media that reveals motility information. This media is especially useful if the motility results in SIM media are obscured by the black precipitate generated when H_2S is made.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sulfur-indole-motility (SIM) media tubes (5; 4 to inoculate and 1 as a control)
- ❑ General motility agar tubes (5; 4 to inoculate and 1 as a control)
- ❑ Kovac's reagent
- ❑ Inoculating needles (wire or disposable)
- ❑ Microbial incinerator or Bunsen burner (if not using disposable inoculating needles)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Micrococcus luteus*
  - ☐ *Proteus mirabilis* (BSL-2)[3]
  - ☐ *Streptococcus* species or *Staphylococcus epidermidis*

[3] ***Note to instructor:*** Approximately 75% of *Citrobacter freundii* strains are positive for sulfur reduction and it may serve as a suitable BSL-1 replacement for *P. mirabilis*. *Aeromonas hydrophila* may also work as a substitute.

Media and Reagent Recipes

Sulfur-Indole-Motility (SIM) Medium

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	20.0 g
Peptic digest of animal tissue	6.1 g
Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$)	0.2 g
Sodium thiosulfate ($Na_2S_2O_3$)	0.2 g
Agar	3.5 g
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

Kovac's Reagent

Amyl alcohol	75.0 mL
Hydrochloric acid, concentrated	25.0 mL
p-dimethylaminobenzaldehyde	5.0 g

General Motility Agar

Beef extract	3.0 g
Pancreatic digest of gelatin	10.0 g
Sodium chloride (NaCl)	5.0 g
Agar	4.0 g
Triphenyltetrazolium chloride (TTC)	0.05 g
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

PROCEDURE

6

You will use an inoculating needle (wire or disposable as directed by your instructor) to stab inoculate SIM medium with four organisms demonstrating a variety of results. If your instructor directs you to do so, you will also stab inoculate the same four bacteria onto general motility agar. When reading your SIM test results, make the motility and H_2S determinations before adding the Kovac's (indole) reagent. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** **Follow BSL-2 precautions if using *P. mirabilis*.**

1 **Obtain Your Samples and Media:** Obtain the four bacterial stock cultures that you are going to test and five tubes of each media, SIM and (if directed) general motility agar. Four of the five tubes of each media will be inoculated; one of each media type will remain uninoculated. Label your tubes with the appropriate media name (e.g., SIM or motility), your name, the date, and the sample descriptor (e.g., organism name or uninoculated).

2. **Inoculate the Media:** Use an inoculating needle (disposable or wire) to stab inoculate four tubes of each media type (SIM and motility) with the provided test organisms. Insert the needle into the agar to within 1 cm of the bottom of the tube. Be careful to remove the needle along the original stab line so that you obtain a straight inoculation. Do not inoculate the control(s).
3. **Incubate Your Samples:** Incubate all tubes aerobically at 35±2°C for 24 to 48 hours.
4. **Clean up:** Save or dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples for Sulfur Reduction and Motility:** Before you proceed to the indole test, examine the SIM media for sulfur reduction and motility. If you performed the general agar motility test, then also make your observations for those samples.
3. **Examine Your Samples for Indole Production:** Add Kovac's reagent to each SIM media tube (including the control) to a depth of 2–3 mm (*not mL*). After several minutes, observe for the formation of red color in the reagent layer.
4. **Record Your Result:** Refer to Tables 36.1, 36.2, and 36.3 along with Figures 36.5, 36.7, 37.8, and 36.10 for assistance with interpreting test results. Record your results in the tables provided in the data sheet at the end of this exercise.
5. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 36

DATA SHEET

Assessing Sulfur Reduction, Indole Production, and Motility (SIM Media and General Motility Agar)

During the Lab

1 Using Table 36.1 and Figure 36.5 as guides, record your results and interpretations for sulfur reduction in the following table.

Sulfur Reduction (H_2S Production)			
Organism	**Black PPT** (Y/N)	(+/–)	**Interpretation** (include enzyme)
Uninoculated control			

2 Using Table 36.2 and Figure 36.7 as guides, record your results and interpretations for indole production in the following table.

Indole Production			
Organism	**Red Color ?** (Y/N)	(+/–)	**Interpretation** (include enzyme)
Uninoculated control			

3 Using Table 36.3 and Figures 36.8 and 36.10 as guides, record your results and interpretations for motility in the following tables.

Motility (SIM Media)			
Organism	**Growth Location**	**(+/–)**	**Interpretation**
Uninoculated control			

Motility (General Motility Agar)			
Organism	**Result (location of red TTC)**	**Motility (+/–)**	**Interpretation**

Lab Related Questions

1 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ General motility test agar can reveal if a bacterium uses flagella for motility.

_____ Thiosulfate reductase reduces H_2S.

_____ Sulfur reduction occurs during putrefaction.

_____ SIM media is an example of selective media.

2 The IMViC battery of tests includes:

a. Testing for motility.
b. Testing for indole production.
c. Testing for variable sulfur reduction.
d. Testing for constant sulfur reduction.
e. None of the above.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 36
DATA SHEET
(continued)

3 Choose the true statement(s) about sulfur reduction (select all that apply).

a. If sulfur is reduced, then a red layer is evident upon adding Kovac's reagent to SIM media.
b. Cysteine desulfurase performs sulfur reduction.
c. Sulfur reduction can help differentiate *Shigella* from *Salmonella*.
d. An organism that reduces sulfur will generate a fuzzy growth in SIM media.
e. Sulfur reduction can obscure motility test results in SIM agar.

4 Choose the true statement(s) about general motility agar (select all that apply).

a. This test is not technically a biochemical test.
b. The pH indicator methyl red is present in the media.
c. The test can differentiate members of the genera *Klebsiella* from other coliform genera.
d. The test is performed using semisolid agar.
e. 2,3,5-triphenyltetrazolium chloride serves as a color indicator in this test.

5 Choose the true statement(s) about the indole production test (select all that apply).

a. The test will be positive if the bacterium produces thiosulfate reductase.
b. The test is usually developed before 24 hours of incubation.
c. 2,3,5-triphenyltetrazolium chloride serves as a color indicator in this test.
d. Indole is made as a byproduct of tryptophan catabolism.
e. Kovac's reagent is added to detect indole production.

6 The sulfur reduction test is not able to differentiate H_2S produced by anaerobic respiration and H_2S produced by putrefaction. This inability is the result of:

a. Limited test sensitivity.
b. Limited test specificity.
c. Limited activity of thiosulfate reductase.
d. Limited bacterial growth on SIM agar.
e. Limited activity of cysteine desulfurase.

Critical Thinking

1 Consider the TTC indicator in the general motility agar. Why is it essential that the reduced TTC be insoluble?

2 Which ingredient(s) could be eliminated if SIM media were strictly used for testing indole production? Explain.

— Lab Notes —

EXERCISE 37

Triple Sugar Iron Agar (TSIA)/Kligler Iron Agar (KIA)[1]

After doing this exercise, you should be able to:

1. Describe how to use TSIA and/or KIA to test for a bacterium's sulfur reduction ability.
2. Describe how TSIA and/or KIA help us determine a bacterium's ability to ferment glucose, lactose, and/or sucrose.
3. State the differences between TSIA and KIA.
4. Describe how to perform and interpret TSIA and KIA tests.
5. Discuss why assessing fermentation and H_2S production properties is useful.
6. Perform TSIA and/or KIA tests and interpret the results.

Background

This exercise is performed using either triple sugar iron agar (TSIA) or Kligler iron agar (KIA). Each is an enriched medium that differentiates bacteria (especially enterics, *Enterobacteriaceae*) based on sulfur reduction and sugar fermentation. Leading up to this exercise, we reviewed pathways for carbohydrate fermentation (Ex. 27) and hydrogen sulfide (H_2S) production (Ex. 36). If you wish to review those biochemical processes in more detail, refer to those exercises. However, as a basic reminder, recall that hydrogen sulfide (H_2S) is a common byproduct of sulfur reduction pathways, and acids are common byproducts of carbohydrate fermentation. TSIA and KIA tests rely on detecting these key pathway products (H_2S and acids) to test for fermentation and sulfur reduction in one inexpensive and easily performed test.

Media Formulations

As the name implies, TSIA or *triple* sugar iron agar contains three sugars: lactose, sucrose, and glucose. It also contains animal proteins as carbon and nitrogen sources and sodium thiosulfate as an oxidized sulfur source. Phenol red serves as the pH indicator; it appears yellow at pH less than 6.8 and reddish above pH 7.4, so it readily indicates if acidic fermentation byproducts are present. Ferrous sulfate allows us to determine if the tested bacteria reduce sulfur to make H_2S; if they do, then the generated H_2S reacts with ferrous sulfate to form a black precipitate. KIA is almost the same formulation as TSIA, the difference is that KIA contains two sugars (lactose and glucose) versus the three sugars in TSIA.

Both TSIA and KIA are prepared as a shallow agar slant with a deep butt. This format provides both aerobic and anaerobic growth environments (we'll review the implications of this in the next section). Both media are double inoculated, first by a stab in the agar butt and then by a fishtail streak of the slant.

There are two incubation windows for these tests: one time frame is for detecting carbohydrate fermentation and the second is for assessing H_2S production. Cultures are incubated for18–24 hours before interpreting carbohydrate fermentation. After interpreting the carbohydrate fermentation results, samples are re-incubated for an additional 24 hours before making a final H_2S determination.

Test Interpretations

There are two environmental situations established in the medium, an aerobic environment exists in the slant whereas the butt of the tube provides an anaerobic environment. This difference in oxygen levels, combined with differences in sugar concentrations (10 times as much lactose and sucrose as glucose in the medium), facilitates a differential coloration of the slant and/or butt based on what sugar(s) the bacterium ferments. Many reactions in various combinations are possible. Table 37.1 summarizes the possible color changes you may observe and what each observation indicates in terms of fermentation reactions that occurred and/or H_2S production.

- **Yellow Butt and Red Slant = Glucose Fermentation Only:** When glucose-only fermenters grow on TSIA or KIA, acidic products lower the pH and turn the entire medium yellow within just a few hours. However, if you were to recheck your sample at the recommended 18- to 24-hour mark, you'd see that *only* the butt of the tube was yellow. This phenomenon of the medium's slant changing from red (the original color at inoculation) to yellow (the color after a few hours of incubation) and then *back* to red (the final color at 18–24 hours) is called **reversion**.

[1] This exercise is written for either triple sugar iron agar (TSIA) or Kligler iron agar (KIA) use. In form and function, the two media are virtually identical except KIA includes two sugars versus TSIA's three sugars. The results for TSIA and KIA are read the same way. In results indicating fermentation of more than one carbohydrate, TSIA is reported as *glucose and lactose and/or sucrose fermentation*; KIA (because it contains no sucrose) is reported as *glucose and lactose fermentation*.

The reversion process only occurs in the slant because bacteria metabolize glucose under the aerobic conditions of the slant much faster than they metabolize glucose under the anaerobic conditions deeper in the medium. And because the medium has a low glucose concentration (0.1%), bacteria metabolize most (if not all) of the glucose in the slant within about 12 hours. As the glucose in the slant diminishes, bacteria growing in that region switch to metabolizing proteins in the medium as an energy source. As the bacteria break down amino acids (the building blocks of proteins), ammonia is made. Ammonia is a base, so as it accumulates, it alkalizes the slant (pH increases), restoring the slant to its *original* red color.

In contrast, bacteria grow slowly in the anaerobic environment at the butt of the tube and therefore they do not burn through the glucose as fast as bacteria growing in the aerobic slant environment. Because bacteria growing deep in the medium have plenty of glucose to meet their growth needs, they will not switch to metabolizing proteins for energy. This means bacteria in the butt of the tube will not produce ammonia, so the butt remains acidic and therefore remains yellow. Thus, if your TSIA or KIA sample has a red slant and yellow butt at the 18- to 24-hour mark, then the tested bacteria ferments glucose, but not lactose and/or sucrose (see the tube on the far left of Fig. 37.1). If the bacteria make gas during fermentation, you may see fissures in the medium or the agar may lift slightly off the bottom of the tube (see the tube on the far right of Fig. 37.1).

- **Yellow Slant and Yellow Butt = Glucose *and* Lactose *and/or* Sucrose Fermentation:** Organisms that ferment glucose *and* lactose *and/or* sucrose turn TSIA and KIA yellow throughout. (Refer to Fig. 27.1 for the biochemical relationship between these three fermentations.) Because other sugars in the medium (lactose and sucrose for TSIA/lactose for KIA) are 10 times more concentrated than glucose, these sugars remain plentiful in the slant even after glucose is depleted. Therefore, the bacteria do not need to resort to protein catabolism to survive and can instead continue to ferment sugars throughout the 18- to 24-hour incubation period. This means the slant and butt will remain yellow (Fig. 37.1, far right tube).

  As such, a TSIA sample with a yellow slant and butt at the 18- to 24-hour mark indicates the inoculated organism ferments glucose and at least one (though possibly *both*) of the other two sugars. That is, glucose fermented *and* lactose and/or sucrose also fermented.

For KIA, because there are only two sugars present (glucose and lactose), if the slant and butt are both yellow, you can deduce that both sugars fermented. As described in the previous section, if the bacteria make gas as a byproduct of fermenting any of the sugars, the medium may exhibit fissures or the agar may slightly lift from the bottom of the tube.

- **Red Slant and Red Butt = No Fermentation:** An organism that does not ferment any of the carbohydrates but utilizes peptone and amino acids will alkalinize the medium and turn it red (usually a brighter red than uninoculated medium). If the organism uses the peptone aerobically *and* anaerobically, both the slant and butt will appear a bright red. An obligate aerobe will turn only the slant bright red. The difference between a red butt and a butt unchanged by the organism may be subtle; therefore, comparison with an uninoculated control is always recommended.
- **Black Coloration = H_2S Production (Sulfur Reduction):** Bacteria that reduce thiosulfate present in the medium or break down the amino acid cysteine in the medium's peptone will make H_2S (Figs. 36.1 and 36.2). Ferrous sulfate reacts with any generated H_2S to form a black precipitate that, when formed, is easily detectable in the butt of the tube. Acidic conditions must exist for thiosulfate reduction. Therefore, black precipitate in the medium is an

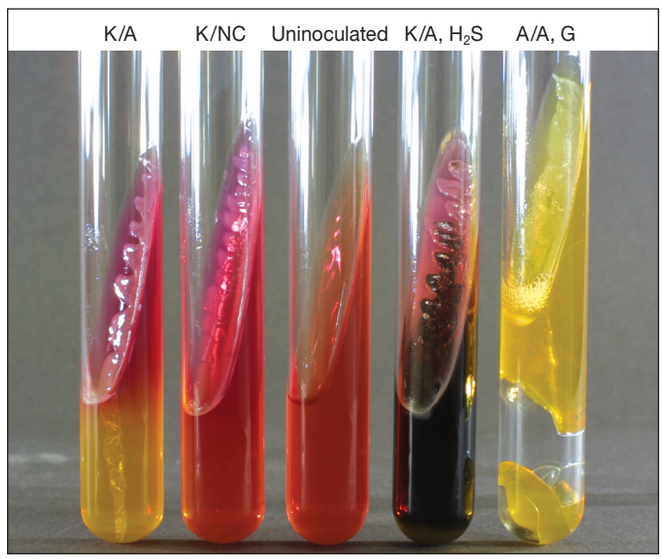


FIGURE **37.1** **Results in Kligler Iron Agar Slants** • These KIA slants from left to right illustrate: alkaline slant/acid butt (K/A); alkaline slant/no change in the butt (K/NC); uninoculated control; alkaline slant/acid butt, hydrogen sulfide present (K/A, H_2S); and acid slant/acid butt, gas (A/A, G). TSIA tubes with the same appearances would be scored the same. Refer to Table 37.1 for interpretations.

indication of sulfur reduction *and* at the very least, glucose fermentation. If the black precipitate obscures the color of the butt, you can reasonably assume the organism fermented glucose. However, you must still note the color of the slant to determine if any other sugars were fermented.

A Note on Test Result Observation Timing

Because we are observing a delicate balance between slant versus butt acidification or alkalinization, timing is critical when reading TSIA and KIA results. An early reading could reveal yellow throughout the medium, leading you to believe that the organism ferments more than one sugar when it simply has not yet exhausted the glucose. A reading after all the sugars have been depleted could reveal a yellow butt and red slant, leading you to falsely believe that the organism is a glucose-only fermenter. Interpreting the H_2S production results too early could lead you to wrongly conclude the organism is unable to form H_2S.

TABLE **37.1** TSIA and KIA Test Results and Interpretations

Result	Interpretation	Symbol
Yellow slant/yellow butt—KIA	Glucose and lactose fermentation with acid accumulation in slant and butt	A/A
Yellow slant/yellow butt—TSIA	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt	A/A
Red slant/yellow butt	Glucose fermentation with acid production; proteins catabolized aerobically (in the slant) with alkaline products (reversion)	K/A
Red slant/red butt	No fermentation; peptone catabolized aerobically and anaerobically with alkaline products; isolate is not from *Enterobacteriaceae*	K/K
Red slant/no change in the butt	No fermentation; peptone catabolized aerobically with alkaline products; isolate is not from *Enterobacteriaceae*	K/NC
No change in slant/no change in butt	Organism is growing slowly or not at all; isolate is not from *Enterobacteriaceae*	NC/NC
Black precipitate in the agar	Sulfur reduction (an acid condition from glucose fermentation exists in the butt, even if the yellow color is obscured by the black precipitate)	H_2S
Cracks in or lifting of agar	Gas production	G

Clinical Applications

Recall that the family *Enterobacteriaceae* is comprised of rod-shaped, Gram-negative bacteria that includes harmless bacteria as well as familiar pathogens (e.g., *Escherichia coli*, *Salmonella*, and others). TSIA and KIA are primarily used to differentiate members of *Enterobacteriaceae* based on sugar fermentation profiles and H_2S production. Many of the bacteria that can ferment sugars in the butt of TSIA and KIA are enterobacteria, so these tests can also help us distinguish *Enterobacteriaceae* from other Gram-negative rods such as *Pseudomonas aeruginosa*, which does not ferment sugars in the butt of the tube.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Triple sugar iron agar (TSIA) and/or Kligler iron agar (KIA) slants (5)
- ❑ Wire inoculating needle and loop or disposable inoculating needle and loop
- ❑ Microbial incinerator or Bunsen burner (if not using disposable inoculating needles)
- ❑ Fresh agar slant cultures of these recommended organisms[2]:
  - ☐ *Escherichia coli*
  - ☐ *Morganella morganii* (BSL-2)
  - ☐ *Pseudomonas aeruginosa* (BSL-2)
  - ☐ *Salmonella typhimurium* (BSL-2)
  - ☐ *Streptococcus* species or *Staphylococcus epidermidis*

6

[2] ***Note to instructor:*** If your lab is not equipped to handle BSL-2 organisms, these will be adequate substitutes for the recommended BSL-2 organisms: *Alcaligenes faecalis*, *Citrobacter freundii* (Hardy Diagnostics #0315P), and *Citrobacter koseri* (Hardy Diagnostics #0106P).

Medium Recipes

Triple Sugar Iron Agar (TSIA)

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	10.0 g
Peptic digest of animal tissue	10.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Sucrose ($C_{12}H_{22}O_{11}$)	10.0 g
Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$)	0.2 g
Sodium chloride (NaCl)	5.0 g
Sodium thiosulfate ($Na_2S_2O_3$)	0.2 g
Agar	13.0 g
Phenol red	0.025 g
Distilled or deionized water	1.0 L

Adjust to pH 7.1–7.5 at 25°C

Kligler Iron Agar (KIA)

Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	10.0 g
Peptic digest of animal tissue	10.0 g
Lactose ($C_{12}H_{22}O_{11}$)	10.0 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Ferric ammonium citrate ($C_6H_5O_7 \cdot Fe \cdot NH_3$)	0.5 g
Sodium chloride (NaCl)	5.0 g
Sodium thiosulfate ($Na_2S_2O_3$)	0.5 g
Agar	15.0 g
Phenol red	0.025 g
Distilled or deionized water	1.0 L

Adjust to pH 7.2–7.6 at 25°C



PROCEDURE

Today, you will inoculate four TSIA or KIA slants. Use a large inoculum and try not to introduce excessive air when stabbing the agar butt. All tubes should be removed from the incubator after no more than 24 hours. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** If you are studying any BSL-2 organisms, follow BSL-2 precautions.

1. **Obtain Your Samples and Media:** Obtain five slants and the sample bacteria that you will inoculate onto the media. Label four of the slants with the names of the organisms, your name, and the date. Label the fifth slant "control."
2. **Inoculate the Media:** Heavily inoculate four slants with the test organisms using an inoculating needle (not a loop). First stab the agar butt, and then streak the slant. Do not inoculate the control. Be sure to properly dispose of the inoculating needle (if a disposable one is used). Otherwise, incinerate the inoculating needle.
3. **Incubate Your Samples:** Incubate all tubes aerobically at 35±2°C for 18–24 hours (no more or less). Be sure the caps are loose enough to allow gas exchange.
4. **Clean up:** Save or dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples).
2. **Examine Your Samples:** Examine the tubes for characteristic color changes and gas production. The proper format for recording results is: slant reaction/butt reaction, gas production, hydrogen sulfide production. For example, a tube showing yellow slant, yellow butt, fissures in or lifting of the agar, and black precipitate would be recorded as: A/A, G, H_2S.
3. **Record Your Result:** Using Table 37.1 as a guide, record your results in the tables provided in the data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 37

DATA SHEET

Triple Sugar Iron Agar (TSIA)/ Kligler Iron Agar (KIA)

During the Lab

1 Using Table 37.1 and Figure 37.1 as guides, record your results and interpretations in the following table.

Organism	Color and Gas Result	Symbol	Interpretation (include enzyme when known)
Control			

Lab Related Questions

1 Assume that after 18 hours of incubation your sample has a black precipitate that obscures your view of the underlying butt color. What is the best course of action?

a. Repeat the test and observe the results at 15 hours.
b. Assume the butt was alkalinized and would have appeared red.
c. Assume the butt was alkalinized and would have appeared yellow.
d. Assume the butt was acidified and would have appeared red.
e. Assume the butt was acidified and would have appeared yellow.

2 Choose the true statement(s) (select all that apply).

a. TSIA is a selective media.
b. KIA does not reveal information about sucrose fermentation.
c. There is more glucose present in TSIA than in KIA.
d. KIA must be incubated longer than TSIA.
e. Sulfur reduction can obscure fermentation results in TSIA, but not KIA.

3 When interpreting the TSIA test, if we observe a yellow slant, then we use the phrasing that lactose and/or sucrose was fermented. Why is this done?

a. Because a test limitation is that we can't know if one or both sugars were fermented.
b. Because if sucrose was fermented, it is likely that lactose was fermented first.
c. Because if lactose was fermented, it is likely that sucrose was as well.
d. Because the media may contain either sucrose or lactose.
e. Because sucrose is derived from lactose fermentation.

4 What is the role of the ferrous sulfate in the media?

a. It selects for the growth of enteric bacteria.
b. It promotes anaerobic fermentation reactions.
c. It serves as an indicator of H_2S production.
d. It serves as a pH indicator.
e. It is added as a nutrient.

5 The term *reversion* as used in the TSIA and KIA tests best describes:

a. The switching of metabolism from lactose fermentation to sucrose fermentation.
b. The switching of metabolism from sucrose fermentation to glucose fermentation.
c. A change in the butt color from red to yellow.
d. A change in the slant color from yellow to red.
e. A change in the slant color from red to yellow.

6 Under what condition(s) may you observe a red butt and red slant of a TSIA tube after 18–24 hours of incubation? (Select all that apply.)

a. Glucose levels were so high in the media that the tested bacterium couldn't ferment it all in 18–24 hours.
b. The organism failed to ferment glucose.
c. The organism fermented lactose, but not sucrose.
d. The pH indictor was not added to the media.
e. The organism failed to ferment lactose and sucrose.

Critical Thinking

1 How would the test results be affected if TSIA medium was prepared with the same concentration of lactose and sucrose as glucose, but the test was still observed at 18–24 hours?

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 37
DATA SHEET
(continued)

2 Your lab partner obtained the following results for TSIA: A/A.
You obtained the following results for KIA: K/A.
Assuming you used the same test organism, what best explains the difference in observed results?

_____

_____

— Lab Notes —

EXERCISE 38

Coagulase Test

After doing this exercise, you should be able to:

1. Describe how the coagulase test is performed.
2. Explain what the coagulase test reveals about a bacterial species.
3. State the applications of the coagulase test.
4. Perform a coagulase test and interpret the test results.

Background

This test detects if bacteria can make **coagulase**, an enzyme that most pathogenic Staphyococcus aureus make. There are two forms of coagulase: **free coagulase** and **bound coagulase** (also called **clumping factor**). Free coagulase is secreted (released from the cell) as an extracellular enzyme whereas clumping factor is attached to the bacterial cell wall.

Upon release into the extracellular environment, free coagulase reacts with **coagulase-reacting factor** (**CRF**), a protein found in plasma (the liquid portion of blood). This interaction triggers a process that resembles the normal blood clot formation mechanism, namely, the soluble protein fibrinogen is converted into an insoluble and highly polymerizable protein, fibrin. As fibrin proteins interact, they form a mesh-like protein network. Normally, this fibrous network would serve as a blood clot scaffold, capturing platelets and red blood cells to stifle bleeding.

However, in this case, the fibrous protein barrier shields the coagulase-producing bacteria from host immune system protections (e.g., elimination by phagocytic white blood cells) and it also promotes biofilm production[1], a situation associated with recurring infections that can be challenging to eradicate due to decreased drug susceptibility. Consequently, coagulase-positive *Staphylococcus aureus* tends to be more virulent (more likely to cause severe disease) than coagulase-negative *Staphylococcus* species.

Bound coagulase generates the same protective fibrin protein barrier as free coagulase, but the mechanisms of barrier generation differ. In contrast to free coagulase, bound coagulase *directly* converts fibrinogen to fibrin without CRF. When bound coagulase reacts with plasma fibrinogen, the precipitated fibrin links the cells to produce visible clumps.

[1] A biofilm is a microbial community of cells attached to a surface via a protective matrix.

Performing and Interpreting the Tube Coagulase Test

There are two forms of the coagulase test: the tube test and the slide test. The tube test detects the presence of either bound or free coagulase whereas the slide test only detects bound coagulase. Both tests utilize rabbit plasma treated with anticoagulant to help reduce the likelihood of false-positive results. The most common anticoagulant added to coagulase tube media is ethylenediaminetetraacetic acid (EDTA), which binds calcium ions thereby interrupting blood clotting unless coagulase is present. In this lab exercise we will only perform the tube test.

To perform a tube coagulase test, we inoculate rabbit plasma with a pure culture of the test bacterium. Plasma coagulation (including any thickening or formation of fibrin threads) within 24 hours is a positive reaction (Fig. 38.1). During the first 4 hours of incubation, it is common practice to periodically observe the inoculated tubes for clotting. If clotting does not occur within the first 4 hours, then samples undergo an overnight incubation, but incubation should never exceed a total of 24 hours. Adhering to the maximum 24-hour incubation is important because after 24 hours, staphylococcal fibrinolytic enzymes may dissolve the fibrin network leading to a false-negative result. Table 38.1 summarizes how to interpret tube coagulase results.

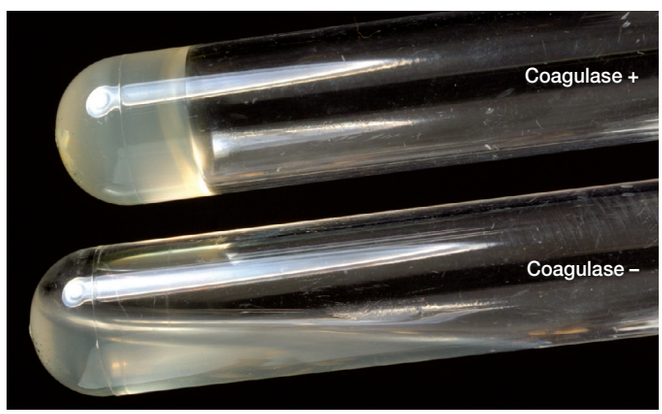


FIGURE **38.1** **Coagulase Tube Test Results in Rabbit Plasma** • Note the liquid nature of the coagulase-negative sample versus the media solidification seen in the case of coagulase-positive bacteria.

TABLE **38.1** Coagulase Tube Test Results and Interpretations

Result	Interpretation	Symbol
Medium solidified within 24 hours or there is evident clumping/coagulation (even if the media did not entirely solidify).	Free or bound coagulase present; plasma coagulated; probable *Staphylococcus aureus*	+
Medium remains liquid after 24 hours; no signs of coagulation are evident	Plasma did not coagulate; coagulase was not detectable; unlikely to be *S. aureus*	–

Clinical Applications

The coagulase test differentiates coagulase-positive *Staphylococcus aureus* from other usually less virulent coagulase-negative *Staphylococcus* species.

Staphylococcus species such as *S. aureus, S. epidermidis,* and *S. saprophyticus* are common members of the normal human microbiome (the collection of microbes that reside in and on humans). These species are most prevalent on the skin and in the upper respiratory tract (especially the nose). When these bacteria remain in their normal microbiome niches, they are not usually problematic. However, if they invade the body (often, though not exclusively, via a skin wound), they can cause disease.

S. aureus causes infections ranging from superficial skin infections like impetigo to deeper infections such as cellulitis (deep skin infection), osteomyelitis (bone infection), and bacteremia (blood infection). Certain *S. aureus* strains also make specialized toxins that lead to toxin-mediated syndromes such as scalded skin syndrome, toxic shock syndrome, and staphylococcal food poisoning.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile rabbit plasma tubes with EDTA (4) (0.5 mL in 12 mm × 75 mm test tubes)
- ❑ Sterile 1-mL transfer pipettes (2)
- ❑ Sterile wooden applicators or disposable plastic loops (6)
- ❑ Sterile saline (0.9% NaCl)
- ❑ Microscope slides (2)
- ❑ Fresh agar slant cultures of these recommended organisms:
  - ☐ *Staphylococcus aureus* (BSL-2)
  - ☐ *Staphylococcus epidermidis*

PROCEDURE

You will perform the coagulase tube test. Read the tube test no later than the following day. It would be most efficient if you inoculate the tube tests as early in your lab period as possible, with the hope that you may observe a positive result before class is over. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** Follow BSL-2 precautions throughout this lab.

1. **Obtain Your Samples:** Obtain three coagulase tubes. Label two tubes with the names of the organisms, your group name, and the date. Label the third tube "control."
2. **Inoculate the Rabbit Plasma:** Using a sterile wooden applicator or disposable loop, inoculate two tubes of rabbit plasma with the test organisms. There should be slightly visible turbidity. Dispose of the stick/loop immediately and properly. Then mix the contents by gently rolling the tube between your hands. Do not inoculate the control.
3. **Incubate the Samples:** Incubate all tubes at 35±2°C for up to 24 hours, checking for coagulation periodically for the first 4 hours or remainder of your lab. Do not exceed 24 hours of incubation. If you do not meet within the next 24 hours, the samples should be removed to a refrigerator until your next lab period.
4. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Remove all tubes from the incubator no later than 24 hours after inoculation.
2. **Examine the Samples**: Examine each tube for clotting of the plasma and record your results on the data sheet at the end of this exercise. Refer to Figure 38.1 and Table 38.1 when making your interpretations.
3. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 38

DATA SHEET

Coagulase Test

During the Lab

1 Refer to Table 38.1 when recording and interpreting your results in the following table.

Tube Test Results		
Organism	**Result**	**Interpretation** (include coagulase type and provisional identification)
Uninoculated control		

Lab Related Questions

1 Why is EDTA included in the medium?

a. It promotes coagulation if coagulase is present.
b. It inhibits coagulation if coagulase is present.
c. It promotes coagulation if coagulase is absent.
d. It inhibits coagulation if coagulase is absent.
e. It serves as an activator of the enzyme coagulase.

2 Which of the following would you expect to make coagulase?

a. All *Staphylococcus* species
b. *Staphylococcus aureus*
c. *Staphylococcus epidermidis*
d. *Staphylococcus saprophyticus*
e. None of the above

3 Choose the false statement(s) about free coagulase (select all that apply).

a. It generates a fibrin matrix by indirectly triggering fibrin polymerization.
b. It generates a fibrin matrix by directly polymerizing fibrin.
c. It is a secreted extracellular enzyme.
d. It is not associated with increased virulence.
e. It is only detectable via the slide coagulase test.

4 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Clumping factor is also known as free coagulase.

_____ Bacteria that make coagulase are typically less virulent than bacteria that lack coagulase.

_____ Coagulase may help bacteria form biofilms.

_____ Bacteria that make coagulase should liquify the test medium within 24 hours.

5 If an organism does not have catalase then (select all that apply):

a. It is not able to cause disease.
b. It will not solidify the test medium.
c. It is likely to be *S. aureus*.
d. It is unlikely to be *S. aureus*.
e. It is unlikely to be found on the skin.

Critical Thinking

1 You and your lab partner each inoculated the rabbit serum media with the same test bacterium. However, their sample appears to manifest clumping by 2 hours whereas yours does not manifest clumping until the 4-hour mark. What is the most likely explanation for this observed time difference?

2 A negative coagulase test, even if performed correctly, does not rule out the presence of *S. aureus*. Why?

EXERCISE 39

Gelatin Hydrolysis (Gelatinase Test)

After doing this exercise, you should be able to:

1. Explain what the gelatinase test reveals about a bacterial species.
2. Describe how to perform and interpret the gelatin hydrolysis test.
3. State the applications of the gelatin hydrolysis test.
4. Perform a gelatin hydrolysis test and interpret the test results.

Background

The gelatin hydrolysis test (or gelatinase test) reveals if a bacterium makes extracellular enzymes called **gelatinases** that can hydrolyze the protein gelatin. Gelatinases are often virulence factors because they can help certain bacteria invade deeper tissues in animals. Gelatin, which is derived from collagen (a fibrous protein abundant in animal connective tissues), is hydrolyzed in two stages (Fig. 39.1). First, gelatinases break down the larger gelatin protein into polypeptides—shorter stretches of amino acids. Next, the polypeptides are further hydrolyzed to release individual amino acids. Bacteria may then import the individual amino acids and further metabolize them as nutrients or use them as building blocks to make proteins.

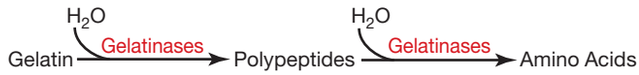


FIGURE **39.1** **Gelatin Hydrolysis** • Gelatinases hydrolyze gelatin to release amino acids.

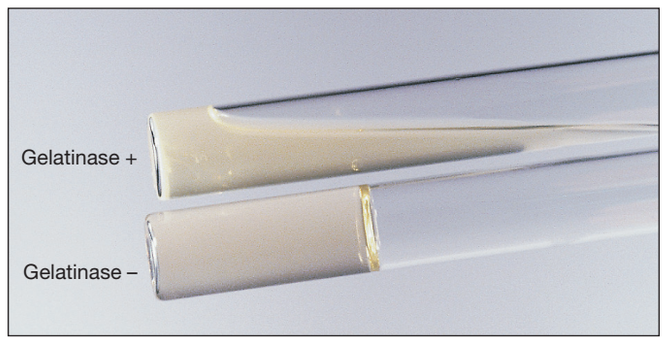


FIGURE **39.2** **Gelatin Hydrolysis Test Results** • Slightly tilting the sample tube reveals if the media is solid or liquid. Before making your observations, confirm that the uninoculated control's media is solid. If it is not, then refrigerate any liquid samples and the uninoculated control until the media for the control is solidified. Gelatinase-positive bacteria will liquefy the media (**top**). Gelatinase-negative bacteria do not liquefy the media (**bottom**).

Gelatin Medium Formulation and Test Result Interpretation

To test for gelatinase, a pure culture of the test bacterium is inoculated into a tube of nutrient gelatin, a test medium composed of gelatin, peptone, and beef extract. Nutrient gelatin differs from most other solid media in that the solidifying agent (gelatin) is also the substrate for enzymatic activity. Consequently, when a tube of nutrient gelatin is stab inoculated with a gelatinase-positive organism, secreted gelatinase (or gelatinases) will liquefy the medium. Gelatinase-negative organisms do not secrete gelatinases; therefore, the medium will remain solid (Figs. 39.2 and 39.3).

A slight disadvantage of nutrient gelatin is that it liquifies at 28°C (82°F). Therefore, inoculated tubes are typically incubated at 25°C (77°F) along with an uninoculated control to verify that any resulting liquefaction is not temperature related. At this lower incubation temperature, a 7-day incubation period is usually sufficient to see liquefaction of the medium. However, gelatinase activity is very slow in some organisms. All tubes still negative after 7 days should be incubated an additional 7 days.

If time is of the essence, you can incubate the samples at 37°C for 2–7 days. However, because this higher temperature will liquify the gelatin medium, an

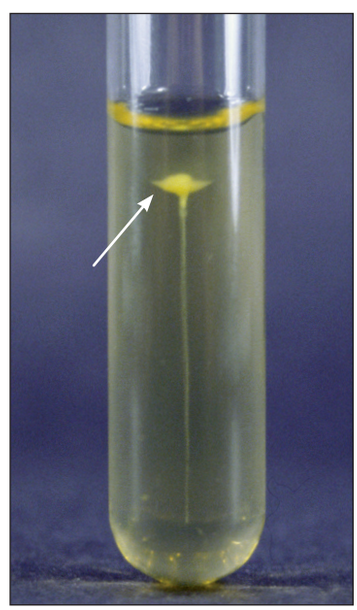


FIGURE **39.3** **Crateriform Liquefaction** • This form of liquefaction also may be of diagnostic use because not all gelatinase-positive microbes liquefy the gelatin completely. Shown here is an organism liquefying the gelatin in the shape of a crater (**arrow**).

uninoculated control must be incubated along with inoculated samples and before making observations, *all* samples must be refrigerated/iced until the uninoculated control solidifies. ***Note:*** Gelatinases usually act at the medium's surface, so shaking the tube while it is warm can generate a false-negative result. Use Table 39.1 to help you interpret your test results.

TABLE **39.1** Gelatin Hydrolysis Test Results and Interpretations

Result	Interpretation	Symbol
Gelatin is liquid (control is solid)	Gelatinase is present.	+
Gelatin is solid	Gelatinase is absent.	–

Clinical Applications

Gelatinases help certain bacteria invade host tissues to establish infection; therefore, these enzymes are important virulence factors for certain pathogens. The gelatin hydrolysis test can help us differentiate *Staphylococcus aureus,* which is gelatinase-positive, from gelatinase-negative *S. epidermidis*. It also helps us to distinguish *Serratia* and *Proteus* species—gelatinase-positive members of *Enterobacteriaceae*—from others in the family, which are usually gelatinase negative. *Bacillus, Clostridium,* and *Clostridioides* species are often gelatinase positive, so this test can help identify important pathogens like *B. anthracis, B. cereus*, *C. tetani*, *C. perfringens*, and *C. difficile*.

6

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Inoculating needles (wire or disposable)
- ❑ Tubes of solidified nutrient gelatin (3)
- ❑ Ice bath or refrigerator
- ❑ Fresh cultures of:
  - ☐ *Bacillus subtilis* or *B. cereus*
  - ☐ *Escherichia coli*

Medium Recipe

Nutrient Gelatin

Beef extract	3.0 g
Peptone	5.0 g
Gelatin	120.0 g
Distilled or deionized water	1.0 L

Adjust to pH 6.6–7.0 at 25°C

PROCEDURE

You will inoculate two nutrient gelatin tubes and incubate them for up to a week. Your instructor may have you incubate samples at 25°C or warmer, depending upon the time constraints for your course and their experiential preferences. To determine if media liquefaction is due to gelatinase activity or a temperature increase, you must include an uninoculated control among the samples. If the media of the uninoculated control is liquid after incubation, then refrigerate the liquified samples until the control is solidified. The control will usually solidify within 15–20 minutes of refrigeration. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

1. **Obtain and Label the Media:** Obtain three tubes of nutrient gelatin. Label two tubes with the name of the organisms that you'll inoculate into each tube, your name, and the date. Label the third tube "control."
2. **Inoculate the Media:** Use an inoculating needle to stab inoculate the appropriate sample tubes. For best results, use a heavy inoculum, an amount of bacteria roughly equivalent to a couple of colonies. Do not inoculate the control.
3. **Incubate Your Samples:** Incubate all tubes at 25°C for up to 1 week. Your instructor may direct you to incubate the samples at a higher incubation temperature (e.g., 35–37°C), in which case you will need to refrigerate the samples and the uninoculated control before making your observations.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

→ Next Lab Session

1. **Obtain Your Samples:** Put on your lab safety gear, and then collect your cultures from the incubator (or wherever your instructor placed your samples). If the uninoculated control is liquified, then all liquified samples must be refrigerated or placed in an ice bath before making observations. If the uninoculated control is solid, the test results for the other samples can be read without refrigeration.
2. **Examine Your Samples:** When the control has solidified (it shouldn't take more than 15–20 minutes, but it must solidify before proceeding), examine the inoculated media for gelatin liquefaction. Carefully tilt the tubes to determine if the media is liquid or solid. Be careful that you do not spill liquified gelatin when tipping the tubes. If you

do spill the media, follow your lab's clean up protocols and disinfect the space before proceeding.

3 **Record Your Result:** Refer to Figure 39.2 and Table 39.1 when interpreting your results. Record your results in the table provided in the data sheet at the end of this exercise.

4 **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 39

DATA SHEET

Gelatin Hydrolysis (Gelatinase Test)

During the Lab

1 Using Table 39.1 and Figure 39.2 for guidance, enter your findings in the following table.

Organism	Observations	Test Result (+/–)	Interpretation
Control			

Lab Related Questions

1 Choose the true statement(s) about the enzyme(s) detected in this test (select all that apply).

a. They are exoenzymes.
b. They are hydrolytic.
c. They are inactive at body temperature.
d. They break down amino acids.
e. They are proteins.

2 You stab inoculate 10 solidified tubes of nutrient gelatin, each with different bacteria, and leave one of the tubes uninoculated. You then incubate each sample at 25°C for a week. Upon making your observations, you notice that all the tubes are liquified, including the uninoculated control. Which of the following is the most likely explanation for the results?

a. The media was prepared with too little gelatin.
b. The media was contaminated during preparation.
c. You used too much inoculum when you inoculated the samples.
d. Each of the tested bacteria make gelatinase.
e. You accidentally inoculated what was supposed to be the uninoculated control.

3 Select the scenarios that could lead to a false-negative test result (select all that apply).

a. You placed the sample in the fridge for a full day.
b. You inoculated a sample twice with the same bacterium.
c. You didn't incubate the samples long enough.
d. You forgot to chill the sample before analyzing the test result.
e. You shook the warm tubes before placing them in the refrigerator.

4 Choose the true statement(s) about the gelatinase test (select all that apply).

a. The media used in the test is selective.
b. It is a differential test.
c. It can help distinguish *Staphylococcus aureus* from *S. epidermidis*.
d. For a positive test result, the media must be fully liquified.
e. The media liquifies at room temperature.

5 Assume you inoculate four tubes of nutrient gelatin with a pure culture of the same bacteria. After 2 days you observe that half of the inoculated samples are partially liquified, but half of your samples remain completely solid. The uninoculated control remained solid. What is the best explanation for your observations?

a. In half of the samples, the tested bacteria mutated and lost the ability to make gelatinase(s).
b. The liquification was due to increased temperature rather than gelatinase activity.
c. The tested bacterium is gelatinase negative.
d. The inoculated tubes that remained solidified were underinoculated.
e. The gelatin media was improperly prepared.

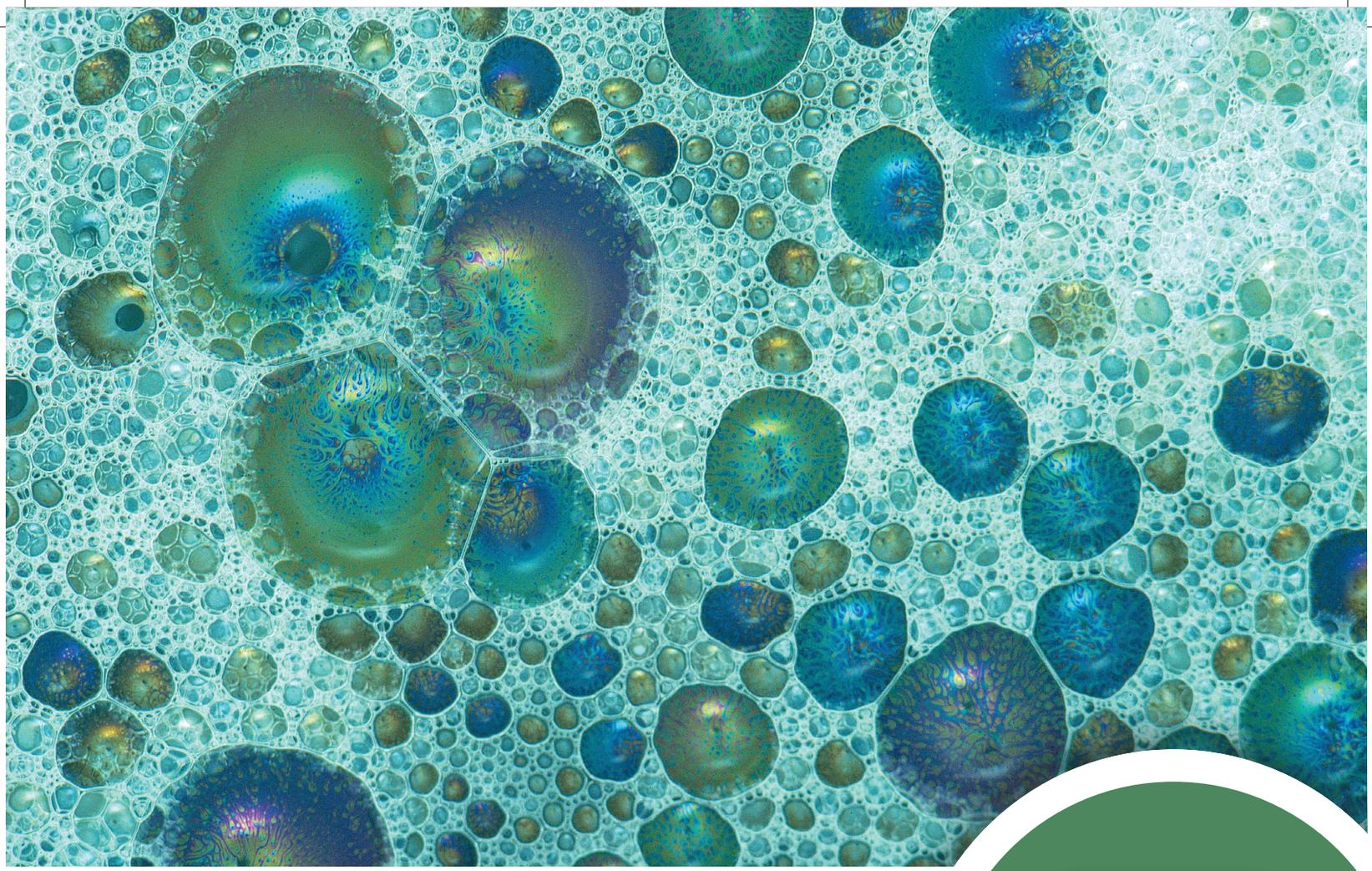
6 Choose the true statement(s) (select all that apply).

a. Gelatinases may allow bacteria to invade host tissues to establish an infection.
b. Gelatinases may allow bacteria to live at warmer temperatures.
c. All pathogens make gelatinases.
d. *Enterobacteriaceae* are usually gelatinase negative.
e. Gelatinases are usually most active at the bottom of the inoculated tube.

Critical Thinking

1 If the control is solid and an inoculated tube is liquid, is it acceptable to read the result before the complete incubation time has elapsed? Explain your reasoning.

2 What effect would doubling the amount of gelatin in the media have on test sensitivity?



SECTION

7

Controlling Bacterial Growth

In This Section

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EXERCISE 41 Effectiveness of Chemical Germicides: The Use-Dilution Test for Disinfectants and Antiseptics **373**

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EXERCISE 44 Bacitracin, Novobiocin, and Optochin Susceptibility Tests **401**

— Lab Notes —

EXERCISE 40

A Comparison of Hand-Cleansing Agents[1]

After doing this exercise, you should be able to:

1. Define sanitization and state how it differs from sterilization.
2. Discuss the differences between disinfectants and antiseptics.
3. Explain the proper method of hand washing for general patient contact.
4. State the goal of antisepsis washing.
5. Describe the difference between microbiocidal and microbiostatic agents.
6. Describe the three tiers of germicides.
7. Outline and evaluate an experimental design intended to illustrate the relative effectiveness of assorted hand-cleansing agents.

Background

Microbiological laboratory techniques and safe health care both depend on sanitization and sterilization procedures. In prior labs, we defined **sterilization** as a process that eliminates all microbial agents from a substance. In contrast, here we discuss the practice of **sanitization**, a process that reduces the number of microbes on a living or nonliving surface. In general, most germicides achieve sanitization; only high-level germicides, which we'll discuss later in this lab, can achieve sterilization.

There are two main categories of germicides: **disinfectants**, which we can use on nonliving surfaces (countertops, equipment, etc.), and **antiseptics**, which we can apply to living tissue such as skin. In this exercise you will compare the relative effectiveness of commercially available *antiseptics* used to sanitize hands (or skin in general) to reduce bacteria levels.

The concept of proper hand hygiene as protective against infections has gone from a controversial beginning (in the early 1800s) to an accepted practice. However, only properly performed hand washing is effective at preventing disease transmission, thus it's important to understand exactly what proper hand washing entails.

What is Proper Hand Washing?

The COVID-19 pandemic brought hand washing into the public health spotlight and led many to wonder how and when to wash up. The U.S. Centers for Disease Control and Prevention (CDC) advised that hand washing should be performed "after being in public and touching an item or surface that may be frequently touched by other people" as well as "before touching your eyes, nose, or mouth."

The hand-washing approach used depends on the goal. For example, hand washing before surgery (surgical scrubbing) to achieve **antisepsis** (a reduction of the normal resident microbiota, the elimination of transient microbes picked up by normal daily functioning, and the blocking of rapid rebound growth of microbes) is far more rigorous than the basic hand washing pursued before and after a general patient encounter. The steps for general patient encounter hand washing are as follow:

1. Wet your hands with cold or warm water.
2. Then, into the palm of your hand, apply the amount of cleansing product the manufacturer recommends.
3. Vigorously rub your hands together for at least 20 seconds. Be sure to cover all surfaces of your hands and wash well between your fingers and up your wrists.
4. Use a disposable towel to dry your hands.
5. Use the same towel that you used for drying to turn off the water.

What Works Best?

In the early stages of the COVID-19 pandemic, there was a shortage of alcohol-based hand rubs to limit transmission of the SARS-CoV-2 virus. This prompted health officials to remind people that proper hand washing with soap and water is just as effective at reducing disease transmission as alcohol-based agents. But the question, "What works best?" remains an interesting topic to explore since so many hand-cleansing agents are commercially available.

In this exercise, you will compare the relative effectiveness of several hand-cleansing agents, each of which has its own appropriate application. Some are for routine household use, such as "washing up before dinner." Others, while available to the public, are best reserved for clinical settings where infection prevention is a matter of life and death. And yet others fall somewhere in between. Figure 40.1 shows examples of products that we test in this exercise.

[1] Thanks to reviewers Johana Melédez-Santiago and Janice Yoder Smith for their suggestions on developing this laboratory exercise.

No matter what the product is, each contains a germicide that is either **microbiocidal** (kills certain microbes) or **microbiostatic** (inhibits the growth of certain microbes).[2] A germicide's decontamination efficacy is ultimately due to its ability to damage and/or inactivate essential cellular components. The more damage the germicide does to the microbial target, the higher the decontamination potential. The cell component targeted could be as small as an enzyme, as large as the entire cytoplasmic membrane, or anything in between.

In general, germicides fall into three categories. High-level germicides destroy all bacteria, fungi, viruses, and low levels of bacterial endospores thereby rendering objects sterile. Intermediate-level agents have the same span of action as high-level agents, but are not effective against bacterial endospores and therefore are not sterilizing agents. Similarly, low-level germicides are not sterilizing compounds; despite destroying most bacteria, fungi, and viruses, they are not effective against bacterial endospores or *Mycobacterium* species.

[2] The suffix "cidal" means killing where as "static" means inhibitory. Thus, an agent that is fungicidal and bacteriostatic will kill fungi, but only inhibit bacterial growth rather than kill bacteria.

Clinical Applications

It is a well-established fact that hand washing reduces disease transmission and good hand hygiene can help *everyone* stay healthier by reducing exposure to bacterial and viral pathogens. However, when it comes to health-care workers, food handlers, and laboratory personnel, proper hand-washing techniques can save lives. Thus, training in how to properly perform hand washing is important.

When health-care workers follow proper hand-washing methods they protect themselves from pathogens found in health-care settings and they protect patients from **health care-associated infections** (**HAIs**), infections patients may develop while receiving treatment for an unrelated medical condition. Similarly, food handlers can limit foodborne illness transmission by frequent and proper hand washing. Lastly, by employing proper hand-washing methods, laboratory workers who handle pathogens (or potential pathogens) can minimize self-inoculation, reduce the risk of spreading infectious agents to others, and lower the chances of culture contamination.



FIGURE **40.1** **Hand-Cleansing Agents** ● From left to right are: Betadine scrub (10% povidone iodine), alcohol-based hand sanitizer (65% ethyl alcohol), chloride-based hand sanitizer (0.13% benzalkonium chloride), antibacterial soap (0.13% benzalkonium chloride), and Hibiclens (4% chlorhexidine gluconate). This exercise uses these agents, but others can be substituted, such as Dawn antibacterial dish soap (0.3% chloroxyenol). If a student has skin sensitivities, they can perform the exercise with just tap water.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Nutrient agar (NA) or tryptic soy agar (TSA) plate (1)
- ❑ Access to a sink and paper towels for hand washing
- ❑ Permanent marking pen
- ❑ Bottles of as many as 5 of the following or suitable replacements*:
  - ☐ Antibacterial liquid soap containing 0.3% chloroxyenol (e.g., Dawn antibacterial dish soap)
  - ☐ Alcohol-based hand sanitizer containing at least 60% ethyl alcohol (ethanol)
  - ☐ An alcohol-free hand sanitizer containing 0.13% benzalkonium chloride (e.g., Hy5 brand of hand sanitizer)
  - ☐ Betadine scrub (10% povidone iodine)
  - ☐ Hibiclens (4% w/v chlorhexidine gluconate)

* Students should not ingest or get any of these products in their eyes. Follow package inserts for agent use. Students should not work with agents for which they have a known allergy or sensitivity.



PROCEDURE

In this exercise, you will evaluate the relative effectiveness of a variety of hand-washing antiseptics. Although you will not wear gloves while sampling your fingers during the first lab session (the session where this lab is set up), you should wear your other safety gear: your lab coat and eye protection. During the second lab session, when you make your growth observations, wear your lab coat, eye protection, *and* gloves. It's important to note that you may have grown a pathogen in this experiment. As such, follow all BSL-2 precautions when handling the plates in your second lab session. To reduce exposure to possible BSL-2 level bacteria, keep all plates *closed* during the second laboratory session.



CAUTION!

The U.S. Centers for Disease Control and Prevention cautions, "Either nonpathogenic or attenuated bacterial strains should be used when possible, especially in teaching laboratories." Before assigning this exercise, faculty should check institutional policies regarding cultivating unknown bacteria from environmental sources and from human subjects. Anytime such sampling is performed, BSL-2 agents that are *not* attenuated could be isolated. Faculty are strongly cautioned to review their institution's policies on students handling such samples. This lab exercise should only be performed in laboratories approved to handle BSL-2 agents.

1. **Obtain and Label the Media:** Label the base of a nutrient agar or tryptic soy agar plate with your name and the date. Then use the marking pen to draw a line down the middle of the plate, thereby dividing the plate in half. Label one half "Before" and the other half "After." Then, write the numbers 1 through 5 on each side of the dividing line so the numbers line up. That is, number 1 on the "Before" side should be opposite number 1 on the "After" side, and so on. Spread the numbers out from one another as much as possible since you will eventually press a finger to each number.
2. **Get Your Hands Dirty:** Sample an area of your choosing by rubbing your left-hand fingertips (all five of them) against the target area. Ideas for sampling include the floor, a tabletop, your backpack, or your forehead.
3. **Inoculate the Medium:** Then, one at a time, gently press your thumb tip next to the "Before" number 1, your index finger next to the "Before" number 2, and so on. Your middle, ring, and pinky fingers are numbered 3, 4, and 5, respectively.
4. **Wash Your Hands**: Wash your hands with soap and water.
5. **Record the Names of the Agent(s) You'll Test for Each Finger:** Use the data sheet at the end of this exercise to record the hand-cleansing agent(s) you will apply to each finger. You will assign a cleansing agent to each finger of your right hand (e.g., thumb-1= triclosan; index finger-2 = alcohol-based hand sanitizer, etc.)
6. **Testing the Cleaning Agent(s)**: Now sample the same site used in Step 2 with the fingertips of your *right* hand, but this time you'll only "dirty" one fingertip at a time. Then, you will rub the dirtied fingertip in a few milliliters of the hand-cleansing agent in your left palm (see Steps a and b for further directions). If the cleansing agent requires rinsing and drying, perform this at a sink. Agents that only require air-drying can be applied/tested at your table.
   a. Place a quarter-size drop of the first hand-cleansing agent in the palm of your left hand. Next, dirty your right thumb by rubbing it on the area to be sampled. Then, rub your dirtied thumb into the cleansing agent for 10 seconds.[3] Once your thumb is dry, gently press it next to the number 1 on the "After" side of your plate. Wash both hands with soap and water, and dry them.
7.

[3] The recommended time for hand washing is about 20 seconds, but because you are only washing the fingertips and concentrating on those, that time has been reduced to see representative results.

b To test the remaining four hand-cleansing agents, repeat Step a using a different finger for each cleansing agent. Be sure to press each finger onto the agar next to its correct number and to wash your hands between each surface sampling step.

7 **Seal and Incubate Your Plate:** Seal your test plate by taping the lid to the base. Then, incubate your plate at 25°C (slightly warmer than average room temperature) until your next lab session.

8 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples**: Examine your plates for growth. (***Note:*** Do not remove the plate's lid unless given permission to do so.) Each colony you see is likely the product of a single cell that was deposited onto the agar. Different microbial species often produce distinctly different colonies, so note colony diversity by inspecting colony features such as color, visual sheen, etc. Refer to Exercise 6 for a review of colony morphology features.

2 **Record Your Observations:** Fill in the table provided at the end of this lab exercise as you assess the relative amount of growth and microbial diversity (based on colony differences) on the "Before" and "After" sides of the plate for each agent. Recognize that you are comparing "apples and oranges" with respect to the intended uses of each cleansing agent. That is, the products tested range from surgical scrubs to over-the-counter soaps intended for household use. A surgical scrub may have better antimicrobial activity than hand soap, but that doesn't make it "better" for household use!

3 **Clean up:** Dispose of the plates as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 40

DATA SHEET

A Comparison of Hand-Cleansing Agents

During the Lab

1 To complete the **relative growth** column in the table provided, use this qualitative scale for evaluation:

+ + + means "a lot of growth"

+ + means "moderate growth"

+ means "little growth"

0 means "no growth"

Note: There is not an absolute cutoff between any of these categories. What you call "moderate growth" might be called "little growth" by another student. Just try to be consistent within your evaluation.

For recording colony **diversity**, look for different colony colors, shapes, textures, sizes, and anything else that is indicative of a different organism growing, and record the number of different organisms present. For the **interpretation** column, describe the relative effectiveness of the tested agent (excellent, good, fair, poor).

Number on Plate	Hand-Cleansing Agent	"Before"		"After"		Interpretation
		Relative Growth	Diversity	Relative Growth	Diversity	
1						
2						
3						
4						
5						

Lab Related Questions

1 Which of the following represents a breach in proper hand-washing protocols for general patient encounters? (Select all that apply.)

a. Failing to wash after the patient encounter.

b. Washing for 10 seconds.

c. Turning on the faucet with a bare hand before hand washing.

d. Turning off the faucet with a bare hand after hand washing.

e. Washing between the fingers and up the wrists.

2 If you were to reuse the same finger for each cleansing agent, which of the following would you most likely observe? (Select all that apply.)

a. The bacterial growth observed for the last agent would be the lowest regardless of the nature of the cleansing agent.
b. The bacterial growth observed for the last agent would be the highest regardless of the nature of the cleansing agent.
c. The bacterial diversity observed for the last agent would be the highest regardless of the nature of the cleansing agent.
d. The bacterial diversity observed for the last agent would be the lowest regardless of the nature of the cleansing agent.
e. You would not observe bacterial growth for any of the cleansing agents.

3 Define the term *microbiostatic*.

4 Define the term *microbiocidal*.

5 Select the true statement(s) (select all that apply).

a. The less damage that a germicide does to a cell, the higher the decontamination potential.
b. Hand washing reduces HAI transmission.
c. A bactericidal agent will lead to less bacterial growth on the experimental plates in the exercise as compared to a bacteriostatic agent.
d. A bactericidal agent is less likely to kill bacteria than a bacteriostatic agent.
e. If hand washing with soap and water is properly performed, it is just as effective at reducing disease transmission as alcohol-based hand sanitizers.

6 Select the true statement(s) (select all that apply).

a. Sterilization is the removal of all microbial agents from a substance.
b. Antisepsis washing achieves sterilization.
c. Sanitization is the same as sterilization.
d. High-level germicides can sterilize a surface.
e. Hand washing does not achieve sterilization.

7 Which of the following would be appropriate for use on a living surface? (Select all that apply.)

a. Germicides
b. Disinfectants
c. Antiseptics
d. Microbiocidal agents
e. Microbiostatic agents

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 40
DATA SHEET
(continued)

8 Select the false statement about antisepsis washing.

a. It leads to a permanent reduction in normal microbiota levels.
b. It removes transient microbiota from the hands.
c. It is a form of hand sanitization.
d. It is performed before surgery.
e. It blocks the rapid rebound growth of microbes on the area treated.

9 Which of the following would you expect for a low-level germicide? (Select all that apply.)

a. It would destroy bacterial endospores.
b. It would not achieve sterilization.
c. It would not eliminate *Mycobacterium tuberculosis* from a surface.
d. It would eliminate most viruses.
e. It would achieve sanitization.

Critical Thinking

1 What other variables besides the cleansing agent might be responsible for any differences noted between fingers or students?

2 Do some research and fill in the following table. Your answer to "Antibacterial Effect" should address the structural and/or functional part of the cell affected, as well as whether the agent is bactericidal (kills bacteria) or bacteriostatic (stops bacterial growth, but doesn't kill bacteria). Under "Other Antimicrobial Effects," note any antiviral, antiprotozoal, and/or antifungal properties of the agent.

Active Ingredient	Antibacterial Effect	Other Antimicrobial Effects
Chloroxyenol		
Isopropanol/ethanol (isopropyl/ethyl alcohol)		
Benzalkonium chloride		
Povidone iodine scrub		
Chlorhexidine gluconate		

— Lab Notes —

EXERCISE 41

Effectiveness of Chemical Germicides: The Use-Dilution Test for Disinfectants and Antiseptics[1]

After doing this exercise, you should be able to:

1. Define the term *germicide*.
2. Define the term *disinfectant*.
3. Define the term *antiseptic*.
4. Describe the application and theory behind this procedure.
5. Perform and interpret the results of this procedure.

Background

As reviewed in Exercise 40, **germicides** are substances that reduce microbial levels on surfaces. They include **disinfectants** for use on nonliving surfaces (e.g., countertops) and **antiseptics** for use on living surfaces (e.g., skin). Also, we reviewed that based on their degree of efficacy, there are three general categories of germicides: high-level, intermediate-level, and low-level agents. Of the three germicide tiers, high-level germicides are the most effective against bacterial endospores, dormant structures that certain spore-forming bacteria such as *Bacillus*, *Clostridioides*, and *Clostridium* genera make. Before a new substance is marketed, the Food and Drug Administration (FDA) and the environmental protection agency (EPA) require that the agent is tested and classified according to its effectiveness against pathogens. The Use-Dilution Test, published by the Association of Official Analytical Chemists (AOAC), is one of many commonly used tests for this purpose.

The Use-Dilution Test is a standard procedure used to measure the effectiveness of disinfectants specifically against *Staphylococcus aureus*, *Salmonella enterica* serovar Choleraesuis, and *Pseudomonas aeruginosa*. In the standard procedure, glass beads or stainless-steel cylinders coated with living bacteria are exposed to varying concentrations (dilutions) of test germicides and then transferred to a growth medium.

After an incubation period, the medium is examined for growth. If a solution prevents microbial growth at least 95% of the time, then it meets the required standards and is considered a usable dilution of that germicide for a specific application. Today's exercise is an adaptation of this method.

The Experimental Process

Today you will examine the effectiveness of four germicides: two common household disinfectants (bleach and Lysol Brand II Disinfectant) and two over-the-counter antiseptics (hydrogen peroxide and isopropyl alcohol). The standard test bacteria are BSL-2 agents, but here you'll perform the protocol with BSL-1 bacteria: *Staphylococcus epidermidis* and *Escherichia coli*.

First, you will coat presterilized ceramic or glass beads with bacteria. You will then expose the coated beads to three concentrations of your assigned germicide. After germicide exposure, you will use the treated beads to inoculate sterile nutrient broth samples. If all the bacteria on the bead are killed during exposure to the germicide, the broth inoculated with that bead will remain clear. If any of the bacteria survive the germicide exposure, they will reproduce during incubation and turn the broth turbid (cloudy). Based on your observations, you will be able to determine the effective concentration (dilution) of your assigned germicide.

The tasks for the exercise are divided among eight student groups (or a convenient number for your lab size). Each group will be responsible for one organism and three dilutions of a specific germicide. Refer to Table 41.1 for your group assignment.

TABLE **41.1** Group Assignments

Germicide	*Staphylococcus epidermidis*	*Escherichia coli*
Bleach	Group 1	Group 2
Lysol	Group 3	Group 4
Hydrogen peroxide	Group 5	Group 6
Isopropyl alcohol	Group 7	Group 8

Finally, this is an interesting exercise with a moderate amount of work involved. If you take your time and carefully apply your aseptic techniques, you will be rewarded with reliable data.

[1] This procedure is modified from its original form and should therefore only be used for instructional purposes.

Clinical Applications

The procedure presented is a modification of the standard Use-Dilution Test protocol used to test the effectiveness of germicides against *Staphylococcus aureus*, *Salmonella enterica* serovar Choleraesuis, and *Pseudomonas aeruginosa*. These are all pathogens that can be found in health-care settings. Knowing what germicides work best against a specific pathogen and knowing the necessary concentrations for maximal efficacy is important to promote effective sanitization procedures. Guarding patient health relies heavily on proper sanitizing of medical equipment and clinical spaces.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Timer or stopwatch
- ❑ 100 mL flask of sterile deionized water
- ❑ Concentrations (3) of one germicide (listed in Table 41.1)
- ❑ Sterile 60 mm petri plates (5)
- ❑ Sterile glass 100 mm petri plate containing filter or bibulous paper (1)
- ❑ Container of sterile ceramic or glass beads* (1)
- ❑ Sterile transfer pipette
- ❑ Sterile nutrient broth (NB) tubes (7)
- ❑ Needle-nose forceps (or appropriate device for aseptically picking up beads)
- ❑ Small screw-cap jar with alcohol (for flaming forceps)
- ❑ Small beaker containing 10 mL of disinfectant (for broth culture disposal)
- ❑ Fresh broth cultures of these recommended organisms (only 1 per group):
  - ☐ *Escherichia coli*
  - ☐ *Staphylococcus epidermidis*

  Disinfectants
  - ☐ 0.01%, 0.1%, and 1% household bleach
  - ☐ 25%, 50%, and 100% Lysol Brand II Disinfectant

  Antiseptics
  - ☐ 0.03%, 0.3%, and 3% hydrogen peroxide (3% is full strength as purchased at the pharmacy)
  - ☐ 10%, 30%, and 50% isopropyl alcohol (70% or 90% is full strength as purchased at the pharmacy)

* Sterilized #8 seed beads from a craft store will work for this purpose.

PROCEDURE

Timing is important in this procedure. Read through the entire procedure before you start. You need to develop a plan with your group *before* you begin your transfers. Having a plan will make it more likely that you'll have uniform soaking times and processes consistent with what other groups are doing. During the lab exercise, you should wear your lab coat, eye protection, and gloves.

1. **Obtain and Label the Media:** As shown in the diagram in Figure 41.1 (Step 1) obtain seven tubes of broth media and label each with your group name/number. Label three of the broths "Broth #1, #2, and #3" and four broths "Control #1, #2, #3, and #4."
2. **Obtain Your Stock Culture:** Your group was assigned a specific bacterium to test, either *Staphylococcus epidermidis* or *Escherichia coli*. Obtain the stock culture and note the name of the organism here: _____.
3. **Set up Your Treatment Stations:** Your group was assigned a specific disinfectant or antiseptic to test. Write down the name of the agent your group will test: _____. Now, obtain five sterile 60 mm petri plates. Label three of the plates with the three test dilutions of your germicide. Label the remaining two plates "Sterile Water." Add enough of each germicide concentration to its respective plate (Plates #1 through #3) to cover the beads (approximately 15 mL). Add an equal volume of sterile water into Plates #4 and #5.
4. **Set up Controls 1 and 2:** Per Figure 41.1 (Step 2), carefully mix your bacterial culture until uniform turbidity is achieved. Take care not to splash into the cap. Use an inoculating loop to transfer a loopful of bacteria from the stock sample into Control Tube #2. Control Tube #1 is your uninoculated control, so just set it aside along with the inoculated Control Tube #2.
5. **Coat the Beads with Bacteria:** Per Figure 41.1 (Step 3), alcohol-flame your forceps and aseptically drop six beads into the stock culture. (You will only use four beads; the other two are extras in case you drop one during the procedure.) After the beads have soaked for 1 minute, decant the liquid portion into a beaker of disinfectant. Remove as much of the broth as possible without losing the beads in the disinfectant. Then, so that the beads can dry, carefully pour the beads onto a piece of filter paper in a sterile 100 mm petri plate. Allow the beads to air-dry for 10 minutes.

CAUTION!

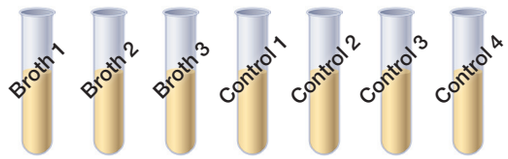
Store your forceps in the alcohol jar between transfers. When it is time to make a transfer, remove them from the alcohol. Then pass them through the flame to burn off the alcohol, holding them away from the alcohol jar while doing so. If the alcohol jar catches on fire, smother the flame with the lid. When finished, return the forceps directly to the jar without flaming.

6. **Treat the Beads:** Transfer one bacteria-coated bead from Step 5 to each treatment as shown in Figure 41.1 (Step 4A). Then, as shown in Figure 41.1 (Step 4B), re-flame the forceps and transfer a sterile bead (not a bacteria-coated bead) to a separate plate of sterile water that has not been used to treat bacteria-coated beads (Plate #5 in your set up). Allow the beads to soak in their respective treatments for 10 minutes.
7. **Add the Treated Beads to Your Broth Tubes for Incubation:** As shown in Figure 41.1 (Step 5), use flame-sterilized forceps to transfer each bead to a separate tube as indicated.
8. **Incubate the Tubes:** Once all the beads have been transferred to their respective broth tubes, incubate all seven tubes at 35±2°C for 48 hours.
9. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

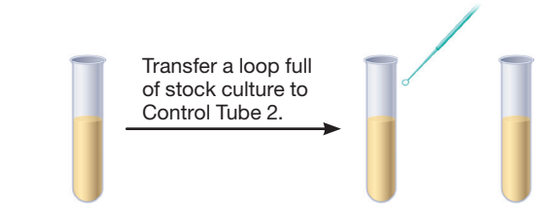
Next Lab Session

1. **Examine the Samples:** Remove all broth tubes from the incubator. Gently mix Control #1 and Control #2 and examine them for evidence of growth. Enter your results on the data sheet at the end of this exercise. (***Note:*** Control #1 should have no growth and Control #2 should show turbidity. If both conditions are met, you may proceed with your comparison. If not, see your instructor.)
2. **Record Your Observations:** Using Controls #1 and #2 as comparisons, examine broths containing beads exposed to germicide (Broths #1, #2, and #3) and examine Controls #3 and #4. Using "G" to indicate growth and "NG" to indicate no growth, enter your results in both the individual data table and the class data table on the data sheet at the end of this exercise.
3. **Record Data from Other Groups:** Your instructor will provide you with a means to share data obtained by all eight groups. Record these data on your data sheet at the end of this exercise.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

1. Label 7 tubes with your group name and the labels as shown.



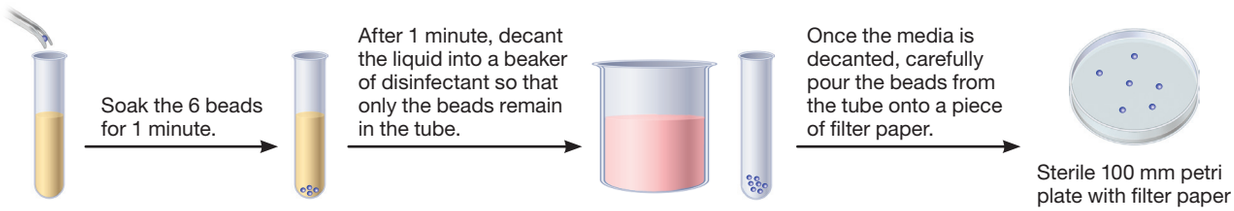
2. Mix your stock culture and inoculate Control 2. Also, set Control 1 aside as your uninoculated control.



Set Controls 1 and 2 aside until it is time to incubate all the tubes.

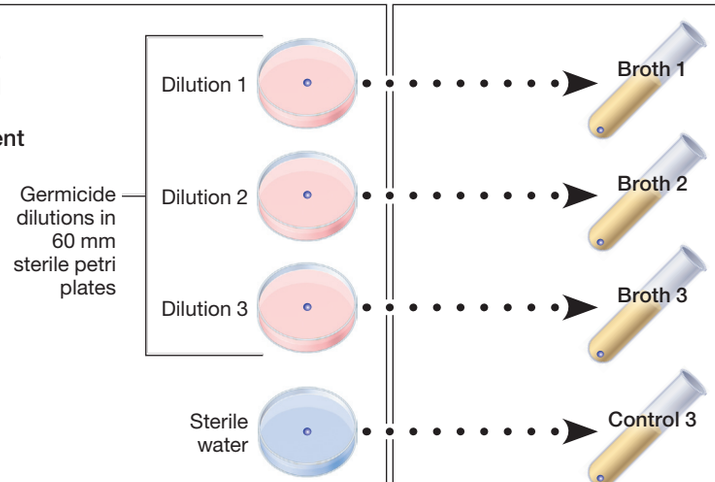
3. Coat the pre-sterilized beads with bacteria and then transfer them to filter paper to dry.

Use flame-sterilized forceps to add 6 sterile beads to the stock culture. You'll only use 4 beads, but the others are in case you drop a bead.

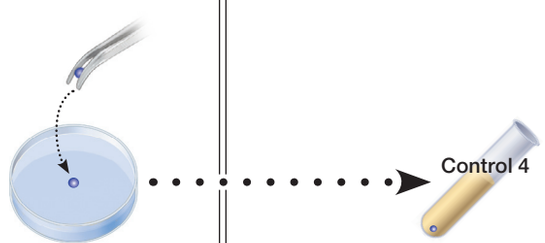


Once the 6 beads are on the paper, let them air-dry for 10 minutes. Four of these beads will be transferred to treatments (germicide dilutions and sterile water) in the next step.

4A. Flame-sterilize the forceps and transfer one bacteria-coated bead from the prior step to each treatment as shown.



4B. Re-flame the forceps to sterilize them. Then transfer a *sterile bead* (not a bacteria-coated bead) to a separate plate of sterile water that has not been used to treat bacteria-coated beads.



Once all beads are in their designated treatment, set the timer for 10 minutes.

5. After treating the beads in germicide or water for 10 minutes, use flame-sterilized forceps to transfer each to a separate tube as indicated.

NOTE: **Flame-sterilize the forceps between every bead transfer.**



Incubate all tubes (including Controls 1 and 2 from Step 2) at 35°C for 48 hours.

FIGURE **41.1** **Procedural Diagram for Chemical Germicides**

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 41

DATA SHEET

Effectiveness of Chemical Germicides: The Use-Dilution Test for Disinfectants and Antiseptics

During the Lab

1 Enter your individual data in the following table.

Controls	Growth (G) or No Growth (NG)
#1	
#2	
#3	
#4	

2 Enter your data as well as other group data in the following table. Use "G" for "Growth" and "NG" for "No Growth."

Organism	Household Bleach			Lysol Brand II Disinfectant			Hydrogen Peroxide			Isopropyl Alcohol		
	0.01% Broth 1	0.1% Broth 2	1% Broth 3	25% Broth 1	50% Broth 2	100% Broth 3	0.03% Broth 1	0.3% Broth 2	3% Broth 3	10% Broth 1	30% Broth 2	50% Broth 3
S. epidermidis												
E. coli												

Lab Related Questions

1 Select the true statement(s) about antiseptics (select all that apply).

a. Disinfectants can be used as antiseptics.

b. Antiseptics are germicides.

c. Antiseptics may be used on skin.

d. The same antiseptic may be effective against one microbial species at one concentration but require a different concentration to be effective against a different species.

e. Bleach is considered an antiseptic.

2 Which of the following should lack growth? (Select all that apply.)

a. Control #1
b. Control #2
c. Control #3
d. Control #4

3 A student failed to allow the coated beads to dry before transferring them to the germicide treatment. Which of the following experimental outcomes would be *most* likely to occur due to this error?

a. Moisture from the beads would overly dilute the germicides and render the treatments ineffective for all the tested samples.
b. None of the germicide concentrations would appear to be effective because the bacterial levels on the beads would be too high.
c. The moisture would prevent the germicide from interacting with the bacteria on the bead thereby leading us to conclude the germicide was less effective than it truly is.
d. The bacteria would wash off the beads as soon as they were placed in the germicide leading to less growth even if the germicide was not effective against the test bacteria.
e. An effect of this error is unlikely to occur.

4 Select the false statement about the Use-Dilution Test.

a. All the beads are sterile at the start of the test.
b. The test bacteria used in the exercise are the same as used in the standard protocol.
c. The test is used to measure the effectiveness of disinfectants.
d. The test requires use of a pure bacterial culture.
e. The test helps manufacturers comply with FDA and EPA standards.

5 Which of the following scenarios would you be *most* likely to encounter if you forgot to flame-sterilize the forceps between transferring each bead to the tubes of broth?

a. Broth Tube #2 may exhibit growth even if Broth Tubes #1 and #3 did not.
b. Broth Tube #2 may exhibit growth even if Broth Tube #3 did not.
c. Broth Tube #3 may exhibit growth even if Broth Tube #1 did not.
d. Only Control Tube #2 would exhibit growth.
e. All the control tubes would exhibit growth.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 41
DATA SHEET
(continued)

Critical Thinking

1 Compare your results with the class data.

a. Which germicide was most effective and at what concentration? Defend your choice.

b. Which was least effective? Defend your choice.

2 Which organism seemed to be more resistant to the germicides?

— Lab Notes —

EXERCISE 42

The Effect of Ultraviolet Radiation on Microbial Growth

After doing this exercise, you should be able to:

1. Describe some basic principles regarding UV light and state why UV-C is germicidal.
2. State some pros and cons of UV decontamination methods.
3. Discuss the application and theory behind this procedure.
4. Perform and interpret the results of this procedure.

Background

In Exercises 40 and 41, we reviewed chemical germicides, disinfectants, and antiseptics as agents that help us reduce microbial levels. In this exercise, you will examine control of microbial growth by physical means. Examples of physical microbial control include temperature changes (heat/cold), radiation, and filtration. Here we will illustrate the germicidal effects of ultraviolet (UV) radiation.

UV radiation is a type of **electromagnetic energy**. Like all electromagnetic energy, UV travels in waves and is distinguishable from all others by its **wavelength**. Wavelength is the distance between adjacent wave crests and is typically measured in nanometers (nm) (Fig. 42.1). Based on wavelength, ultraviolet light is divided into three groups:

1. UV-A, the longest wavelengths, ranging from 315 nm to 400 nm;
2. UV-B, wavelengths between 280 nm and 315 nm; and
3. UV-C, wavelengths ranging from 100 nm to 280 nm.

Of these three categories, UV-C is the most detrimental to microbes. Bacterial exposure to UV-C (specifically 240–280 nm) for more than a few minutes usually results in irreparable DNA damage and cell death. A few minutes of UV-C exposure also tends to irreversibly damage viral genetic material, making these disinfection methods broadly useful to protect against viral contamination. For example, during the earlier stages of the COVID-19 pandemic, N95 respirator masks were in short supply, so many health-care workers periodically placed their masks under UV light so they could get multiple uses out of what would typically have been a single-use mask. The germicidal effect of UV-C is related to time of exposure, lamp intensity, and distance to the target (intensity *decreases* as distance from the source to the target *increases*). The UV

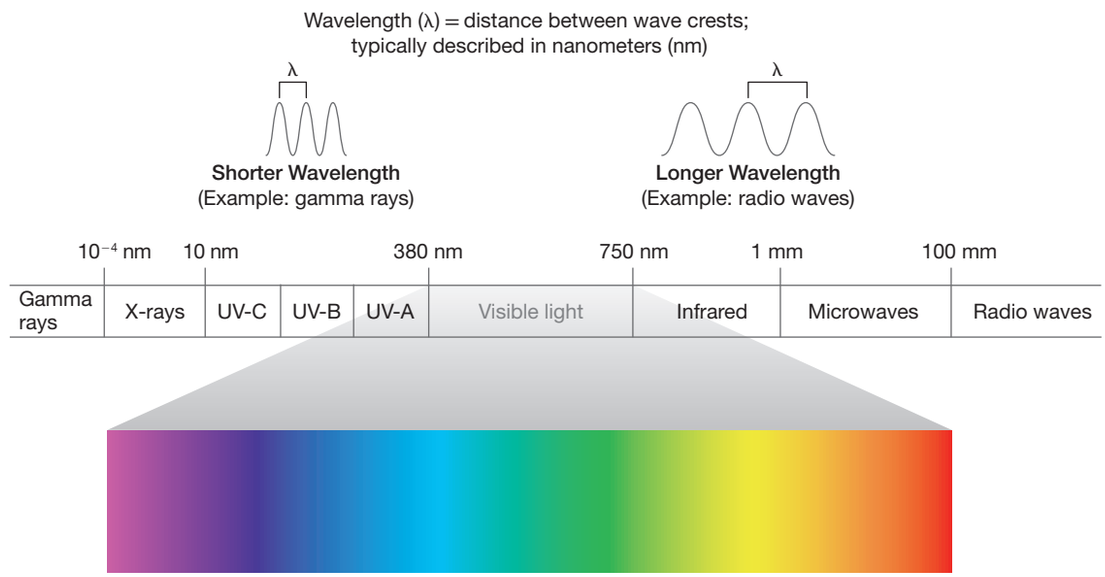


FIGURE **42.1** **Electromagnetic Spectrum** ● Gamma rays have the shortest and highest energy wavelengths (λ less than 10^{-4} nm). In contrast, radio waves have long wavelengths that can be 1 kilometer or longer. Between about 100 nm and 380 nm (just shorter than visible light) is the portion known as ultraviolet light.

light must also have "line of sight" to the surface being decontaminated. That is, UV-C doesn't penetrate materials well nor does it readily bend around corners or trickle into crevices. This means substances such as dust, organic matter, or any other material that may shield microbes from direct exposure to the UV-C waves will diminish the disinfecting efficacy of a UV treatment.

Lastly, the exposure time and intensity of the UV treatment must be optimized for the microbes being targeted. For example, a UV treatment that may eliminate certain viruses may not destroy bacterial endospores, hearty structures that are challenging (though not impossible) to destroy via chemical and physical methods. Taken together, this means that UV-C as a germicidal agent has its limited applications. However, the upsides of UV-C as a decontamination measure are also worth noting. Namely, UV-C decontamination approaches are inexpensive, these measures do not require the storage of chemicals that can expire or spill, and UV-C can decontaminate air and water without introducing chemicals into the environment.

The Experimental Process

The tasks for the exercise are divided among six student groups (or a convenient number for your lab size). Each group will be responsible for exposing one organism to UV light for specific time points. Refer to Table 42.1 for your group assignment. ***Note:*** Two *Bacillus subtilis* cultures are used in this experiment, one is a fresh culture and the other is 1 week old. As a reminder, *Bacillus* species can form endospores when stressed. Older cultures are more likely to have undergone sporulation than fresh cultures. Therefore, as you perform the experiment and analyze the results, think about the susceptibility of these two cultures to UV-C treatments.

Clinical Applications

Ultraviolet light is useful for disinfecting laboratory and health-care environment work surfaces and surrounding air. It is also used to treat water and food packaging to reduce water and foodborne illnesses.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Short wavelength ultraviolet lamp (UV-C) with appropriate shielding and support
- ❑ Cardboard to cover plates (see Fig. 42.2)
- ❑ Tryptic soy agar (TSA) plates (4 per group; 8 for group 1)
- ❑ Stopwatch or electronic timer
- ❑ Sterile cotton swabs (4 per group; 8 for group 1)
- ❑ Fresh broth cultures of these recommended organisms (only 1 per group):
  - □ *Bacillus subtilis*
  - □ *Bacillus subtilis* 7-day culture
  - □ *Deinococcus radiophilus*
  - □ *Escherichia coli*

PROCEDURE

Today you will compare the effect of UV exposure on four cultures: *Bacillus subtilis* (24-hour and 7-day cultures), *Deinococcus radiophilus,* and *Escherichia coli*. Because of the large number of plates to be treated, the work will be divided among six groups of students. Refer to Table 42.1 for assignments. ***Note:*** If the class doesn't divide conveniently into six groups, then the groups assigned to the shorter exposure times can do more than one set of plates. During the lab exercise, you should wear your lab coat, eye protection, and gloves.

1. **Setting up:** Enter your group number and exposure time (from Table 42.1) on the data sheet at the end of this exercise. Then, obtain four TSA plates and label the bottom of each with the names of the organisms to be inoculated and your group number. Draw a line to divide the plates in half, and label the sides "A" and "B."
2. **Obtain Your Stock Culture:** Your group was assigned a specific bacterium to test. Obtain the stock culture and note the name of the organism here:

   _____.
3. **Inoculate the Plates:** Dip a sterile cotton swab into the broth culture and wipe the excess on the inside of the tube so that the swab is not dripping wet. Inoculate the appropriate plate by spreading the organism over the entire surface of the agar. Do this by streaking the plate surface completely three times, rotating it by one-third between streaks. There should be no gaps between streaks. When incubated, this will form a bacterial lawn. Inoculate each of your four plates in this manner.

4 **Arranging Inoculated Plates for UV-C Treatment:** Per Figure 42.2, for each UV time point, you will arrange inoculated plates under the UV lamp with the agar surface exposed. Set the lids of each plate open side down on a disinfectant-soaked towel. You'll put the lids back on the plates after completing the UV treatment. Once you have all plates arranged, place a piece of cardboard over the plates so that Side B of each plate is shielded.

5 **Turn on the UV Lamp:** Be sure the UV lamp is the same distance from the plates for all exposures (we recommend between 8 and 12 inches). Then, turn on the lamp for the prescribed time. ***Caution!*** Be sure the protective shield is in place and do not look at the light while it is on!

6 **Incubate the Plates:** After the UV treatment is finished, turn off the UV light and immediately replace the lids for each plate. Then invert the plates and incubate them as follows:

- *B. subtilis* and *E. coli*, 35±2°C for 24 to 48 hours.
- *D. radiophilus*, 30°C for 72 to 96 hours.

7 **Repeat the Process:** Repeat the plate exposure process (Steps 4–6) for each UV treatment time point.

8 **Clean up:** Save or dispose of the stock cultures as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove your plates from the incubator and observe for growth. Side B should be covered with a bacterial lawn (confluent growth). If this is not the case, see your instructor.

2 **Record Your Observations:** Note the extent of growth on Side A of each plate. Record your observations in the table in the data sheet at the end of this exercise. So that groups can easily compare data, record the level of growth on each of your group's plates as "heavy confluent growth," "sparse confluent growth," "individual colonies," or "no growth."

3 **Record Data from Other Groups:** Your instructor will provide you with a means to share data obtained by all groups. Record these data on your data sheet at the end of this exercise.

4 **Graph the Data:** On the graph paper provided with the data sheet, construct a graph representing growth versus UV exposure time for all three organisms. If you need help with graphing, see pages 10–12.

5 **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

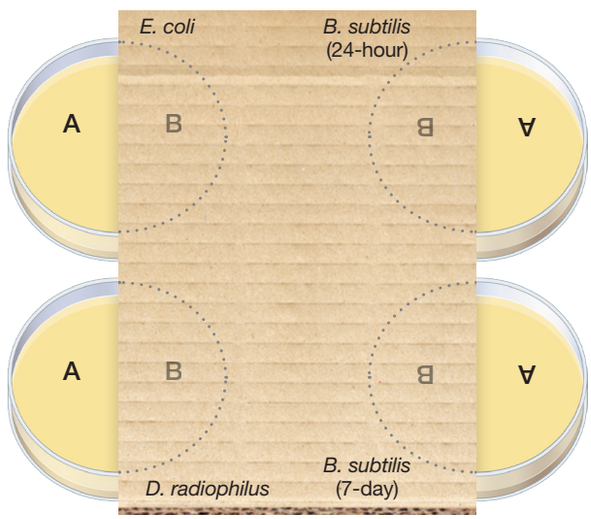


FIGURE **42.2** **Plates Shielded for UV Exposure** • Place the plates under the UV lamp with the lids removed and the cardboard shield covering half of each plate as shown. Make sure the petri plate lids are placed open side down on a disinfectant-soaked towel.

TABLE **42.1** **Group Assignments** •

Organism	No UV	1 Minute	2 Minutes	4 Minutes	8 Minutes	12 Minutes	16 Minutes
B. subtilis (24-hour culture)	1	1	2	3	4	5	6
B. subtilis (7-day culture)	1	1	2	3	4	5	6
Deinococcus radiophilus	1	1	2	3	4	5	6
E. coli	1	1	2	3	4	5	6

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 42

DATA SHEET

The Effect of Ultraviolet Radiation on Microbial Growth

During the Lab

1 Enter your class data in the table provided using the following descriptions:

0 = no growth

1 = individual colonies

2 = sparse confluent growth

3 = heavy confluent growth

UV Exposure (minutes)	*B. subtilis* (24-hour culture)		*B. subtilis* (7-day culture)		*D. radiophilus*		*E. coli*	
	Side A	Side B	Side A	Side B	Side A	Side B	Side A	Side B

Lab Related Questions

1 Why were you told to remove the plate covers prior to exposing them to UV?

a. If the lids were left on, the heat generated during the treatment would be trapped, which would cause the temperature of the cultures to increase to a level that would kill the bacteria on the plates. This temperature-induced killing would therefore eliminate perceivable differences among the treated plates.

b. If the lids were left on, they would block UV-C rays, thereby sparing all bacteria and eliminating perceivable differences among the treated plates.

c. If the lids were left on, they would intensify the UV-C rays, to the point of killing all the inoculated bacteria on the plates and thus a difference among the treatments would not be evident.

d. The removal of the lids was necessary to avoid differences in UV exposure due to variances in lid thickness.

e. If the lids had been left on, condensation would have formed on the inner lid surface and spilled back onto the cultures during the UV exposure, thereby contaminating the plates.

2 What is the general relationship between bacterial growth and UV exposure time?

a. As UV exposure time increases, growth decreases.
b. As UV exposure time increases, growth increases.
c. As UV exposure time decreases, growth decreases.
d. UV exposure time does not affect bacterial growth.

3 The purpose of this exercise is to demonstrate the comparative effect of UV on four bacterial populations. This could have been accomplished without the cardboard cover. What statement best explains why the cover was used?

a. It allowed you to conduct the experiments using fewer plates.
b. It reduced the time for conducting the experiment.
c. It enhanced the UV intensity for Side A because a smaller surface area was exposed to the light.
d. It allowed for side-by-side comparisons of an experimental and a control all on the same plate.
e. It allowed you to conduct the experiment in less time.

4 Assume the UV lamp is bumped and, as a result, it is located twice as far from the plates for the last time point (16 minutes) as compared to each of the earlier time points. What result would you least expect?

a. The time 0 plate would have more growth than the 16-minute plate.
b. The 1-minute plate would exhibit more growth than the 16-minute plate.
c. The 12-minute plate would exhibit less growth than the 16-minute plate.
d. The 12-minute plate would exhibit more growth than the 16-minute plate.

5 The intensity of a UV exposure _____ as the distance from the target decreases.

6 List the pros and cons of UV-C radiation as a means of achieving disinfection.

7 The germicidal action of UV-C is primarily due to:

a. Damage to the bacterial cell wall.
b. Heat generated by the UV radiation.
c. Destruction of cellular proteins.
d. Interference with biochemical pathways.
e. Induced nucleic acid damage.

Name _____ Date _____

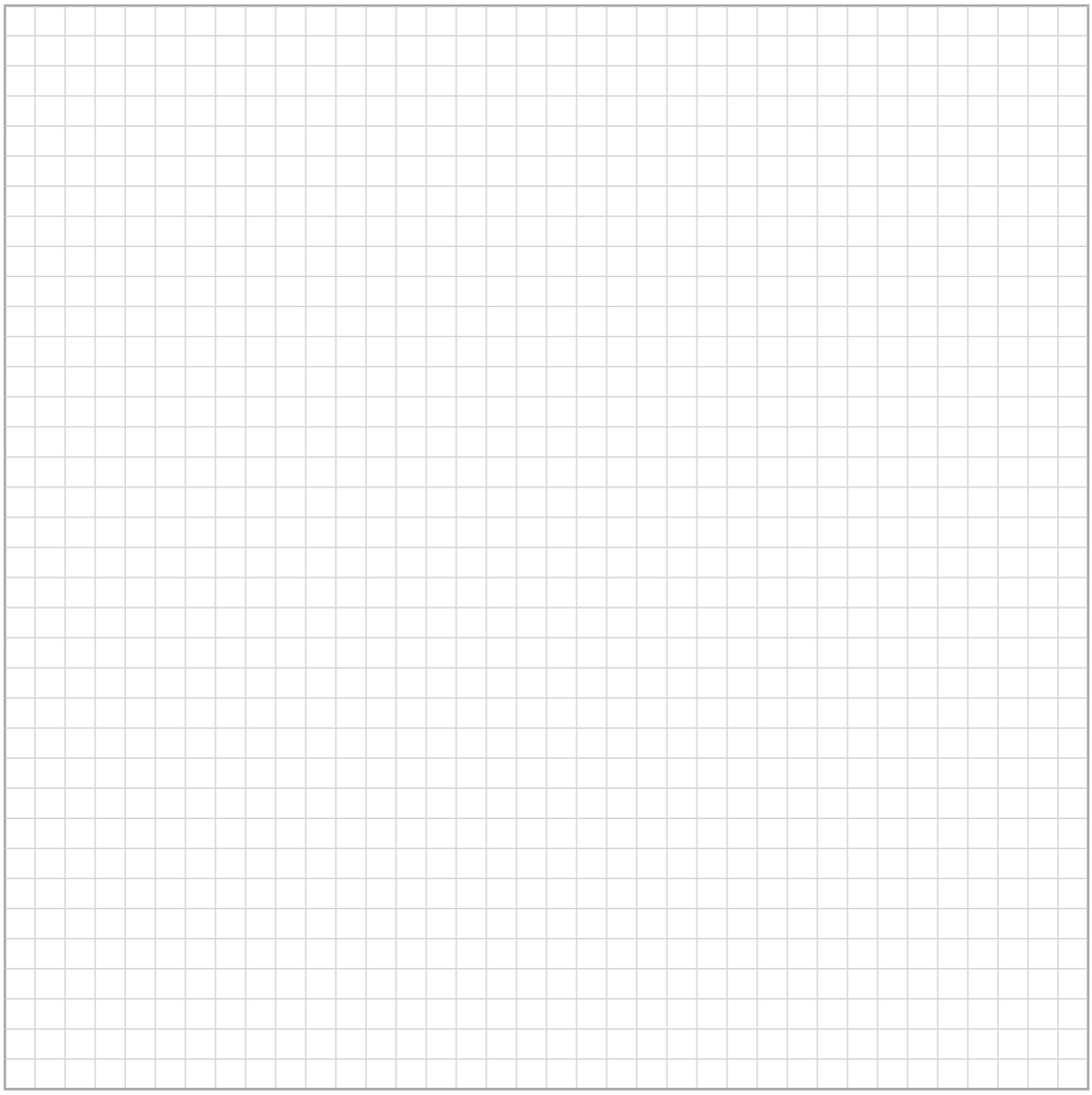
Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 42
DATA SHEET
(continued)

Critical Thinking

1 Which organism survived the longest exposure? Why do you suppose it did?

2 Using the graph paper provided, construct a single graph of relative growth (0, 1, 2, 3) versus UV exposure time for the four cultures. Include a legend defining the numbers and symbols/line colors for the different cultures. See pages 10–12 for proper graphing technique.



— Lab Notes —

EXERCISE 43

Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method

After doing this exercise, you should be able to:

1. Describe how the disk diffusion test for antimicrobial drug susceptibility testing works and what it reveals.
2. Define what a zone of inhibition is and describe how it is evaluated in the disk diffusion test.
3. State a key limitation of the disk diffusion test.
4. Define the terms *bactericidal* and *bacteriostatic*.
5. Explain the standardized aspects of the Kirby-Bauer procedure.
6. Describe what an antibiogram is and discuss why it is clinically useful.

Background

In this exercise, you will perform an antimicrobial susceptibility test known as the **disk diffusion (Kirby-Bauer) method.** This test is useful for assessing a pure culture's susceptibility to various antimicrobial drugs.[1] In 1966, Alfred Bauer, William Kirby, and associates first published a standardized disk diffusion method of testing the effectiveness of more than 20 antimicrobial agents. Their methodology, which bears their names, is a valuable tool for establishing the effectiveness of antimicrobics against pathogenic microorganisms in clinical laboratories.

In the test, antimicrobic-infused paper disks are placed on an inoculated agar plate (Fig. 43.1). Once the disks are in position, the plates are incubated at 35±2°C for 16 to 18 hours. During incubation, the bacteria grow as a **confluent lawn** (a uniform spread of bacterial growth on an agar plate's surface as opposed to streaks, patches, or colonies on the media's surface). Also, during incubation, the drugs infused into the paper disks diffuse into the agar. As each drug diffuses through the agar away from the disk, a concentration gradient develops in which the drug concentration *decreases* as the distance from the disk *increases*. Following incubation, a clear zone called a **zone of inhibition (ZOI)** may be visible around some of the disks. These clearings are areas where the antimicrobial drug concentration was high enough to inhibit bacterial growth (Fig. 43.2). The margin of the ZOI reflects the **minimum inhibitory concentration (MIC)** of the drug needed to interfere with bacterial growth. As performed in this exercise, we will not be able to quantify the MIC; however, there are modified versions of the diffusion test—namely, the **E-test**—that can quantify the MIC.

If a ZOI is *not* observed around a disk, then the bacterium is by default considered resistant to the drug. However, the mere presence of a ZOI does not

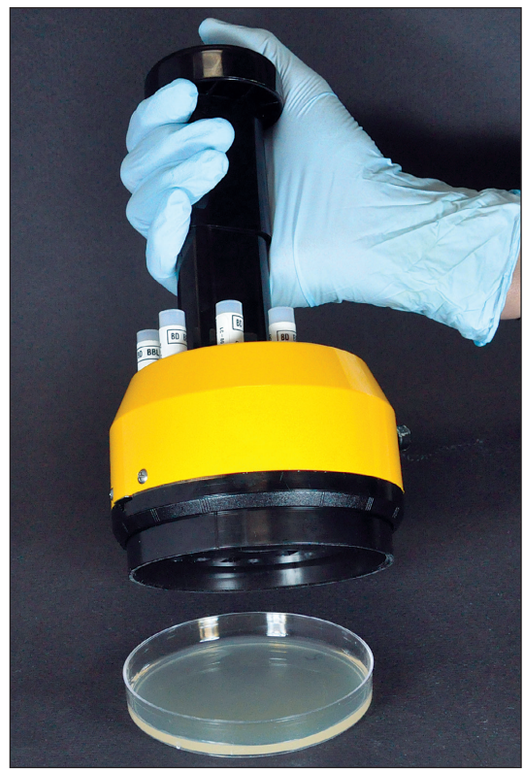


FIGURE **43.1** **Disk Dispenser** • A disk dispenser, like the one shown, is sometimes used to uniformly deposit disks on a Mueller-Hinton agar plate.

[1] Notice that here we refer to antimicrobial drugs rather than antibiotics. This is because the term *antibiotic* technically only refers to naturally occurring antimicrobial agents produced by microorganisms. For example, the mold *Penicillium notatum* naturally makes the antibiotic penicillin. Today many of the antibacterial agents that treat bacterial infections are synthetic, so the terms *antimicrobial*, *antimicrobic*, or *antibacterial* drug are arguably more appropriate ways of referring to these agents. That said, it's common for people (including many scientists and clinicians) to simply use the term *antibiotic* to refer to *all* antibacterial drugs (both natural and synthetic) as antibiotics. The main point is that *you* know the difference for an exam should your instructor highlight this point!

automatically mean the bacterium is susceptible to the drug that generated the ZOI. Instead, the diameter of the zone, which is measured in mm and includes the disk diameter, must be measured (Fig. 43.3) and compared to standardized criterion measurements to determine if the tested bacterium is resistant (R), susceptible (S), or intermediate (I) in its response to the given drug (Table 43.1).

It is important to appreciate that the cleared zone devoid of bacterial growth does not necessarily mean that the drug killed the bacteria in that area; you cannot assume the drug is **bactericidal.** The clear zone may have developed because the drug is **bacteriostatic** and only inhibiting the bacterium's growth. An important limitation of the disk diffusion (Kirby-Bauer) method is that it does not reveal if the drugs used in the test are bactericidal or bacteriostatic against the assessed bacterium. It is worth clarifying that from a clinical standpoint, bactericidal drugs are not necessarily considered superior to bacteriostatic drugs.

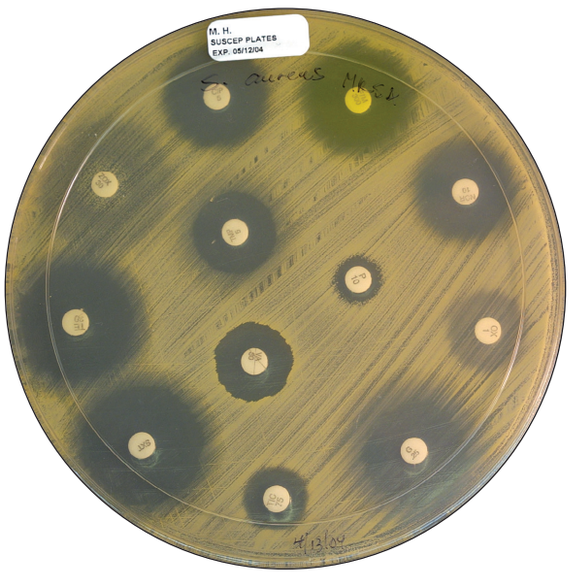


FIGURE **43.2** **Disk Diffusion Test of Methicillin-Resistant *Staphylococcus aureus* (MRSA)** ● This plate illustrates the effect of (clockwise from top outer right) Nitrofurantoin (F/M300), Norfloxacin (NOR 10), Oxacillin (OX 1), Sulfisoxazole (G 0.25), Ticarcillin (TIC 75), Trimethoprim-Sulfamethoxazole (SXT), Tetracycline (TE 30), Ceftizoxime (ZOX 30), Ciprofloxacin (CIP 5), and (inner circle from right) Penicillin (P 10), Vancomycin (VA 30), and Trimethoprim (TMP 5) on Methicillin-resistant *Staphylococcus aureus* isolated from a patient. ***Note:*** This petri plate is 150 mm in diameter to accommodate 12 disks.

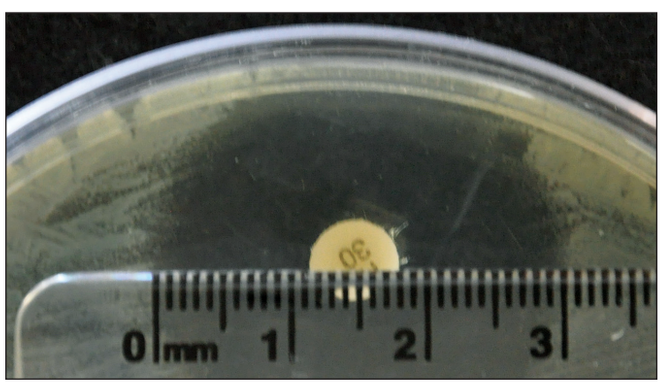


FIGURE **43.3** **Measuring Zones of Inhibition** ● Using a metric ruler and a dark, nonreflective background, measure the diameter of each clearing in millimeters (mm). This zone is 29 mm in diameter.

Standardized Aspects of the Kirby-Bauer Procedure

All aspects of the Kirby-Bauer procedure are standardized to ensure reliable results. Therefore, care must be taken to adhere to these standards.

Agar pH and Depth

Mueller-Hinton agar pH is between 7.2 and 7.4. This pH must be strictly adhered to when making the media because fluctuations either above or below the stated pH levels can affect drug activity. Also, the agar is always poured to a depth of 4 mm; this is true regardless of the plate size used (e.g., 150 mm or 100 mm petri plates). The agar depth is important to standardize because it can affect drug diffusion. Thick agar slows lateral diffusion and thus produces smaller zones than plates held to the 4 mm standard. In contrast, if the agar is thinner than the 4 mm standard, then the drugs will diffuse further than normal and may lead to artificially larger ZOIs, which may lead us to inaccurately conclude the tested bacterium is susceptible to the given drug.

Sample Turbidity

Another aspect of the Kirby-Bauer procedure that's standardized is the density of the bacterial culture inoculated onto the media. Typically, the bacterial sample to be tested is grown as a broth culture that is incubated for 4 to 6 hours to bring the cells into the **exponential phase of growth,** or **logarithmic growth stage** (log stage), the stage at which the bacteria are typically the healthiest and actively dividing. Because many antibiotics target the cellular processes of actively growing cells, it's essential to have the cells in this phase of growth before testing their susceptibility to antibacterial drugs. Failure to do so may lead to inaccurate data regarding drug susceptibility. After the incubation period, the culture is subsequently diluted to match a **0.5 McFarland turbidity standard** (Fig. 43.4). A McFarland Standard is a solution of barium chloride and sulfuric acid that react to form a fine precipitate of barium sulfate, which optically mimics a turbid bacterial sample. The 0.5 McFarland Standard corresponds to a cell density between 1 and 2×10^8 CFU/mL (colony forming units/mL). An alternative to the McFarland approach is to use a spectrophotometer

TABLE **43.1** Zone Diameter Interpretive Chart ●

Antibiotic	Organism(s)	Code	Disk Potency	Zone Diameter Interpretive Standards (mm)		
				Resistant	Intermediate	Susceptible
Chloramphenicol	*Enterobacteriaceae* and *Staphylococcus*	C 30	30 µg	≤ 12	13–17	≥ 18
Ciprofloxacin	*Enterobacteriaceae* and *Staphylococcus*	CIP 5	5 µg	≤ 15	16–20	≥ 21
Penicillin	*Staphylococcus*	P 10	10 U	≤ 28		≥ 29
Streptomycin	*Enterobacteriaceae*	S 10	10 µg	≤ 11	12–14	≥ 15
Tetracycline	*Enterobacteriaceae* *Staphylococcus*	TE 30 TE 30	30 µg 30 µg	≤ 11 ≤ 14	12–14 15–18	≥ 15 ≥ 19
Trimethoprim	*Enterobacteriaceae* and *Staphylococcus*	TMP 5	5 µg	≤ 10	11–15	≥ 16

This table includes the antibiotics used in this exercise and contains data provided by the Clinical and Laboratory Standards Institute (CLSI). Permission to use portions (specifically Tables 2A and 2C) of *M100 Performance Standards for Antimicrobial Susceptibility Testing, 30th edition* (2020) has been granted by CLSI. The interpretive data are only valid if the methodology in CLSI Standard *M02 Performance Standards for Antimicrobial Disk Susceptibility Tests*, 13th edition, (2018) is followed. CLSI updates the interpretive tables in M100 annually through new editions of the supplement. Users should refer to the most recent editions. The most current editions of both documents may be obtained from CLSI, 950 West Valley Road, Suite 2500, Wayne, PA 19087. Contact also may be made via the website (www.CLSI.org), email (customerservice@clsi.org), and by phone (1.877.447.1888).

to bring the broth culture to an absorbance of 0.08 to 0.10 at 625 nm in a 1 cm cuvette. This absorbance corresponds to the 0.5 McFarland standard.

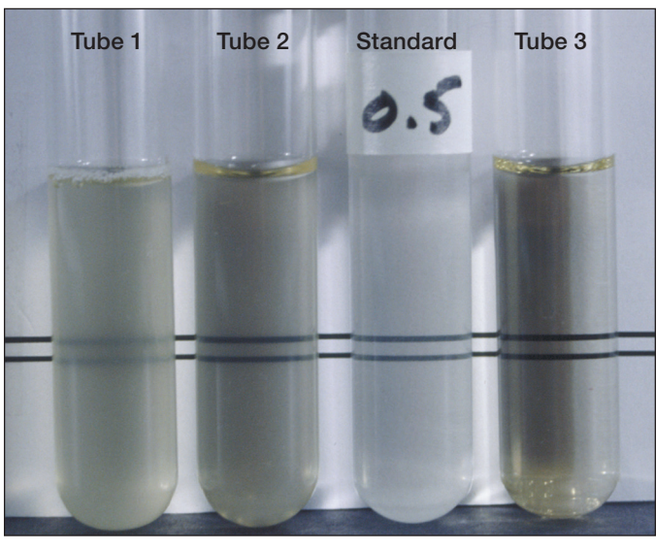


FIGURE **43.4** **McFarland Standards** ● This image shows a comparison of a 0.5 McFarland turbidity standard to three broths of varying turbidity. A visual comparison of turbidity is made by placing a card with sharp black lines behind the tubes. Notice that Tube 2's turbidity matches the McFarland standard exactly, whereas Tube 1 is too turbid and Tube 3 is too clear.

Number and Spacing of Disks on a Plate

The number of disks used on a given plate depends on the plate size. For 150 mm plates, place no more than 12 disks on the agar surface. For 100 mm plates, the size of the plates used in this exercise, a maximum of 6 disks should be used. The positioning of the drug-infused disks is also standardized. There must be a spacing of at least 24 mm between disks (as measured from one disk's center to the adjacent disk's center).

Drug Concentrations and Interpretive Standards

The U.S. Food and Drug Administration establishes the disk concentration of each agent, which is printed on the disk along with a code indicating the chemical agent. The Clinical Laboratory Standards Institute (CLSI) in Wayne, Pennsylvania, has performed extensive research (and continues to do so) to establish the ZOI diameter interpretive standards like those presented in Table 43.1. ***Note:*** Many more antimicrobial agent/pathogen combinations exist than are represented in the provided table. Instead, the full collection of data is housed in a resource called the *M100 Performance Standards for Antimicrobial Susceptibility Testing*. This reference is annually updated and is the source of the information in Table 43.1.

The CLSI establishes the interpretive criteria regarding zone diameter breakpoints for resistance and susceptibility. The cutoff criteria are based on zone of inhibition diameters obtained for bacteria known to be susceptible to the given drug. If an observed ZOI's diameter is *less* than the ZOI diameter obtained for related susceptible bacterium, then the ZOI diameter is said to fall within the **resistance breakpoint.** This means the tested bacterium grew even when exposed to a higher drug concentration than that which inhibits the growth of a related bacterium known to be susceptible to the drug. Likewise, the ZOI diameter above which all susceptible strains fall is the **susceptibility breakpoint.** In some cases, there may be an intermediate range of zone diameters in which some resistant and susceptible strains fall.

A Note on Bacterial Susceptibility to Antimicrobial Drugs

Bacterial resistance to antimicrobial drugs is a complex matter, and increasingly problematic in health care. Although a full discussion of drug resistance mechanisms is beyond the scope of this exercise, you're encouraged to review Table 43.2, which presents the modes of drug action for the drugs used in this exercise and examples of how bacteria may develop resistance to the tested drugs.

Clinical Applications

The disk diffusion test is a standardized method used to assess if microorganisms are susceptible to specific antimicrobial drugs. Ultimately, susceptibility testing provides important information that helps clinicians decide which antimicrobial drug(s) would be best for treating a patient's infection.

To improve patient care, clinical microbiology labs produce drug susceptibility reports called **antibiograms.** An antibiogram can be based on a specific isolate for a single patient sample, or it can be a broader report (e.g., institutional antibiogram) that conveys drug susceptibility data for bacterial species isolated from patients at a specific health-care facility (e.g., hospital, long-term care center, etc.). Because bacteria are constantly evolving drug resistance, CLSI recommends that health-care facilities update their institutional antibiogram data at least once per year. This helps to ensure that clinicians have accurate data regarding the usefulness of specific antimicrobial drugs against bacterial strains circulating in the given health-care setting. For example, if a clinician knows their patient has an *E. coli* infection and the hospital's most recent antibiogram shows most *E. coli* isolates from patients in the hospital are resistant to a

TABLE **43.2** Antibiotic Targets and Resistance Mechanisms •

Antibacterial Drug	Cellular Target	Resistance Mechanism
Chloramphenicol	Prevents peptide bond formation during translation	1. Poor uptake of drug 2. Inactivation of drug
Ciprofloxacin	Interferes with DNA replication	1. Altered target 2. Poor uptake of drug
Penicillin	Inhibits cross-linking of the cell wall's peptidoglycan	One or more of: 1. Altered target 2. Poor uptake of drug 3. Production of β-lactamases
Streptomycin	Blocks initiation complex formation in protein synthesis	1. Altered target
Tetracycline	Blocks attachment of aminoacyl tRNA to A site on ribosome	1. Efflux mechanism
Trimethoprim	Inhibits purine and pyrimidine synthesis	1. Altered target

Not all antimicrobial drugs affect cells in the same way. Some attack the bacterial cell wall, and others interfere with biosynthesis reactions. There are three main drug resistance mechanisms: (a) **target alteration:** the cell may alter the drug's target such that the antibacterial drug is less effective at interacting with its target; (b) **drug inactivation:** the cell may break down or alter the antibacterial drug rendering it less effective; and (c) **reducing the drug's concentration inside the cell:** a cell can limit or block drug entry or even pump the drug out of the cell via efflux pumps.

particular drug—say, tobramycin—then the clinician would avoid prescribing tobramycin and would instead select a more effective drug.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ 100 mm Mueller-Hinton agar plates (2)
- ❑ Penicillin, chloramphenicol, tetracycline (optional), trimethoprim, streptomycin (optional), and ciprofloxacin antibiotic disks (and/or other disks as available)
- ❑ Antibiotic disk dispenser or forceps for disk placement
- ❑ Screw-capped jar of alcohol (for sterilizing forceps, if used)
- ❑ Sterile cotton-tipped swabs (2)
- ❑ Metric ruler (1)
- ❑ Sterile saline (0.85%)
- ❑ Sterile transfer pipettes (2)
- ❑ McFarland 0.5 standard with card (1)
- ❑ Black, nonreflective poster board (8.5" × 11")
- ❑ Fresh broth cultures of these recommended organisms:
  - ☐ *Escherichia coli*
  - ☐ *Staphylococcus aureus* (BSL-2)
  - ☐ *Staphylococcus epidermidis* (a BSL-1 substitute for *S. aureus*)

Optional Materials (necessary if not using McFarland standard)

- ❑ Spectrophotometer
- ❑ Matched, sterile, 1 cm cuvettes (3)
- ❑ Sterile transfer pipettes (3)
- ❑ Capped, sterile 12 mm test tubes (2); and 75 mm test tubes (2)
- ❑ Tryptic soy agar (TSA) (or nutrient agar, NA) plates (2; for optional procedure)

Media Recipe

Mueller-Hinton II Agar (MHA)

Beef extract	2.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled or deionized water	1.0 L

Adjusted to pH 7.2–7.4 at 25°C

PROCEDURE

You will test the susceptibility of *Escherichia coli* and either *Staphylococcus aureus* or *S. epidermidis* to penicillin, chloramphenicol, tetracycline (optional), trimethoprim, streptomycin (optional), and ciprofloxacin. These antimicrobial drugs were chosen because they exhibit different modes of action on bacterial cells (Table 43.1). Wear your lab coat, eye protection, and gloves throughout the procedure. ***Caution!*** Follow BSL-2 precautions if using *S. aureus*.

1. **Obtain the Stock Cultures and MHA Media:** Gently mix the stock cultures until they reach their maximum turbidity. You'll also need two plates of MHA media. Label the bottom of each MHA plate with your name and the date. Then, add the label "*E. coli*" to one plate and designate the second one as "*S. aureus*" or "*S. epidermidis*," depending on which bacteria your instructor has available for your lab session.

2. **Check the Stock Culture Turbidity:** In some cases, your instructor may provide prediluted stock bacterial samples that are ready for you to inoculate. If that is the case, you may skip to Step 3. However, if your instructor does *not* provide you with samples that are prediluted to the proper turbidity, you must adjust the sample concentrations before inoculating the media. Following are two methods for doing this. Use the method your instructor selects.

   - ***McFarland Method:*** Holding the culture and McFarland standard upright in front of you, place the card behind them so you can see the black line(s) through the liquid in the tubes. As you can see in Figure 43.4, the line becomes distorted by the sample turbidity. Use the black line to compare the turbidity level of the two tubes. Dilute the broth with sterile saline until it appears to have the same turbidity as the standard. If it is not turbid enough, incubate it until it reaches that level. Repeat this process for all bacterial cultures you will use in this exercise.
   - ***Spectrophotometer Method:*** Rather than using the McFarland approach, your instructor may prefer that you use a spectrophotometer to bring the samples to the proper growth density. To do this, you need to first prepare a "blank sample" to zero out the spectrophotometer before you read your stock bacterial sample turbidity. You can set up your blank sample by using a sterile transfer pipette to transfer some sterile broth (the same as that used to grow the bacterial stock cultures) into a cuvette. Then, follow your instructor's directions regarding how to "zero out," or blank, the spectrophotometer in your lab. Next, mix the *E. coli* culture and use a sterile pipette to aseptically transfer some of the stock culture into a

second cuvette. You should determine the sample absorbance at 625 nm. Depending upon the reading, either dilute the stock culture or let it grow longer to bring its absorbance to 0.08 to 0.10. Repeat this process for all bacterial cultures you will use in this exercise.

3 **Inoculate the Medium with *E. coli*:** Dip a sterile swab into the *E. coli* stock culture that is at the correct turbidity. As you remove the swab, press and rotate the cotton tip against the side of the tube to remove excess broth so that the swab is not dripping wet. Then, use the swab to streak the entire surface of the agar. Your goal is to achieve a confluent lawn of growth, so make the streaks right next to each other and cover the entire surface of the media. ***Caution!*** To avoid contamination, use the lid to guard your plate's surface. Never take the lid completely off and never set the lid on the bench surface!

To ensure full coverage, once you have streaked the entire plate surface, you should rotate the plate by one-third and repeat the process with the same swab (do *not* redip the swab in the culture). Once you've completed this second pass, you'll perform a third and final streaking (do *not* redip the swab in the culture) by again rotating the plate by one-third and then restreaking the media's entire surface. When finished, properly dispose of the swab and replace the plate's lid.

4 **Inoculate the Medium with *S. aureus* or *S. epidermidis*:** Repeat Step 3, but this time use the *S. aureus* or *S. epidermidis* culture.

5 **Apply Disks to the Media:** Apply penicillin-, chloramphenicol-, ciprofloxacin-, and trimethoprim-infused disks to the agar surface of each plate. If you do not have a disk dispenser, you can apply the disks singly using alcohol-flamed forceps[2]. Be sure to space the disks sufficiently apart (at least 24 mm between disks) to prevent zones of inhibition from overlapping. You also must keep the disks away from the plate edge. Using your forceps, gently tap the disks so they make uniform contact with the agar surface, but do *not* push the disks into the agar.

6 **Incubate Your Plates:** Incubate your inverted plates aerobically at 35±2°C for 16 to 18 hours.

7 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples**: Remove the plates from the incubator (or refrigerator). Hold each plate over the black, nonreflective poster board and examine the plate with reflected light. The edge of a zone is where no growth is visible to the naked eye. Measure the diameter of each zone of inhibition in millimeters (Fig. 43.3).

2 **Record Your Observations:** Use Table 43.1 to help you record your results in the data table at the end of this exercise. If you used additional antibiotics, then refer to the ZOI diameter data that the manufacturer includes with all antibiotic disks.

3 **Clean up:** Dispose of the plates as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Optional Procedure

You would start this optional procedure on the same day that you make your ZOI observations. This procedure is to examine if the drugs that generated ZOIs were bacteriostatic or bactericidal in their action toward the tested bacteria. Wear a lab coat, gloves, and eye protection when performing this procedure. ***Caution!*** Follow BSL-2 precautions if using *S. aureus*.

1 **Obtain and Label TSA or NA Media:** Obtain two TSA or NA plates. With your marking pen, divide the plates into four sectors (or more if you used more antibiotics). Label both plates with your group name. Label one plate *E. coli* and the other *S. aureus* (or *S. epidermidis*). Label each sector with the name of the antibiotic that generated a ZOI. It is easiest if you label your plate in the same order as the drugs are placed on your MHA plates.

2 **Inoculate the Media:** Using a sterile loop for each transfer, obtain a sample from each antibiotic's zone of inhibition on the *E. coli* plate, and spot-inoculate the corresponding sector on the TSA (or NA) plate. If there is no zone for a particular antibiotic, no transfer is necessary. Repeat with the *S. aureus* (or *S. epidermidis*) MHA plate.

[2] Keep the forceps in the alcohol jar until ready to use them. Then, close them as you remove them from the alcohol. This will trap some alcohol between the tips. Pass the forceps *through* the flame and allow the alcohol to burn off *away from the alcohol jar and the flame*. When the flame burns out, give the forceps a few seconds to cool, and then place the disk on the plate. Return the forceps to the alcohol jar until needed. ***Caution!*** Keep the flaming forceps away from the alcohol jar. If the alcohol in the jar catches fire, extinguish it by replacing the jar's lid.

3 **Incubate Your Plates:** Incubate the inverted plates aerobically at 35±2° for 24–48 hours.

4 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1 **Examine the Samples:** Remove the plates from the incubator and examine each sector for growth.

2 **Record Your Observations:** Record your observations and answer the questions on the data sheet provided at the end of this exercise.

3 **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 43

DATA SHEET

Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method

During the Lab

1 Record the zone of inhibition diameters (mm) in the data table provided. Then, use Table 43.1 as a guide to assess if the bacterium was susceptible (S), resistant (R), or intermediate (I) in its response to the tested antimicrobics. (Not all combinations of antibiotics and organisms are presented in Table 43.1. If you used antimicrobics not listed, then refer to the ZOI data that the manufacturer includes with all antibiotic disks.)

Interpreting Zone of Inhibition (ZOI) Diameter Data

Organism	Chloramphenicol		Ciprofloxacin		Trimethoprim		Penicillin		Streptomycin (optional)		Tetracycline (optional)	
	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R
E. coli												
S. aureus (or *S. epidermidis*)												

2 If you did the optional exercise, record "G" for growth and "NG" for no growth in the data table provided. Then, record your conclusion regarding the antimicrobial drug's bactericidal or bacteriostatic effects on the tested bacteria.

Bactericidal or Bacteriostatic Effects on Test Bacteria

Organism	Chloramphenicol		Ciprofloxacin		Trimethoprim		Penicillin		Streptomycin (optional)		Tetracycline (optional)	
	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal
E. coli												
S. aureus (or *S. epidermidis*)												

Lab Related Questions

1 Using Table 43.1, designate the following as resistant (R), intermediate (I), or susceptible (S).

_____ A *Staphylococcus* species with a ZOI of 20 mm around a ciprofloxacin disk.

_____ An *Enterobacteriaceae* species with a ZOI of 18 mm around a chloramphenicol disk.

_____ A *Staphylococcus* species with a ZOI of 10 mm around a trimethoprim disk.

_____ An *Enterobacteriaceae* species with a ZOI of 15 mm around a tetracycline disk.

_____ A *Staphylococcus* species with a ZOI of 27 mm around a penicillin disk.

2 Which of the following is (are) standardized in the disk diffusion test? (Select all that apply.)

a. The type of media used.
b. The depth of the media.
c. The concentration of the bacterial culture used for inoculation.
d. The size of the antimicrobial infused disks
e. The type of bacteria used.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ TSA media is used to perform the Kirby-Bauer test.

_____ The Kirby-Bauer test does not reveal if a drug acts against an isolate in a bactericidal manner.

_____ Bacteria that lack a ZOI are automatically considered resistant to the tested antimicrobial drug.

_____ In the Kirby-Bauer test performed in this exercise, you used either a 1.0 McFarland standard or a spectrophotometer to assess culture density prior to inoculation.

4 Select the true statement(s) (select all that apply).

a. An institutional antibiogram for Hospital A in New York is usable at Hospital B in Colorado.
b. An antibiogram reveals which drugs are most effective in patients with specific underlying conditions.
c. An institutional antibiogram reveals which antimicrobial drugs are most likely to be effective against select species at a specific health-care facility.
d. CLSI recommend that institutions update their antibiograms at least every other year.
e. An antibiogram can be generated for a bacterium isolated from a specific patient sample.

5 Imagine you performed the exercise using MHA plates that were poured 2 mm deep instead of 4 mm deep. What is the most likely experimental outcome?

a. The ZOIs will have an artificially decreased diameter.
b. The ZOIs will have an artificially increased diameter.
c. The drugs will not diffuse from the disks into the agar.
d. The bacteria will not grow.
e. ZOIs will not develop.

6 Select the true statement.

a. The smaller a ZOI, the more concentrated a drug must be to prevent bacterial growth.
b. The smaller a ZOI, the less concentrated a drug must be to prevent bacterial growth.
c. The larger a ZOI, the more concentrated a drug must be to prevent bacterial growth.
d. ZOI diameter is not related to drug concentration; tested drugs are uniformly concentrated throughout a given ZOI.
e. Bactericidal drugs will generate larger ZOIs than bacteriostatic drugs.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 43
DATA SHEET
(continued)

7 In clinical applications of the Kirby-Bauer test, diluted cultures (for the McFarland standard comparison) must be used within 30 minutes. Why is this important?

a. If you delayed inoculation past 30 minutes, the bacterial growth will decrease due to nutrient limitation rendering the data inaccurate.

b. If you delayed inoculation past 30 minutes, the bacterial growth will develop resistance to the drugs in the media rendering the data inaccurate.

c. If you delayed inoculation past 30 minutes, the McFarland standard's density will be inaccurate.

d. If you delayed inoculation past 30 minutes, the bacterial growth will be too dense, and the data may therefore be inaccurate.

e. If you inoculate within 30 minutes, the bacterial growth will be too dense rendering the data inaccurate.

8 Was there a general difference in susceptibility between the tested Gram-positive bacterium (*S. aureus* or *S. epidermidis*) and the tested Gram-negative bacterium (*E. coli*)? If so, what difference(s) did you see?

Questions Related to the Optional Exercise

1 In the optional exercise, why were samples taken from the zones of inhibition for subculturing on TSA or NA plates?

a. To determine if the tested bacteria were susceptible to the tested drug(s).

b. To determine if the tested bacterial samples were contaminated.

c. To determine if the tested drug(s) killed or inhibited the bacteria from growing.

d. To determine if the tested bacteria can grow in the absence of the tested drug.

e. To determine if the tested bacteria are pathogens.

2 In this optional experiment, assume you obtained the following data:

Bacterium	Penicillin	Tetracycline	Ciprofloxacin
E. coli	Growth	Growth	No growth
S. epidermidis	No growth	No growth	Growth

Based on the data, what is the best conclusion?

a. Penicillin and tetracycline are more likely to kill *S. epidermidis* than *E. coli*.

b. *E. coli* is susceptible to ciprofloxacin whereas *S. epidermidis* is resistant to ciprofloxacin.

c. The tested drugs are more likely to kill *E. coli* than *S. epidermidis*.

d. Penicillin and tetracycline are bacteriostatic toward *S. epidermidis*.

e. Penicillin has bactericidal effects on *E. coli* and bacteriostatic effects on *S. epidermidis*.

Critical Thinking

The following is an excerpt from a hypothetical hospital antibiogram. Using the data in the antibiogram, answer the following questions.

	E. coli	*K. pneumoniae*	*P. mirabilis*	*P. aeruginosa*	*E. faecalis*	*S. aureus*	*S. epidermidis*	*S. pneumoniae*
	Gram-Negative Bacteria				**Gram-Positive Bacteria**			
Number of Isolates Analyzed	1654	98	142	190	398	350	202	51
Percent of Isolates Suceptible								
Ampicillin	51%	10%	94%	5%	98%	0%	0%	0%
Cefazolin	92%	99%	70%	0%	0%	99%	35%	0%
Levofloxacin	81%	97%	83%	79%	80%	75%	50%	98%

1 Your patient has a urinary tract infection caused by *E. coli* and wound infections caused by *S. aureus* and *P. aeruginosa*. Which antimicrobial drug(s) would you recommend for treatment and why? (***Note:*** Based on patient history, none of the listed drugs are contraindicated and you can assume for this question that drugs can be prescribed alone or in any combination.)

2 Of the three drugs in the antibiogram, which one has the broadest activity against Gram-positive and Gram-negative bacteria? Why do you suppose it is so broadly effective against diverse Gram-positive and Gram-negative bacteria? (***Hint:*** Consider how the drug you selected works.)

EXERCISE 44

Bacitracin, Novobiocin, and Optochin Susceptibility Tests

After doing this exercise, you should be able to:

1. Compare and contrast the procedure for assessing bacitracin, novobiocin, and optochin susceptibility to the Kirby-Bauer procedure.
2. Describe how bacitracin, novobiocin, and optochin susceptibility testing works and what these tests reveal.
3. Discuss the features of bacitracin, novobiocin, and optochin.
4. Describe what a zone of inhibition is and explain how it relates to assessing a bacterium's susceptibility to bacitracin, novobiocin, and/or optochin.
5. State how assessing bacitracin, novobiocin, and optochin susceptibility is clinically useful.

Background

In this exercise, you will assess bacterial susceptibility to three antimicrobial agents: bacitracin, novobiocin, and optochin. Although these susceptibility tests resemble the Kirby-Bauer procedure (disk diffusion test) reviewed in Exercise 43, they are far less rigorously standardized. The less stringent assessment parameters relate to the point that bacitracin, novobiocin, and optochin susceptibility tests are bacterial identification strategies. Unlike the Kirby-Bauer test, they are not for guiding decisions related to which drug is best for treating a patient infection.

As opposed to the Mueller-Hinton agar used in the Kirby-Bauer procedure, bacitracin, novobiocin, and optochin susceptibility testing procedures usually call for sheep blood agar so that hemolysis activity (Ex. 25) and drug susceptibility of an isolate can be assessed at the same time.[1] Similar to the Kirby-Bauer procedure, you will apply paper disks infused with a specific concentration of an antimicrobial substance onto confluently inoculated plates.[2] Then, following incubation, you will measure the diameter of resulting clear zones (zones of inhibition, or ZOIs) and compare your measurements to a standardized chart. Based on ZOI diameter, you'll decide if the tested bacterium is resistant or susceptible to the tested antimicrobial compound. Unlike the Kirby-Bauer procedure, an intermediate ranking (I) is not applicable in the bacitracin, novobiocin, and optochin susceptibility tests, nor are the cultures assessed for cell density before inoculation.

[1] Blood agar is relatively expensive, so your instructor may have you use TSA (tryptic soy agar) as a blood agar substitute. Such a substitution is acceptable in this exercise because you are using known bacteria and can therefore lookup the hemolytic properties of each bacterium.

[2] Recall from Exercise 43 that a confluent inoculation generates a uniform spread of bacterial growth.

A Review of Bacitracin, Novobiocin, and Optochin

Each of the three agents in this exercise are active against certain bacteria. Bacitracin and novobiocin are both classified as antibiotics (compounds naturally made by other microbes) whereas optochin is not naturally occurring and is therefore considered an antimicrobial drug (or antimicrobic) rather than an antibiotic. Before performing the exercise, it's worth exploring a bit more about each of the three agents you will test.

Bacitracin, produced by *Bacillus licheniformis*, is a powerful peptide antibiotic that inhibits bacterial cell wall synthesis by interfering with transport of peptidoglycan subunits across the cytoplasmic membrane. Thus, it is only effective on actively growing bacteria that have a cell wall. If a zone of inhibition 10 mm or greater develops around a 0.04 U bacitracin disk (called an "A" disk), then the bacterium is bacitracin susceptible (Fig. 44.1). Bacitracin is a common active ingredient in certain over-the-counter topical antibacterial ointments (ointments applied to the skin's surface) such as Neosporin; however, it is rarely used in non-topical medications because when ingested or injected, it is associated with renal failure (kidney failure). Consequently, assessing bacitracin susceptibility is useful for bacterial identification, not to inform decisions about using bacitracin as a therapeutic.

Novobiocin is an antibiotic, produced by *Streptomyces niveus, a bacterium found in soil*. It is chemically related to coumarins, drugs that block blood clotting. Novobiocin interferes with ATPase activity associated with DNA gyrase, an enzyme necessary for DNA replication. A zone of inhibition 16 mm or more around a 5-µg disk is interpreted as novobiocin susceptibility (Fig. 44.2). As with bacitracin, novobiocin has toxic side effects in humans when ingested or injected. Consequently, it is not useful as a medication for humans, although it is occasionally used in veterinary medicine for treating larger animals.

Optochin is an antimicrobial substance derived from quinine. It targets the enzyme ATP synthase and thereby disrupts ATP production. A zone of inhibition 14 mm or more around a 6 mm optochin disk (called a "P" disk) containing 5 µg of optochin is interpreted as optochin susceptibility (Fig. 44.3). In the early 1900s, optochin was developed to combat bacterial pneumonia in humans, but because of its toxicity, it was never approved as a therapeutic.



FIGURE **44.1** **Bacitracin Susceptibility Test on a Sheep Blood Agar Plate** ● The zone diameter cut off for bacitracin susceptibility is 10 mm or more. The organism above has no clear zone and is resistant (R). The organism below has a zone diameter of 19 mm and is susceptible (S). The disks themselves are usually 6 mm and are referred to as "A" disks.



FIGURE **44.2** **Novobiocin Susceptibility Test on a Sheep Blood Agar Plate** ● The zone diameter cut off for novobiocin susceptibility is 16 mm or more. A novobiocin-resistant organism (R) with a zone diameter of 7 mm is on the left; a susceptible organism (S) with a zone diameter of 27 mm is on the right.



FIGURE **44.3** **Optochin Susceptibility Test on a Sheep Blood Agar Plate** ● The zone diameter cut off for optochin susceptibility is 14 mm or more. The zone of inhibition surrounding the "P" disk is 19 mm, indicating susceptibility to optochin. The α-hemolysis coupled with optochin susceptibility leads to presumptive identification of *S. pneumoniae*.

Clinical Applications

Unlike the Kirby-Bauer test, which helps clinicians decide which antimicrobial drug may be most effective against an infectious agent, the susceptibility tests performed in this exercise are used for bacterial identification.

The bacitracin test allows for a presumptive identification of the causative agent of strep throat (streptococcal pharyngitis), the β-hemolytic *Streptococcus pyogenes*. This pathogen is a Group A streptococcus that is bacitracin susceptible and thereby differentiated from other β-hemolytic streptococci that are bacitracin resistant. The bacitracin susceptibility test also differentiates members of the genus *Staphylococcus*, which are almost always resistant to bacitracin, from typically susceptible members of the genus *Micrococcus*. Recall that certain staphylococcus members, such as *S. aureus*, cause a variety of infections but are especially infamous for causing skin infections.

The novobiocin test differentiates coagulase-negative staphylococci (see Ex. 38). Most frequently it presumptively identifies *Staphylococcus saprophyticus*, which is novobiocin resistant. *S. saprophyticus* is a common cause of urinary tract infections.

The optochin test presumptively differentiates a common cause of pneumonia, *Streptococcus pneumoniae* (susceptible), from other α-hemolytic streptococci.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Blood agar plate (1 per test) (commercial preparation of TSA containing 5% sheep blood; TSA may be substituted for blood agar)
- ❑ Sterile cotton-tipped swabs (1 per organism to be tested)
- ❑ Screw-cap jar of alcohol with forceps

Bacitracin Test

- ❑ 0.04 unit bacitracin disks ("A" disks)
- ❑ Fresh broth cultures (18–24 hours old) of these recommended organisms:
  - □ *Staphylococcus aureus* (BSL-2)
  - □ *Micrococcus luteus*

Novobiocin Test

- ❑ 5 µg novobiocin disks
- ❑ Fresh broth cultures (18–24 hours old) of these recommended organisms:
  - □ *Staphylococcus epidermidis*
  - □ *Staphylococcus saprophyticus*

Optochin Test

- ❑ 5 µg (6 mm) optochin disks ("P" disks)
- ❑ Fresh broth cultures (18–24 hours old) of these recommended organisms:
  - □ *Streptococcus pneumoniae* (BSL-2)
  - □ *Streptococcus agalactiae* (BSL-2)

PROCEDURE

In this exercise, you will assess the susceptibility of test bacteria to one or more of the following: bacitracin, novobiocin, and optochin. Usually blood agar is used for these tests; however, for convenience and cost savings, TSA may be substituted in this lab. After inoculating the plates in a fashion that will generate a lawn of bacterial growth, you will place specific antibiotic disks in the center of the inoculum. Following incubation, you will measure the diameter of any resulting clear zones surrounding the disks and compare your findings to Tables 44.1, 44.2, and 44.3 to determine if your sample bacteria are susceptible or resistant to the tested compounds. Wear a lab coat, gloves, and eye protection when performing this procedure. **Use BSL-2 precautions as appropriate** for the organisms assigned. ***Caution!*** Follow BSL-2 precautions if using *S. aureus*, *S. pneumoniae*, or *S. agalactiae*.

TABLE **44.1** Bacitracin Test Results and Interpretations

Result	Interpretation	Symbol
Zone of clearing 10 mm or greater	Organism is susceptible to bacitracin; probable *Streptococcus pyogenes* if a β-hemolytic streptococcus, or *Micrococcus* species if a Gram-positive coccus	S
Zone of clearing less than 10 mm	Organism is resistant to bacitracin; not *Streptococcus pyogenes*; probable *Staphylococcus aureus* if Gram-positive coccus	R

TABLE **44.2** Novobiocin Test Results and Interpretations

Result	Interpretation	Symbol
Zone of clearing 16 mm or greater	Organism is susceptible to novobiocin; not likely *Staphylococcus saprophyticus* if a coagulase-negative staphylococcus	S
Zone of clearing less than 16 mm	Organism is resistant to novobiocin; probable *Staphylococcus saprophyticus* if a coagulase-negative staphylococcus	R

TABLE **44.3** Optochin Test Results and Interpretations

Result	Interpretation	Symbol
Zone of clearing 14 mm or greater	Organism is susceptible to optochin; probable *Streptococcus pneumoniae* if an α-hemolytic streptococcus	S
Zone of clearing less than 14 mm	Organism is resistant to optochin; not *Streptococcus pneumoniae* if an α-hemolytic streptococcus	R

1 **Obtain and Label the Plated Media:** For each drug being tested, you will need one TSA or blood agar plate and two test bacteria. Label the bottom of each plate with your name and the date. Then draw a line down the middle of the plate and label one half of the plate with the name of one of the test bacteria, and the other half with the name of the other bacterium. Make sure you have the correct organisms for each antimicrobial drug you were told to test!

2. **Inoculate Half of the Plate:** Gently mix the stock culture. Avoid splashing it into the tube's cap. Then, dip a sterile cotton-tipped swab into the bacterial stock. Before removing the swab, press the cotton tip against the inside of the tube to squeeze out excess fluid so that the swab is not dripping wet when removed. Inoculate the plate by making back-and-forth streaks that stop short of the line you drew on the bottom of the plate. Then, turn the plate and evenly run the swab over the same half of the plate in strokes perpendicular to those you previously made. Your goal is to thoroughly cover the half of the plate with bacteria so that after incubation it will resemble the growth shown in Figure 44.1.
3. **Inoculate the Other Half of the Plate:** Being careful not to mix the cultures, repeat the inoculation process described in Step 2 to introduce the second test organism to the other half of the plate. Allow the agar to absorb the broth for 5 minutes before proceeding to the next step.
4. **Apply Disks to the Plate:** Alcohol-flame the forceps by placing them in the Bunsen burner flame long enough to ignite the alcohol, and then remove them. ***Caution!*** Do not hold the forceps in the flame and do not hold them near the alcohol jar. If the alcohol jar should ignite, smother the flame with the jar's lid. Once the alcohol has burned off, use the forceps to place the appropriate antibiotic disk in the center of the half containing the first organism. Gently tap the disk into place to ensure that it makes full contact with the agar surface, but do *not* push the disks into the agar. Return the forceps to the alcohol and repeat the process for the other half of the plate.
5. **Repeat for Every Plate:** Repeat the inoculation and disk placement process (Steps 2–4) for the other plates.
6. **Incubate Your Plates:** Incubate your inverted plates at 35±2°C for 24 to 48 hours.
7. **Clean up:** Dispose of the materials as your instructor directs, disinfect your work surface, and thoroughly wash your hands with soap and water before leaving the lab.

→ Next Lab Session

1. **Examine the Samples:** Remove the plates from the incubator (or refrigerator). Hold each plate over a black, nonreflective surface and examine the plate. The edge of a zone is where no growth is visible to the naked eye. Measure the diameter of each zone of inhibition in millimeters.
2. **Record Your Observations:** Use Tables 44.1, 44.2, and 44.3 to help you record your results in the data table at the end of this exercise.
3. **Clean up:** Dispose of the plates as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 44

DATA SHEET

Bacitracin, Novobiocin, and Optochin Susceptibility Tests

During the Lab

1 Record the zone of inhibition diameters (mm) in the data table provided. Then, use Tables 44.1, 44.2, and 44.3 to help you assess if the bacterium was susceptible (S) or resistant (R) in its response to the tested antimicrobics.

Antibacterial Agent	Organism	Zone Diameter (mm)	Susceptible (S) or Resistant (R)
Bacitracin			
Novobiocin			
Optochin			

Lab Related Questions

1 The main application of bacitracin, novobiocin, and optochin susceptibility testing procedures is to:

a. Identify if a bacterium is a pathogen.

b. Determine if the bacterium is Gram positive.

c. Determine which drugs would be best for treating an infection caused by the tested bacterial isolate.

d. Presumptively identify the tested bacterial isolate.

e. Determine if the tested bacterial isolate can break down red blood cells.

2 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ Bacitracin, novobiocin, and optochin susceptibility testing procedures typically call for TSA media.

_____ Bacitracin, novobiocin, and optochin susceptibility testing procedures are performed on mixed bacterial cultures.

_____ Bacteria that have a ZOI around a drug-infused disk are automatically considered susceptible to the tested antimicrobial drug.

_____ Bacteria that lack a ZOI around a drug-infused disk are automatically considered resistant to the tested antimicrobial drug.

3 Why is it important to grow a bacterial lawn in this experiment rather than isolated colonies on the plated media?

4 Select the true statement(s) (select all that apply).

a. Bacitracin is usually administered in an ingested pill format.
b. Bacitracin is associated with renal toxicity.
c. Bacitracin would be useful to treat an *S. aureus* wound infection.
d. Bacitracin is mainly used as a topical preparation.
e. Bacitracin tests allow for a presumptive identification of *Streptococcus pyogenes*.

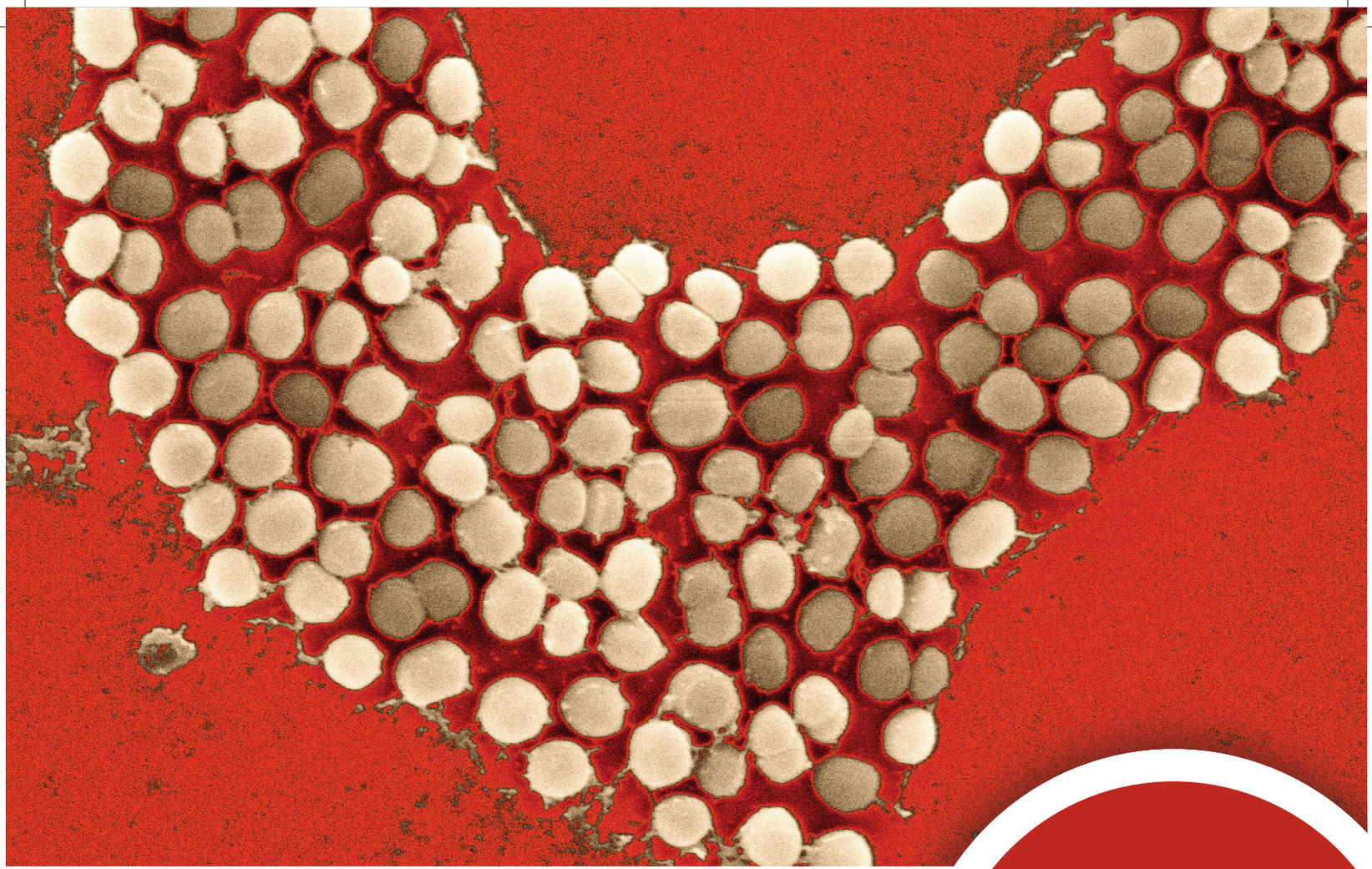
5 Assume you know your tested bacterium is sensitive to bacitracin and α-hemolytic. Based only on this data, what can you rule out as the bacterium's identity? (Select all that apply.)

a. *Streptococcus pyogenes*
b. *Staphylococcus aureus*
c. *Micrococcus luteus*
d. *Streptococcus pneumoniae*

Critical Thinking

1 Does the zone of inhibition's edge indicate the limit of the antibiotic's diffusion into the agar? Explain your reasoning.

2 What effect would using an older culture of *Streptococcus pyogenes* have on bacitracin susceptibility test results?



SECTION

8

Microbes Causing Trouble: Clinical Biofilms and Epidemics

In This Section

EXERCISE 45 Clinical Biofilms **409**

EXERCISE 46 Epidemic Simulation **413**

— Lab Notes —

8

EXERCISE 45

Clinical Biofilms

After doing this exercise, you should be able to:

1. Define the term *biofilm*.
2. State why biofilms represent a challenge in medicine.
3. Simulate the formation of a biofilm.

Background

A **biofilm** is a collection of one or more species of microorganisms and microbial products that have adhered to a surface. Biofilm development begins with formation of a **conditioning film** composed of biomolecules and particles from the environment on the surface. This results in the surface being more conducive to microbial adhesion. As **planktonic** (free-floating) microorganisms adhere to the surface, the biofilm grows.

The precise mechanism for cell adhesion varies depending on the amount of fluid movement and other factors; however, biofilm formation invariably depends upon the microbial community itself through secretion of an extracellular polysaccharide (**glycocalyx**). As more microbes attach, they contribute to the biofilm's chemistry, which in turn may further promote the adhesion of other microorganisms. As the biofilm matures, cells may detach from the film through erosion, abrasion, simple breakage, or physiological signals that promote planktonic cell release.

Biofilms occur in natural environments, but they also form in industrial and medical settings. Biofilm formation on indwelling medical devices (e.g., catheters) can lead to **health care-associated infections** (commonly called HAIs, or nosocomial infections), which are infections that develop while a patient is receiving care in a health-care facility. According to the U.S. Centers for Disease Control and Prevention, on any given day about one in every 31 hospital patients has one or more HAIs. Staphylococcal species are commonly associated with clinical biofilms, especially *Staphylococcus aureus* and *S. epidermidis*, which are normal residents of the skin microbiota and therefore easily transferred to medical devices.

The protective matrix and many layers of cells in a biofilm shelter the microbial residents from antimicrobial compounds and from the patient's immune defenses. Furthermore, microbes in biofilms seem to express antibiotic resistance factors at a greater level as compared to planktonic cells. This point combined with the physical sheltering from antimicrobics renders microbes in biofilms up to 1,000 times more resistant to antimicrobial drugs as compared to planktonic counterparts.[1] These features make biofilm-based infections especially challenging to eliminate. This point is even more alarming when we consider the estimate from the National Institutes of Health that biofilms account for 80% or more of microbial infections in the body.

[1] Gebreyohannes, G., A. Nyerere, C. Bii, and D. B. Sbhatu. "Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms," *Heliyon* 5 no. 8 (2019): e02192.

Clinical Applications

As previously mentioned, biofilms are a serious concern in medical settings, so understanding how they form is clinically important. *S. aureus* and *S. epidermidis* are notorious for forming biofilms on invasive medical devices, resulting in nosocomial infections.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Overnight broth cultures of *S. aureus* (BSL-2) and *S. epidermidis*
- ❑ Tubes of tryptic soy broth (TSB) enriched with 1% glucose (2)
- ❑ 50 mL phosphate buffered saline (PBS) working solution
- ❑ 50 mL 0.1% crystal violet
- ❑ Deionized water bottle
- ❑ Test-tube rack
- ❑ Receptacle for culture media disposal

Media, Stain, and Buffer Recipes

Tryptic Soy Broth (TSB) Supplemented with 1% Glucose

- Tryptic soy broth 1.0 L
- Glucose 10.0 g

0.1% Crystal Violet Stain

- Gram crystal violet (1%) 10.0 mL
- dH_2O 90.0 mL

10× Phosphate Buffered Saline (PBS)

- Na_2HPO_4, anhydrous, reagent grade 12.36 g
- $NaH_2PO_4 \cdot H_2O$, reagent grade 1.80 g
- NaCl, reagent grade 85.00 g

Dissolve ingredients in distilled water to a final volume of 1 L

PBS Working Solution (0.01 M Phosphate, pH 7.6)

- 10× PBS stock solution 100 mL
- dH_2O 900 mL

PROCEDURE

You will grow a mixed culture of *S. aureus* and *S. epidermidis* for up to 1 week in a test tube, stain it, and observe for evidence of a biofilm. Wear a lab coat, gloves, and chemical eye protection when performing this procedure. ***Caution!*** Follow BSL-2 precautions throughout this lab.

1. **Obtain Two Tubes of TSB + 1% Glucose:** Label the first tube of media as "*S. aureus* with *S. epidermidis*." Label the second tube "negative control." Write your name and the date on both tubes.
2. **Inoculate the Media:** Heavily inoculate the first TSB + 1% glucose tube with both *S. aureus* and *S. epidermidis*. ***Caution!*** Use BSL-2 precautions when transferring *S. aureus*. Do not inoculate the second tube of media.
3. **Incubate Your Samples:** Incubate the tubes for 24–48 hours at 35 ± 2°C.
4. **Clean up:** Dispose of stock samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.

8

→ Next Lab Session

1. **Obtain Your Samples and Decant the Liquid:** Place a disposal receptacle on a disinfectant-soaked towel. Then, carefully pour the broth media from each tube into the disposal receptacle. Avoid dripping the culture onto any surfaces. If you do spill or drip culture, clean it up with your lab's disinfectant and thoroughly wash your hands.
2. **Rinse the Tubes:** Gently rinse each tube with 1× PBS. Empty the buffer into the designated disposal receptacle. Repeat so that each tube is rinsed twice.
3. **Dry the Tubes:** Air-dry the tubes in an inverted position on a test-tube rack.
4. **Stain and Rinse the Tubes:** Stain both tubes with 0.1% crystal violet solution for 10 minutes at room temperature. To ensure full coverage of the tube, add more stain than the original volume of broth in the tubes. After 10 minutes, pour the dye into the designated receptacle. Then gently fill each tube with dH_2O and immediately pour the water into the designated receptacle. This rinse will remove unbound crystal violet. Again, air-dry the tubes in an inverted position.
5. **Examine Your Samples:** Compare your results with Figure 45.1. You are looking for a purple film adhering to the inside of the experimental tube. Ignore any dark ring at the top of the tube.
6. **Record Your Result:** Record your results in the data sheet at the end of this exercise.
7. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.



FIGURE **45.1** **Staphylococcal Biofilm** • The faint purple is the biofilm stained with dilute crystal violet. The tube on the right is an uninoculated control.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 45

DATA SHEET

Clinical Biofilms

During the Lab

1 Record your results for the biofilm test in the following table.

Tube	Observations
Control	
Staphylococcus mixture	

Lab Related Questions

1 A biofilm is best described as:

- **a.** A community of microbes in a solution.
- **b.** A community of microbes that causes disease.
- **c.** A community of microbes attached to an indwelling medical device.
- **d.** A community of microbes adhered to a surface.
- **e.** A community of antibiotic-resistant microbes.

2 Free-floating bacteria are called:

- **a.** Plantae.
- **b.** Plankton.
- **c.** Pluralistic.
- **d.** Planktonic.
- **e.** Planktic.

3 List two reasons why microbes in biofilms are difficult to eliminate.

_____

_____

_____

4 Assume you saw biofilm formation in the control tube. What could account for such an observation? (Select all that apply.)

- **a.** Your TSB-glucose media was contaminated.
- **b.** The dye stuck to the glucose in the media.
- **c.** The crystal violet dye solution was contaminated.
- **d.** You left the tubes in the incubator too long.
- **e.** You used non-sterile test tubes.

5 A polysaccharide-enriched extracellular structure called the _____ assists in biofilm formation.

Critical Thinking

1 Assume you forgot to add glucose to the TSB media. What results would you anticipate and why?

2 Suppose you forgot to add *S. epidermidis* to the media and instead double inoculated with *S. aureus*. What result would you expect? Explain your reasoning.

EXERCISE 46

Epidemic Simulation

After doing this exercise, you should be able to:

1. Define the term *epidemiology* as well as other basic terms such as *fomites*, *portal of entry*, and *index case*.
2. Describe the difference between direct and indirect transmission and provide examples of each.
3. Calculate the incidence and period prevalence of a simulated epidemic.
4. Determine the index case of a simulated epidemic.
5. Perform a simulated epidemic and interpret the test results.

Background

Epidemiology is the study of the causes, occurrence, transmission, distribution, and prevention of infectious and noninfectious diseases in populations. While there is no single approach to finding answers to these epidemiological questions, identification of the pathogen frequently happens first followed by an exploration of the pathogen's transmission mode.

Pathogens are transmitted to new hosts through direct or indirect mechanisms. **Direct contact transmission** occurs when a host is infected during a direct physical interaction with an infected host or an environmental source (e.g., person is bitten by an infected animal; sexual contact with an infected individual; vertical transmission from an infected mother to her fetus; bacteria in soil directly entering a wound). In contrast, **indirect contact transmission** does not require a physical encounter with an infected host. Instead, the infection may spread through the air (e.g., respiratory aerosols emitted by the coughs or sneezes of an infected person); via **fomites** (inanimate objects) like contaminated surfaces or dirty needles; through food and water (e.g., norovirus may get into food and spread without ever directly encountering an infected person); and via vectors (e.g., cockroaches, biting insects, etc.). The route the pathogen uses to gain access to the host's body and establish infection is referred to as a **portal of entry** (e.g., through the GI tract or respiratory tract). Some pathogens have more than one portal of entry.

When a disease is transmitted from a specific source to a population (e.g., a cooling system of a building spreading a pathogen to many inside the building), the resulting outbreak is called a **common source epidemic. Propagated transmission** is where the pathogen is transmitted from person to person. The **index case** (or patient 0) is the first documented disease case in an outbreak.

In this exercise, you will determine the index case of a simulated epidemic. You will also gather data and calculate **incidence** and **prevalence rates** for the "disease" spreading through your class.

Incidence rate is essentially a measure of the frequency of *new* disease cases in a population within a specified time period. We use the following equation to calculate incidence rate:

$$\textbf{Incidence rate} = \frac{\text{Number of new disease cases in a specified time period}}{\text{Number of people at risk for the disease at the midpoint of the time period}} \times 10^n$$

Because population size constantly changes, it is conventional to use the population size at the midpoint of the study period. Also, the usually small decimal fraction obtained for the rate is multiplied by some power of 10 (10^n) to get its value up to a number bigger than one. That is, an incidence rate of 0.00003 is multiplied by 100,000 (or 10^5) so it can be reported as three cases per 100,000 people per time period, rather than 0.00003 cases per person per time period.

You also will be calculating one form of prevalence rate called **period prevalence.** This measure helps us understand the burden of disease in a population during a specified period. Period prevalence is calculated as follows:

$$\textbf{Period Prevalence Rate} = \frac{\text{Number of existing cases in a specified time period}}{\text{Number of people at risk for the disease at the midpoint of the time period}} \times 10^n$$

As with incidence rate, the calculated prevalence is multiplied by some power of 10 (10^n) to bring the number up to one or more. Alternatively, if the average duration (D) of a disease and the incidence rate (I) are known, then prevalence (P) can be estimated as follows:

$$P = I \times D$$

Clinical Applications

Epidemiologists have the task of identifying the source of a disease and discovering the mode of its transmission. Further, epidemiologists characterize diseases quantitatively, using measures such as incidence and prevalence. Incidence rate indicates the probability an individual will contract the disease in the stated time, whereas prevalence is a useful indicator of the disease's presence in a population. Based on these and other information, epidemiologists can make recommendations for behaviors that can reduce disease transmission (e.g., boiling water before drinking it, wearing a mask while in public, etc.). Epidemiological data can also help medical scientists as they develop drugs, vaccines, and other therapeutics for specific diseases.

Procedure Modification

We selected *S. marcescens* as the transmissible agent in this lab because it is a BSL-1 bacterium with a distinctive color that is easy to detect upon culturing. ***Note:*** This bacterium is known to be an opportunistic infectious agent and proper precautions must be observed. **To avoid possible health risks, instructors can use Glo Germ powder or Glo Germ gel rather than bacteria.** In such a case, rather than noting bacterial growth as the measure of infection, UV light is used to detect Glo Germ on gloves after all handshakes in the simulation procedure have occurred. The Glo Germ-based procedure requires only one lab session whereas the bacteria-based procedure requires two sessions.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Each student will need a numbered petri plate containing one hard candy. Only one of the plates should contain a contaminated candy (a piece of candy swabbed with a culture of *Serratia marcescens*[1]); the other plates will contain a non-contaminated candy (candy swabbed with sterile saline). Number the petri plates according to the number of students in the class. Do not leave gaps in the numbering sequence.
- ❑ Tube of sterile saline (1 per student)
- ❑ Nutrient agar (NA) or tryptic soy agar (TSA) plate (1 per student)
- ❑ Sterile cotton-tipped swabs (2 per student)
- ❑ Marking pen
- ❑ Containers for swab and glove disposal should be located around the room so all students have easy access.

[1] Your instructor may substitute Glo Germ powder or gel for the "contaminant" on the candy. If this is the case, then sterile swabs and growth media are not necessary. Instead, after the contact transmission simulation, students use UV light to check if their gloved nondominant hand is contaminated with the glowing substance.

MATERIALS FOR GLO GERM ADAPTATION OF THE PROCEDURE

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Glo Germ powder or gel (sold by numerous vendors, including Carolina Biological and Fisher Scientific)
- ❑ Each student will need a numbered petri plate containing one hard candy. Only one of the plates should contain a contaminated candy (a piece of candy dipped in Glo Germ gel or powder) the other plates will contain a non-contaminated candy (candy swabbed with sterile saline). Number the petri plates according to the number of students in the class. Do not leave gaps in the numbering sequence.
- ❑ UV light to check for Glo Germ on gloved nondominant hand after the simulation

PROCEDURE

For your safety and for the safety of those around you, carefully follow the instructions in this lab. For enhanced safety, the instructor should orchestrate the entire lab so that steps are precisely performed, and students do each step at the same time. Before you begin, look at Figure 46.1 to see how to separate gloves safely. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.

Bacteria-Based Simulation Procedure

1. **Obtain the Materials:** Each student should obtain a petri plate containing candy, two sterile cotton-tipped swabs, a tube of sterile saline, and a nutrient agar or TSA plate. Circle the number of your candy plate in the "case" column of the data sheet at the end of this lab exercise. Once you have noted your "case number" put away all your papers and books until the lab is finished.
2. **Label the Media:** Use a marking pen to label the bottom of your petri plate with your name and the number of your candy plate. Then draw a line to divide the plate in half. Label the halves as "Side 1" and "Side 2," respectively.
3. **Open the Applicators:** Open a package of sterile, cotton-tipped swabs so they can be easily removed. You'll only need two swabs for this lab.
4. **Pre-Transmission Sampling of the Palm of Your Nondominant Gloved Hand:** After putting on your gloves, use your dominant hand to remove one

cotton-tipped swab, moisten it in the sterile saline, and sample the palm of your other gloved hand. Do not touch anything else with the palm of the glove. When finished, zigzag inoculate Side 1 of the plate with the cotton-tipped swab (see Fig. 4.6, p. 72). Discard the swab in an appropriate container. This side will serve as your pre-exposure control.

5. **Pick up the Candy:** Pick up the candy with your gloved nondominant hand. Roll the candy around in your hand until a good amount of it is transferred to the glove. Then, drop the candy back into the petri plate and close the lid with your dominant hand. **Touch nothing with your "contaminated" hand!** Just sit with your elbow on the table and your hand in the air. Be mindful that YOU might be the one with *S. marcescens* on your hand.

6. **Transmission Simulation Round 1:** The plate number identifies each student as a case number. Using "contaminated" hands only, Case #1 rubs hands with Case #2, making sure to transfer anything that may be on the palm of the glove to the palm of the other student's glove. Both students must hold on to the cuff of their glove throughout this step so it doesn't come off. After a few seconds of rubbing, gently slide gloved hands apart, as shown in Figure 46.1. **Do not "snap" the gloves as they separate because this will produce aerosols.** Case #1 should go back to their desk and sit with the elbow of their "contaminated" hand on the table so that their hand is in the air and not making contact with anything.

7. **Subsequent Transmission Simulations:** Next, Case #2 rubs gloved hands with Case #3, and #3 with #4, and so on until all students have contacted "contaminated" hands. Continue to be careful not to snap the gloves when you separate. Hold on to the cuff when transferring and separating to prevent the glove from coming off your hand.

8. **Ending the Transmission Simulation:** When all students have rubbed hands, the last (highest numbered) case should rub hands with Case #1.

9. **Post-Transmission Sampling of the Palm of your Gloved Nondominant Hand:** With a second sterile cotton-tipped swab, sample the palm of your "contaminated" hand as before and inoculate Side 2 of the plate. Be sure to sample the area of the glove that contacted other students' gloves. Dispose of the cotton swab in the nearest appropriate autoclave container. Do not walk across the room with the swab.

10. **Remove Your Gloves:** Remove your gloves by inserting the thumb of your dominant hand under the cuff of the "contaminated" glove and rolling it off your hand, inverting it in the process (see Fig. S.4, p. 6). Remove the other glove using the same rolling method. Dispose of the gloves properly.

11. **Wash Your Hands:** Wash your hands thoroughly (preferably with antiseptic soap, if available).

12. **Incubate Your Sample**: Tape the lid on your inoculated plate. Invert the inoculated plate and incubate it at 25°C for 24 to 48 hours.

13. **Clean up:** Discard all materials in appropriate containers. Be sure to tape the lid on your candy plate before you dispose of it. Wipe down your work area with disinfectant.

14. **Wash Your Hands *Again*:** After cleaning up, thoroughly wash your hands again.

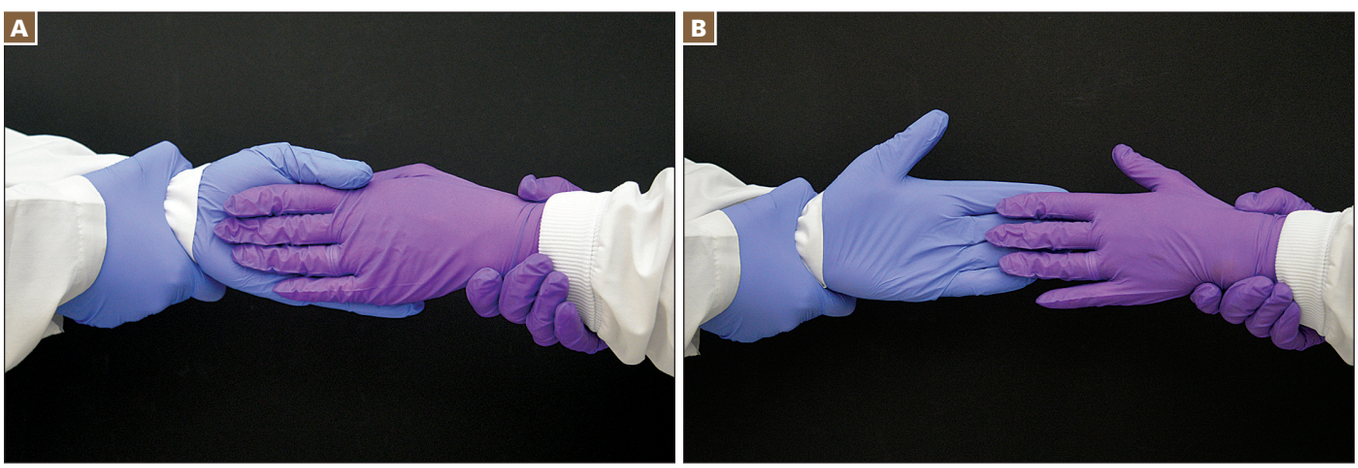


FIGURE **46.1** **Sliding the Gloves Apart** • After rubbing palms, students must separate by gently sliding their gloved hands apart (as shown in **A** and **B**). Hold on to the glove's cuff to prevent it from coming off. These safety precautions must be followed and are designed to prevent the gloves from snapping apart (they will be sticky) and producing aerosols.

Next Lab Session

1. **Obtain Your Samples:** Remove the plates from the incubator and examine them for characteristic reddish growth of *S. marcescens*.
2. **Examine Your Samples:** Examine Side 1 of your plate for the characteristic red-orange growth of *S. marcescens*. Notify your instructor if you see *S. marcescens* growth. If your plate shows *S. marcescens* growth on Side 2, enter a "+" next to your case number on the data sheet at the end of this exercise. If there is no evidence of *S. marcescens* growth, enter a "–" instead.
3. **Share Your Result:** All class members will need to report if they had *S. marcescens* growth on Side 2 of their plate. Your instructor may do a roll call, have you post results on the board, or use some other mechanism for dispensing this information. Enter class results on your data sheet at the end of this exercise, and follow the instructions for establishing the index case, incidence rate, and period prevalence.
4. **Clean up:** Dispose of the samples as your instructor directs, disinfect your work surface, and thoroughly wash your hands.



Glo Germ-Based Simulation Procedure

1. **Obtain the Materials:** Each student should obtain a petri plate containing a candy. Circle the number of your candy plate in the "case" column of the data sheet at the end of this lab exercise. Once you have noted your "case number," put away all your papers and books until the lab is finished.
2. **Pre-Transmission Observation of Nondominant Gloved Hand:** After putting on your gloves, observe your nondominant gloved hand under the UV light. If you observe any glowing, wash your gloved hand with plain water. After air-drying the gloved hand, reobserve it under the UV light to ensure your hand is "glow free" at the start of the simulation. Once you have established that your nondominant hand is free of any glowing substance(s), do not touch anything else with the palm of the glove.
3. **Pick up the Candy:** Pick up the candy with your gloved nondominant hand. Roll the candy around in your hand until a good amount of it is transferred to the glove. Then, drop the candy back into the petri plate and close the lid with your dominant hand. **Touch nothing with your "contaminated" hand!** Just sit with your elbow on the table and your hand in the air. Be mindful that YOU might be the one with the Glow Germ on your hand.
4. **Transmission Simulation Round 1:** The plate number identifies each student as a case number. Using "contaminated" hands only, Case #1 rubs hands with Case #2, making sure to transfer anything that may be on the palm of the glove to the palm of the other student's glove. Both students must hold on to the cuff of their glove throughout this step so it doesn't come off. After a few seconds of rubbing, gently slide gloved hands apart, as shown in Figure 46.1. **Do not "snap" the gloves as they separate because this will produce aerosols.** Case #1 should go back to their desk and sit with the elbow of their "contaminated" hand on the table so that their hand is in the air and not making contact with anything.
5. **Subsequent Transmission Simulations:** Next, Case #2 rubs gloved hands with Case #3, and #3 with #4, and so on until all students have contacted "contaminated" hands. Continue to be careful not to snap the gloves when you separate. Hold on to the cuff when transferring and separating to prevent the glove from coming off your hand.
6. **Ending the Transmission Simulation:** When all students have rubbed hands, the last (highest numbered) case should rub hands with Case #1.
7. **Observation of Your Gloved Nondominant Hand:** Place your gloved nondominant hand under a UV light to check for Glo Germ. If you observe glowing particles on the glove, enter a "+" next to your case number on the data sheet at the end of this exercise. If there is no evidence of *glowing*, enter a "–" instead.
8. **Remove Your Gloves:** Remove your gloves by inserting the thumb of your dominant hand under the cuff of the "contaminated" glove and rolling it off your hand, inverting it in the process (see Fig. S.4, p. 6). Remove the other glove using the same rolling method. Dispose of the gloves properly.
9. **Clean up:** Discard all materials in appropriate containers and thoroughly wash your hands.
10. **Share Your Result:** After the simulation is completed, all class members will need to report if they had Glo Germ on their nondominant hand. Your instructor may do a roll call, have you post results on the board, or use some other mechanism for dispensing this information. Enter class results on your data sheet at the end of this exercise, and follow the instructions for establishing the index case, incidence rate, and period prevalence.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 46

DATA SHEET

Epidemic Simulation

During the Lab

1 **Before You Begin:** Circle your "case number" in the following table. Your case number is written on the petri plate you were given that contains the candy.

Case	Week	Disease (+/–)	Case	Week	Disease (+/–)	Case	Week	Disease (+/–)	Case	Week	Disease (+/–)
1	1		11	3		21	4		31	7	
2	1		12	3		22	4		32	7	
3	1		13	3		23	5		33	7	
4	1		14	3		24	5		34	7	
5	2		15	3		25	5		35	8	
6	2		16	4		26	5		36	8	
7	2		17	4		27	5		37	8	
8	2		18	4		28	6		38	9	
9	2		19	4		29	6		39	9	
10	3		20	4		30	6		40	9	

2 **If you used the Glow Germ procedure:** Note glowing on the gloved nondominant hand with a "+" as an indication of infection. If there is no glowing, consider the person to be healthy and record a "–" in the appropriate box.

3 **If you did NOT use the Glo Germ procedure, analyze the data as follows:**

a. **Observe Side 1 of your plate:** After the plates have been incubated, compile data for your class population (each case is the student's candy number in the exercise). The organism spread during contact between student pairs was *Serratia marcescens*, which produces reddish-orange colonies. Check Side 1 of your plate for evidence of *S. marcescens* growth. Notify your instructor if you see any.

b. **Observe Side 2 of your plate:** You will likely see growth on both sides of the plate, but red colonies are the only sign of "disease." If there is reddish-orange growth on Side 2 of the plate, consider the person to have the "disease" and record a "+" in the appropriate box. If there is no red growth on the plate or there is no difference between the two sides, consider the person to be healthy and record a "–" in the appropriate box.

4 **After reviewing all the class data, deduce which case is the index case**:

Case# _____ was the index case.

5 **Use the class data to calculate the incidence and period prevalence rates of your class population:** For this purpose, assume that the cases have been identified over a 9-week period (or a shorter period, depending on how many students participated in the simulation). Also assume that the duration of the disease is 7 days, so new cases in 1 week are still diseased in the next week but are healthy by the following week.

Record the population size: _____

Use the table provided to record incidence and point prevalence rates for each week. The incidence and period prevalence rates should be reported as "cases per 10^n per week."

Week	Incidence Rate	Period Prevalence Rate
1		
2		
3		
4		
5		
6		
7		
8		
9		

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 46
DATA SHEET
(continued)

Lab Related Questions

1 Choose the true statement(s) (select all that apply).

a. Epidemiologists perform work that reveals the source of a disease.
b. Epidemiology includes the study of noninfectious disease occurrence in populations.
c. Epidemiologists develop vaccines for diseases.
d. Epidemiology includes the study of infectious disease occurrence in populations.
e. Epidemiological data includes information on how diseases are transmitted in populations.

2 Which of the following disease features would contribute to a higher incidence rate? (Select all that apply.)

a. A noninfectious agent was the cause of disease.
b. An infectious causative agent was the cause of disease.
c. The disease is mainly transmitted by indirect contact.
d. The disease is mainly transmitted by direct contact.
e. The disease is transmitted the best before obvious signs and symptoms manifest in an infected person.

3 Assume you conducted the simulation as described in this exercise, but with only 6 total students. The data reveal that only Students #2 and #3 remained uninfected. In such a scenario, who was the index case?

a. Student 1
b. Student 3
c. Student 4
d. Student 5
e. Student 6

4 The index case is best described as:

a. The first patient to die of a particular disease in a population.
b. The first documented disease case in an outbreak.
c. The first patient ever diagnosed with a given disease.
d. The first patient to receive a vaccine against a given disease.
e. The first patient to exhibit symptoms of a specific disease in a population.

5 If you know the period prevalence rate and the duration of a disease then you know/can determine (select all that apply):

a. Incidence rate.
b. Mode of transmission.
c. The number of people at risk for the disease at the midpoint of the time period.
d. The index case.
e. The disease source.

6 During the COVID-19 pandemic, a simple calculation called the *percent positive* was a useful figure for understanding disease burden in a population. In most cases, the recommendation was that health departments work to maintain a percent positive of less than 5%. The percent positive is calculated as follows:

$$\textbf{Percent Positive} = \frac{\text{Number of people who had a positive test result in a specified time period}}{\text{Total number of people tested in the time period}} \times 100$$

Based on this information, which scenario(s) would increase the percent positive measure? (Select all that apply.)

a. Decreased disease cases in a population and increased testing in that same population.
b. Decreased disease cases in a population and decreased testing in that same population.
c. Increased disease cases in a population and increased testing in that same population.
d. Increased disease cases in a population and decreased testing in that same population.

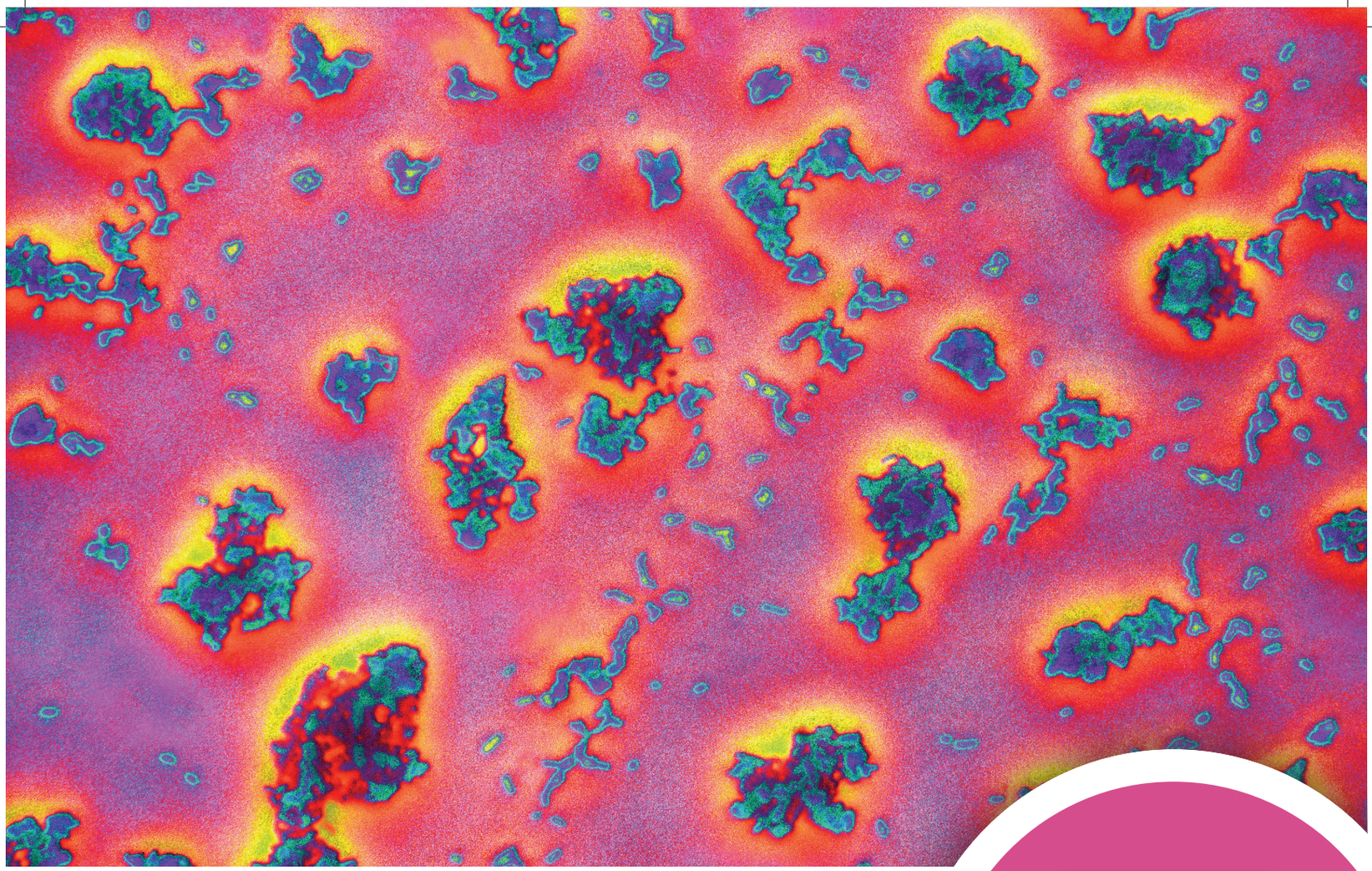
7 Assume 10 students participated in the simulation described in this lab exercise and the index case was Student #10. In such a scenario, who among the following should show evidence of being "infected" in the simulation? (Select all that apply.)

a. Student 1
b. Student 2
c. Student 8
d. Student 9
e. Student 10

Critical Thinking

1 We made numerous assumptions about the nature of the "disease" in this simulation. List three assumptions that were inherent to the simulation.

2 If a disease's average duration remains constant, then how would an effective preventive measure that reduces disease incidence likely affect disease prevalence? Explain your reasoning.



SECTION

9

Identifying Unknowns

In This Section

EXERCISE 47 Case-Based Unknown (Dry Lab) **423**

EXERCISE 48 Bacterial Unknown Exercise and Resources **431**

— Lab Notes —

EXERCISE 47

Case-Based Unknown (Dry Lab)

After doing this exercise, you should be able to:

1. Discuss three misconceptions that you should avoid if using a dichotomous key identification strategy.
2. Explain why a battery of biochemical tests is normally inoculated for identifying an unknown bacterium.
3. State what the API test system is and how it generally works.
4. Apply your knowledge of biochemical tests to determine the identity of a theoretical unknown.

Background

The underlying goal of all clinical microbiology laboratory efforts is to provide accurate information as quickly as possible, delays and inaccurate data cost lives. Data accuracy is in no small part affected by the sample collection process at the point of care; it makes no difference how skilled the microbiology team is if the health-care provider fails to practice proper aseptic techniques and sample collection protocols. During the laboratory part of your microbiology training, you have been practicing aseptic techniques that are essential to saving lives; you'll apply them in patient care as well if you are required to collect a sample for microbiological analysis.

Most of the methods you have learned about in this laboratory course are microscopy- and culture-based. While such methods remain valid assessment tools, they are often accompanied by a wide variety of molecular-based tools (e.g., genetic analysis, serological studies, etc.).

Ultimately, clinical suspicion drives what tests the clinical microbiologist will perform. That is, the clinician collects specific patient samples (e.g., blood, urine, etc.) based on the nature of the patient's signs and symptoms. For example, a urine sample is collected when a urinary tract infection (UTI) is suspected. A throat swab is performed when strep throat (streptococcal pharyngitis) needs to be ruled out. A sample of cerebrospinal fluid is often collected if the patient is experiencing signs and symptoms of meningitis. Because clinical suspicion is really the driving force for clinical microbiological analysis, it's worthwhile for you to perform an "unknown" analysis with some infusion of that context. In this lab, you'll follow a patient from signs and symptoms to definitive diagnosis using microscopy and biochemical test data to inform clinical decisions.

Dichotomous Key Strategies: Avoiding Misconceptions

Dichotomous keys, like that shown in Figure 47.1, are a common approach to teaching students about identifying a bacterial unknown.[1] These branching-style maps are useful because they provide a visual representation of how one species differs from another. In that spirit, some of you may be asked to develop a dichotomous key to show that you understand how certain bacteria differ from one another. While dichotomous keys have their place in bacterial identification, they have the potential to perpetuate several misconceptions about the bacterial identification process:

1. Students may see such keys and conclude that one biochemical test is performed at a time until the bacterium's identity is determined;
2. Dichotomous keys imply a rank-and-file order/hierarchy for performing biochemical tests (always start with test A, then do B, followed by C, etc.); and
3. Students may perceive that tests earlier in the key are more accurate/reliable than those situated lower in the key.

The reality is that once a pure culture is obtained, a battery of biochemical tests is normally inoculated all at once and interpreted as soon as possible. There is not a stepwise, test-by-test inoculation. There are good reasons for this:

1. If clinical lab workers embraced a stepwise approach (inoculated one or two biochemical tests per day based on the data of a previous test), it may be more economical in terms of resources used, but it could *easily* triple the time required to determine the bacterium's identity.

[1] In addition to the dichotomous key in this exercise, there is a collection of dichotomous keys provided in the appendix of this book. Your instructor may have you use them for other lab exercises.

2. The types of tests performed hinge largely on clinical suspicion. For example, the clinical lab team will not analyze a urine sample with the intention of differentiating pathogens that cause UTIs from those that cause strep throat. That is, not all bacteria are reasonable suspects, the analysis is almost always narrowed based on the clinical presentation. In such cases, there is usually an empiric battery of tests—tests that experience has repeatedly shown are likely to lead to the correct identification of the culprit. Of course, if those empiric tests are inconclusive (which *can* happen), then further testing is necessary. That said, such a dead end would be detected in 24–48 hours by the battery approach versus a week of "if/then" testing that occurs in stages; time saved is lives saved.
3. No test is 100% accurate 100% of the time. Based on your experiences in the microbiology lab, this point may seem obvious; after all, you've learned there are always sources of error that can range from media formulations to strain variations to everything in between. Based on this fact alone, it doesn't make sense to hinge your testing workflow on a one-test-at-a-time strategy. What if the first test you performed yielded inaccurate results? You would probably waste a lot of time before figuring out the error, if you figured that out at all. Thus, you are *far* more likely to correctly identify your unknown if you perform a *collection* of tests and see where the closest match lies based on the various pieces of data.
4. Even if a particular bacterial species is noted as being positive for a particular test, that doesn't mean all isolates of the species are positive; the room for variation is often 10%. As many as 10% of the strains of a species listed as positive or negative on a test give the opposite result. That is, 90% of strains (genetic variants of the same species) may have a positive test result for a specific biochemical test while 10% of isolates may generate a negative test result. Indeed, perfect alignment is exceedingly rare. So, embarking on a

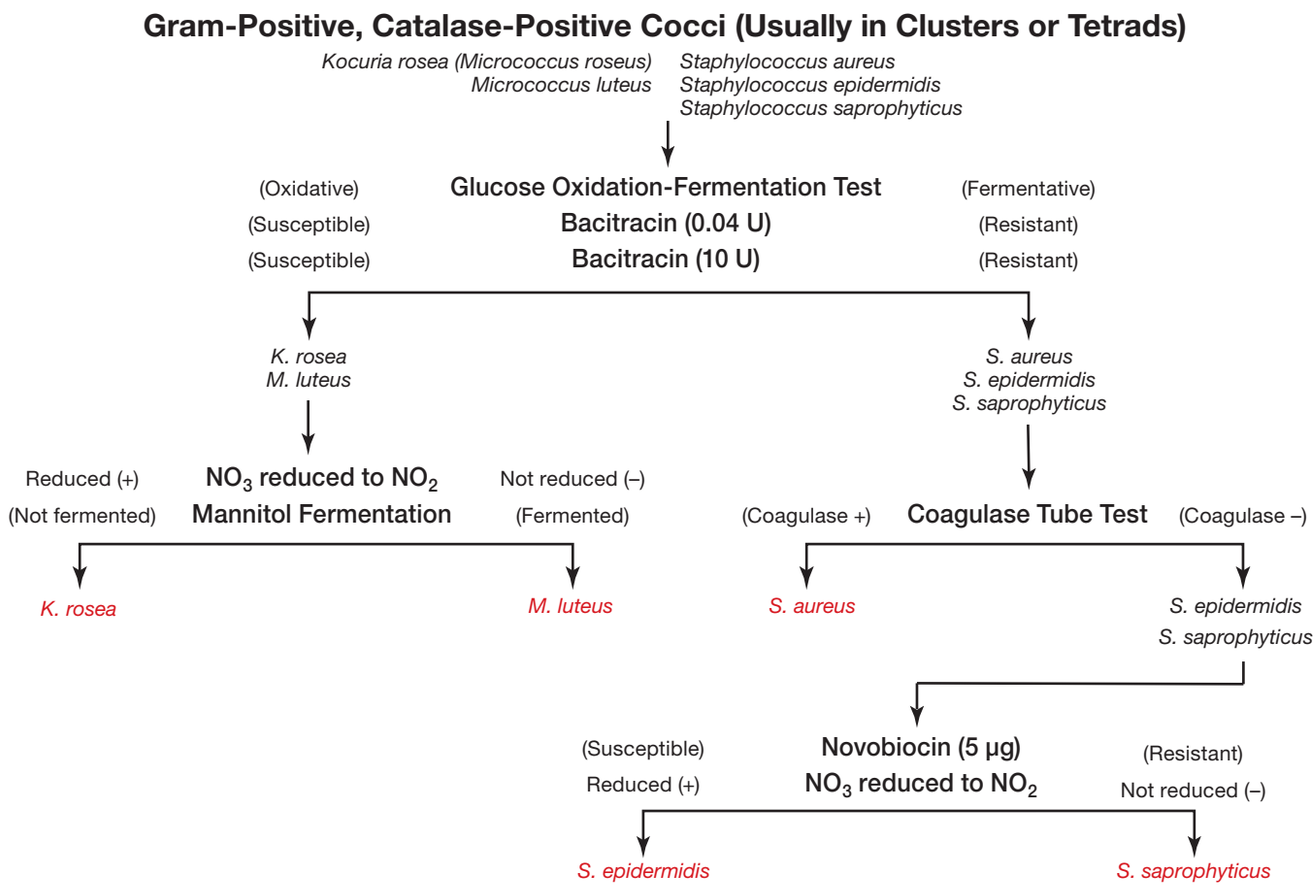


FIGURE **47.1** **Dichotomous Key Flowchart for Gram-Positive, Catalase-Positive Cocci (Usually in Tetrads or Clusters)** • A dichotomous key approach can be useful for bacterial identification. For example, this dichotomous key could be useful for discovering the identity of a pure culture of Gram-positive, catalase-positive, cocci arranged in clusters (or tetrads).

one-test-at-a-time strategy increases the risk of inaccurately identifying the isolate. It's much safer to consider all the data together rather than taking a piecemeal approach.

Based on these four points, when analyzing biochemical test results many clinical labs lean toward a "closest fit" approach rather than a traditional dichotomous key approach. In the closest fit approach, the data from a battery of biochemical tests are entered into a computer program, which then compares the reported data to one or more databases to determine the statistically most likely candidate (or candidates). One of the more widely used examples of this technology is the API system (Analytical Profile Index system manufactured by bioMérieux) (Fig. 47.2).

To more closely simulate the clinical lab bacterial identification strategy, this lab exercise will not use a dichotomous key strategy. You will instead analyze a clinical case, decide which battery of tests are most appropriate to isolate and identify the potential pathogen, and proceed from there with your analysis to determine the specimen's identity.

Clinical Applications

This lab provides a way for you to walk through the clinical specimen identification process. The process of identifying a pathogen in a sample typically begins with a health-care provider making the decision to collect a sample for microbiological analysis. That decision is usually made based on the signs and symptoms the patient is experiencing. Once the sample is collected, it is conveyed to a clinical microbiology lab. There the specimen is typically inoculated onto various media for isolation. Once an isolated candidate is grown, it is usually subjected to staining and microscopy to ascertain features like Gram property, cell shape, and cellular arrangement. Then, if the sample requires further analysis to make a definitive identification, it may be subjected to further biochemical testing.

MATERIALS

Because this is a "dry lab" (meaning the exercise doesn't require reagents or other lab materials), it can be an excellent way to gain a better grasp of the process before you are required to perform a bacterial identification lab with a real sample. Dry labs are also a great way to practice your problem-solving skills before you are called to use those skills in higher-stake settings with possibly dangerous biological and chemical substances.

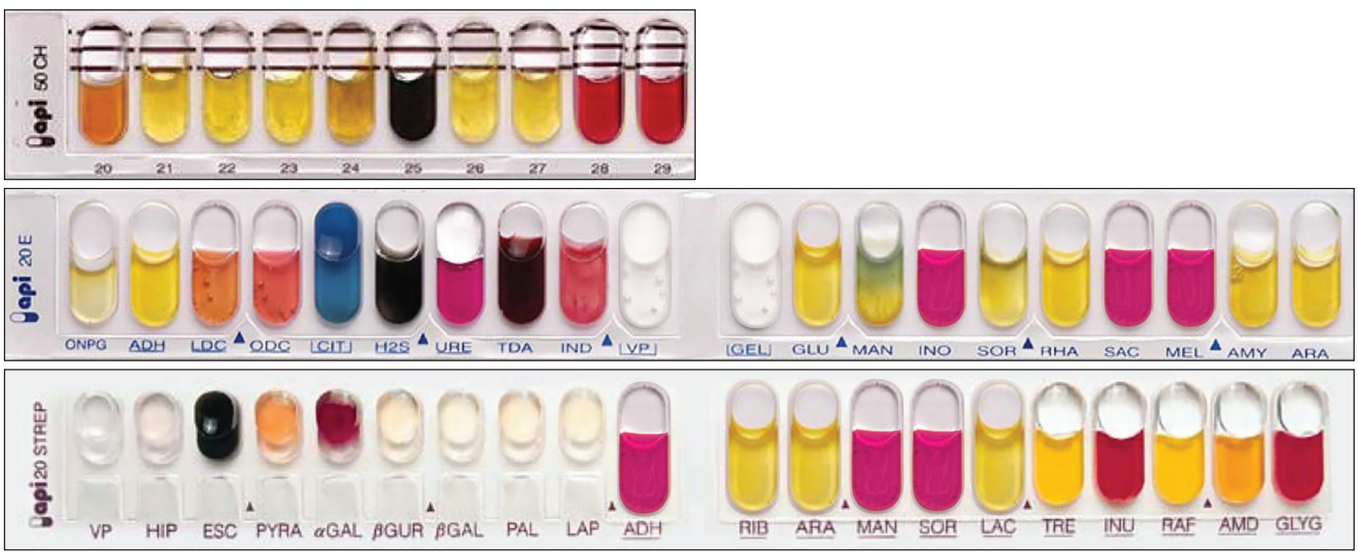


FIGURE **47.2** **API Test Strips** • Although only three examples of API test strips are shown, many other API test strips are available; most deliver test results in fewer than 24 hours. Before reading the test results, the microbiologist processing the samples would add specific reagents to certain cupules on the strip. If you look closely, you may notice that under each cupule there is an abbreviation that represents the test name. You may even recognize some of them. For example, CIT is the citrate test and GLU stands for the glucose fermentation test.

PROCEDURE

Read the case sections and perform the analyses as instructed.

At the Patient's Bedside

A 68-year-old man is brought to the emergency department (ED) of the hospital where you work. He reports a severe stabbing pain in his lower back, nausea without vomiting, and blood in his urine. CT scan imaging revealed two kidney stones (renal calculi) in the left kidney, as well as one stone lodged in the urethra and another stuck in the distal portion of the left ureter. He is running a fever and has underlying health conditions such as diabetes and heart disease. The ED doctor orders urine and blood samples to be collected for microbiological analysis as well as for other tests. Once the samples are collected, the doctor starts the patient on a broad spectrum intravenous antibacterial drug and admits him for inpatient hospital care.

In the Clinical Microbiology Lab

Day_0: Same Day as Sample Collection

You are responsible for analyzing the patient's urine sample and detecting the pathogen of interest. You start with a Gram stain of the centrifuged urine sample. You observe the following via light microscopy (Fig. 47.3):



FIGURE **47.3** **Gram Stain of Centrifuged Urine Sample**

1. Gram property = _____
2. Cell shape = _____
3. Cellular arrangement = _____
4. Assuming oil immersion microscopy with a 10× ocular and 100× objective, what would the final magnification be for the image? _____
5. Based on the Gram-stain and morphological information, you can make an informed decision about your next steps. For each of the following, state if you would or would not perform the procedure and explain the reasoning behind your decision. Remember, performing unnecessary tests takes time and costs your lab money, so you would only perform tests that would help you further narrow down the bacterium's identity.

a Inoculate the sample onto mannitol salt agar (MSA).

b Test the bacteria for coagulase.

c Test the bacteria for oxidase.

d Perform an acid-fast stain.

e Inoculate the sample onto eosin methylene blue agar (EMB).

At the Patient's Bedside

The infectious disease specialist familiarizes themselves with their newly admitted patient's case. In the meantime, the assigned urologist also visits the patient. They consult on the care plan for their shared patient. Their plan primarily centers on removing the lodged kidney stone within the next 12 hours while simultaneously managing the infection that has developed. The infectious disease doctor states that the Gram-stain data suggests a narrower-spectrum drug might be a more desirable option as compared to the broad-spectrum drug started in the ED.

However, until they have more data on the identity of the bacterium causing the infection and its antimicrobial drug susceptibility profile, they will keep the patient on the broad-spectrum antibacterial drug initiated in the ED.

6 Look up information on narrow-spectrum versus broad-spectrum drugs. Explain why a narrow-spectrum drug could be preferable to a broad-spectrum drug?

7 Based on this information, how would you likely describe the coloration of the growth on MacConkey agar? Explain the reasoning behind your response. (***Note:*** To answer this question, assume you are only describing the color of a single colony of the suspected pathogen. Do not concern yourself with colony morphology.)

In the Clinical Microbiology Lab

Day$_1$: Approximately 24 hours after Sample Collection

Upon arriving at the lab today, you check your various cultures from yesterday. You observe that you have successfully isolated a bacterium that could be the pathogen responsible for the patient's urinary tract infection. The suspected bacterium appears to ferment lactose when grown on MacConkey agar.

Today your work will focus on fine tuning the bacterium's identity and performing drug susceptibility testing. Provided all goes well, you should know the bacterium's identity and drug susceptibility data by tomorrow, at which point you can send the microbiology report to the attending health-care team.

The lab where you work has an API system. This helps to reduce the time for identification because the tests are miniaturized and require shorter incubation periods. Some of the API test strip panels can return biochemical test results in as few as 4 hours. To help you decide which API test strip you should use, you refer to the following chart (Fig. 47.4):

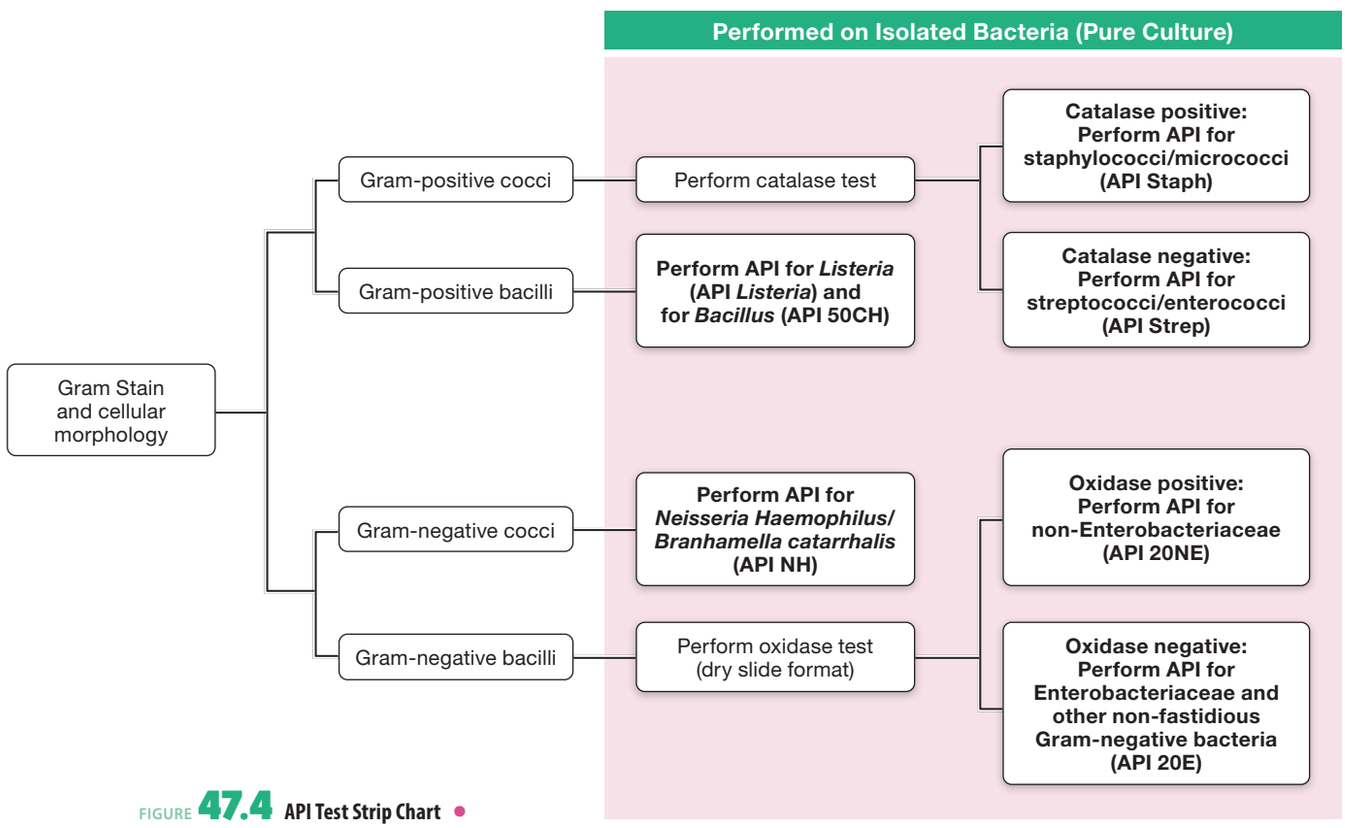


FIGURE **47.4** API Test Strip Chart

8 Based on the data you have thus far and considering the chart information, what is your next step (e.g., what API strip and/or test should you conduct)?

At the Patient's Bedside

The kidney stone was successfully removed. A temporary stent was placed in the ureter to keep it open as it heals from the trauma inflicted by the lodged stone. The urologist and infectious disease specialist are now concerned about the patient's infection and hope that by tomorrow the microbiological data will be ready.

In the Clinical Microbiology Lab

Day_2: Approximately 48 hours after Sample Collection

Today you are ready to analyze the biochemical test data to hopefully arrive at a definitive bacterial identification. You also will have some drug susceptibility data to send to the health-care team.

You retrieve the API test strip from the incubator, add reagents to the cupules per the manufacturer's specification, and observe the results in Figure 47.5.

Table 47.1 presents details for each test on the API strip you used to analyze the isolated bacterium. Unfortunately, your intern just dripped some reagents on the document and blurred a few areas. The good news is you have your lab manual from your undergraduate microbiology course available and your experience with biochemical tests, so you can fill in the few spaces where the information was blurred (numbers 9 through 13 in the table).

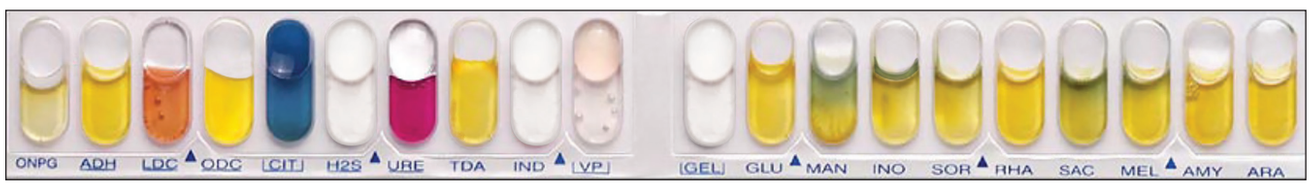


FIGURE **47.5** **API Test Strip Results for Bacterium Isolated from Case Study Patient's Urine**

TABLE **47.1** **Results for Various Biochemical Tests**

Test (Abbreviation)	Detects	Negative Result	Positive Result
ONPG	β-galactosidase (lactose hydrolysis)	Colorless	Yellow (pale color is possible)
ADH	Arginine dihydrolase	Yellow	Red or orange
LDC	Lysine decarboxylase	Yellow	Red or orange
ODC	Ornithine decarboxylase	Yellow	Red or orange
CIT	Citrate utilization	**9.**	**10.**
H_2S	Hydrogen sulfide (H_2S) production	Colorless	**11.**
URE	Urea hydrolysis	**12.**	**13.**
TDA	Tryptophan deamination	Yellow	Brown
IND	Indole production	Colorless/light yellow	Pink or red
VP	Acetoin production (Voges-Proskauer test)	Colorless	Pink or red (pale color is possible)
GEL	Gelatin hydrolysis	Colorless	Black (pigment diffuses)

(continues)

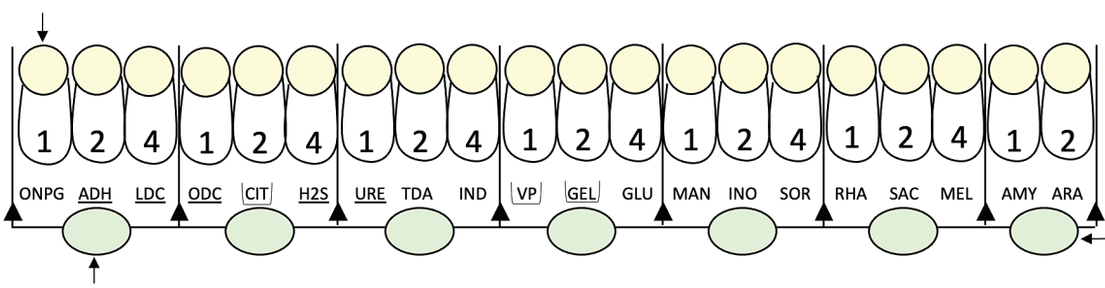
TABLE **47.1** *(continued)* **Results for Various Biochemical Tests**

Test (Abbreviation)	Detects	Negative Result	Positive Result
GLU	Glucose fermentation/oxidation	Blue	Yellow
MAN	Mannitol fermentation/oxidation	Blue	Yellow
INO	Inositol fermentation/oxidation	Blue	Yellow
SOR	Sorbitol fermentation/oxidation	Blue	Yellow
RHA	Rhamnose fermentation/oxidation	Blue	Yellow
SAC	Sucrose fermentation/oxidation	Blue	Yellow
MEL	Melibiose fermentation/oxidation	Blue	Yellow
AMY	Amagdalin fermentation/oxidation	Blue	Yellow
ARA	Arabinose fermentation/oxidation	Blue	Yellow

14 Finally, you're ready to analyze the test results and determine the bacterium's identity. Observe the API test results (Fig. 47.5) and use your completed table (Table 47.1) to fill out the following data recording sheet.

API RESULTS Sample Origin: _____ Date Inoculated: _____

Step 1: In each yellow circle, write a "+" for each positive result and a "–" for each negative result.



If the oxidase test was positive, add 4 to this last number. Note, the oxidase test is not on the test strip, but counts toward the final ID number.

Step 2: Add the numbers for the *positive* (+) test results for each section and write the sum in each green oval. For example, if all three tests in the first section (ONPG, ADH, and LDC) were positive, you'd write a "7" in the first green oval. If only ODC and H2S were positive for the second section, you'd write a "5" in the second green oval. Do this until each green oval has a numerical value. If none of the tests in a section are positive, enter a "0" for the section.

Step 3: To determine the bacterium's identity, look at Table 47.2 and find the 7-digit numerical sequence generated by the green ovals.

Step 4: Bacterium's most likely identity: _____

TABLE **47.2** API Identification Table*

Bacterium	ID number	Bacterium	ID number
Xanthomonas axonopodis	0001000	*Pantoea ananatis*	1245573
Erwinia tracheiphila	0004520	*Pasteurella haemolytica*	1422000
Erwinia amylovora	0005020	*Stenotrophomonas maltophilia*	1422351
Morganella morganii	0174000	*Citrobacter freundii*	1604573
Pseudomonas solanacearum	0201004	*Pseudomonas aeruginosa*	2214006
Erwinia salicis	0204731	*Pasteurella multocida*	3000004
Pseudomonas rubrilineans	0211004	*Vibrio hollisae*	3042140
Abiotrophia adiacens	0402000	*Enterobacter cloacae*	3305773
Streptococcus acidominimus	0470000	*Enterobacter agglomerans*	3307173
Proteus mirabilis	0736000	*Aeromonas hydrophila /caviae*	3577754
Xanthomonas albilineans	1001000	*Hafnia alvei*	4105112
Escherichia adecarboxylata	1004173	*Edwardsiella tarda*	4744000
Erwinia stewartii	1005162	*Streptococcus mutans*	5040710
Corynebacterium fascians	1010000	*Escherichia coli*	5144572
Agrobacterium tumefaciens	1010007	*Klebsiella pneumoniae*	5215773
Agrobacterium radiobacter	1015046	*Serratia marcescens*	5306563
Pasteurella pneumotropica	1020300	*Chryseomonas luteola*	5477761
Bordetella bronchiseptica	1200063	*Aerococcus viridans*	6112711
Serratia rubidaea	1207360	*Salmonella choleraesuis*	6704510
Erwinia chrysantherni	1244173	*Vibrio parahaemolyticus*	7467746

* These API codes are simply examples, they are for teaching purposes only and do not necessarily represent the most recent API code data maintained in the subscription-based API database.

At the Patient's Bedside

The infectious disease doctor reported the microbiology findings to the rest of the health-care team. Based on the microbiology data, the bacterium proved susceptible to several common oral antibiotics. The care providers agreed that the patient could be discharged from the hospital and sent home with a three-day course of ciprofloxacin. The patient fully recovered, although he did experience two more kidney infections over the next 5 years.

15 Look up the bacterium that you identified as the causative agent and describe some of the features that can make it a challenging pathogen to combat.



EXERCISE 48

Bacterial Unknown Exercise and Resources

After doing the unknown exercise students typically are able to:

1. Perform various staining protocols and biochemical tests on a bacterial unknown sample.
2. Use a light microscope to make morphological and staining observations.
3. Record and analyze observations for staining and biochemical tests to discover the identity of an unknown bacterial sample.
4. Report observations and conclusions for a variety of staining procedures and biochemical tests.

Background

A capstone laboratory experience in most undergraduate microbiology courses is the identification of a bacterial unknown. While the approach your instructor uses for this sort of exercise may vary, the general principle is the same: the student applies their newly developed microbiology lab skills to determine the identity of a bacterial sample. The sample may be provided to you as a pure culture or as a mixed culture.

Most instructors have students perform this lab once the class has some experience with staining methods and students have learned how to perform and interpret a variety of biochemical tests. In some courses, you may complete an unknown project at a midpoint in the course and then a more sophisticated unknown project closer to the end of the course. In other courses, you may simply have one unknown project that serves as a laboratory capstone experience.

What to Expect During an Unknown Exercise

You can expect to perform a variety of biochemical tests and stains on a bacterial specimen issued to you and then use the data from those procedures to decide your bacterium's identity. Typically, this exercise is done over two or more lab sessions. You should not expect much guidance from your instructor; rather this is *your* opportunity to solve a real-world problem with your own acquired skills. You've got great new skills and now is your chance to show them off!

In most cases, the better you are at implementing the lab techniques you explored in your course, the more likely you are to correctly identify your bacterial specimen's identity. However, correctly identifying your bacterium's identity is often just one part of your score. Usually your instructor will be looking at your lab writeup or report to see if you truly know how to perform and interpret a variety of biochemical tests and staining protocols. They will also be checking that you are detail-oriented and thorough in terms of how you report your observations and formulate conclusions. And of course, you are always expected to use the proper language and writing conventions for your documentation.

Useful Resources

Refer to the notecards at the end of the book, you'll likely find them useful as you undertake this exercise. These notecards can also be cut out and used as a study resource! Additional resources that you'll likely find useful for this exercise include the Instructional Staining Atlas (pp. 131–135) along with the identification flowcharts in Appendices F–I (pp. 487–500).

MATERIALS

- ❑ Lab coat
- ❑ Gloves
- ❑ Safety glasses
- ❑ Unknown mixture with number (1, your instructor will advise you whether the sample is pure or mixed)
- ❑ Appropriate stains
- ❑ Appropriate biochemical test media and reagents
- ❑ Recommended organisms (samples may be mixed or pure as your instructor decides)

Gram-Positive Candidates

- □ *Bacillus cereus*
- □ *Bacillus coagulans*
- □ *Bacillus subtilis*
- □ *Corynebacterium xerosis*
- □ *Enterococcus faecalis* (BSL-2)
- □ *Kocuria rosea*
- □ *Lactobacillus plantarum*
- □ *Micrococcus luteus*
- □ *Mycobacterium smegmatis* (BSL-2)
- □ *Staphylococcus aureus* (BSL-2)
- □ *Staphylococcus epidermidis*
- □ *Streptococcus salivarius*

(continues)

MATERIALS *(continued)*

Gram-Negative Candidates

- ☐ *Citrobacter freundii*
- ☐ *Enterobacter aerogenes* (BSL-2)
- ☐ *Enterobacter cloacae*
- ☐ *Escherichia coli*
- ☐ *Hafnia alvei*
- ☐ *Klebsiella pneumoniae* (BSL-2)
- ☐ *Morganella morganii* (BSL-2)
- ☐ *Proteus mirabilis* (BSL-2)
- ☐ *Proteus vulgaris* (BSL-2)
- ☐ *Providencia stuartii*
- ☐ *Serratia liquefaciens*
- ☐ *Serratia marcescens*

Other bacteria as decided by your instructor

- ☐ _____
- ☐ _____
- ☐ _____

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 48

DATA SHEET



PROCEDURE

Unknown Report

Unknown Sample # _____

My instructor wants us to work in a group of: _____ (You'll usually work either alone or in a group of two, but this is at your instructor's discretion.)



CAUTION!

Unless your instructor has specified otherwise, assume your sample could be a BSL-2 bacterium and follow all BSL-2 precautions.

STEP 1: OBTAIN A PURE CULTURE AND NOTE COLONY FEATURES

The provided unknown culture is: mixed/pure (select one).
If you are given a mixed culture, your instructor will *likely* tell you how many bacteria are in the sample, you should take note of that information. If you have a mixed culture, then you will need to perform a streak plate isolation to detect the constituents.

Observe the culture characteristics of your pure culture.
Once you have a pure culture with isolated colonies, report features such as the color, shape, elevation, shine or no shine, and borders of the colonies.

Write your observations here (refer to Ex. 6 for help).

Size: _____

Shape (form): _____

Margin (edge): _____

Elevation: _____

Consistency: _____

Surface Texture: _____

Other useful features (e.g., color, optical properties): _____

STEP 2: PERFORM STAINS ON YOUR PURE CULTURE AND MAKE OBSERVATIONS

Place a checkmark next to the stains your instructor wants you to perform and write your observations and conclusions in the following table for each stain you perform.

Stain	Observations
Simple (See Ex. 11)	
Negative (See Ex. 12)	
Gram (See Ex. 13)	
Acid-Fast (See Ex. 14)	
Capsule Stain (See Ex. 15)	
Endospore Stain (See Ex. 16)	

Based on your observations, write a final description of the cellular morphology and specific structures and/or cell wall properties of your bacterium:

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

STEP 3: PERFORM BIOCHEMICAL TESTS ON YOUR PURE CULTURE AND MAKE OBSERVATIONS

Place a checkmark next to the plated media and various biochemical tests your instructor will have available for you to assess your sample.

Differential/Selective Media

On pages 441–443, you will find four summary notecards of differential and selective media. These can be cut out and mounted onto 4 × 6 or 5 × 8 notecards as a reference tool during the lab.

Media	Observations	Conclusions
Mannitol Salt Agar (MSA) (See Ex. 22)		
MacConkey (MAC) Agar (See Ex. 23)		
Eosin Methylene Blue (EMB) Agar (See Ex. 24)		
Blood Agar (See Ex. 25)		

Biochemical Tests

On pages 445–461, you will find 13 summary notecards of biochemical tests. These can be cut out and mounted onto 4 × 6 or 5 × 8 notecards, or other cardstock as a reference tool during the lab.

Test	Observations	Conclusions
Glucose (Dextrose) Fermentation Phenol Red Broth (See Ex. 27)		
Lactose Fermentation Phenol Red Broth (See Ex. 27)		
Sucrose Fermentation Phenol Red Broth (See Ex. 27)		
Methyl Red and Voges-Proskauer (MR-VP) Tests (See Ex. 28)	**MR:** **VP:**	**MR:** **VP:**
Catalase Test (See Ex. 29)		
Oxidase Test (See Ex. 30)		
Nitrate Reduction Test (See Ex. 31)		
Citrate Utilization (See Ex. 32)		
Lysine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		
Ornithine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		
Arginine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

Test	Observations	Conclusions
Starch Hydrolysis (Amylase Test) (See Ex. 34)		
Urea Hydrolysis (Urease Test) (See Ex. 35)		
SIM Medium: Sulfur, Indole, and Motility Tests (See Ex. 36)	**H_2S:** **Indole:** **Motility:**	**H_2S:** **Indole:** **Motility:**
Triple Sugar Iron Agar/Kligler Iron Agar (See Ex. 37)		
Coagulase Test (See Ex. 38)		
Gelatin Hydrolysis (Gelatinase Test) (See Ex. 39)		

OTHER TESTS

Test name	Observations	Conclusions

STEP 4: USE YOUR DATA TO DETERMINE THE BACTERIUM'S IDENTITY

Use the flow charts in Appendices F–I (pp. 487–500) to determine which bacterium you most likely have. Note that you are unlikely to have a perfect match, your task is to find the best match.

Bacterium's Identity: _____ (include genus and species)

STEP 5: REFLECTIVE ANALYSIS

1 What test results for this exercise did *not* align with your conclusion about the bacterium's identity?

2 Regarding the data you stated were incongruent with your conclusion, explain what may have gone wrong.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

3 Provide some background about the bacterium you identified. Include the following in your discussion:

a. Morphological features

b. Gram property

c. Places the bacterium can naturally be found

d. Clinical, environmental, and/or industrial significance of the bacterium

e. Three additional interesting facts about the bacterium

— Lab Notes —

A Summarized Guide of Differential/ Selective Media for the Identification of an Unknown Bacterial Sample

Notecards

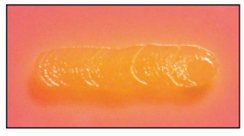
Mannitol Salt Agar (MSA) (From Ex. 22)

Purpose: This media is selective for salt-tolerant organisms such as *Staphylococcus*, *Bacillus*, *Enterococcus*, *Micrococcus*, and others. MSA also contains the sugar mannitol, so those bacteria that grow on the media can be differentiated based on their ability to ferment mannitol.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the media's color around the colonies.

Results	Interpretations
Poor growth (P) or no growth (N)	The organism's growth is inhibited by NaCl; the bacterium is unlikely to be a *Staphylococcus* species.
Good growth (G)	The organism's growth is not inhibited by NaCl. It's possible the bacterium is a *Staphylococcus* species, or some other bacterium known to grow well on MSA (e.g., a *Bacillus*, *Enterococcus*, *Micrococcus* species).
Yellow growth or halo (Y) 	The organism fermented mannitol; the pH was lowered by acids generated during mannitol fermentation. The bacterium may be pathogenic *Staphylococcus aureus* (further testing needed to make this conclusion; many *Staphylococcus* species ferment mannitol and some ferment none; *Staphylococcus* bacteria may ferment mannitol).
Red growth (no halo) (R) 	The organism does not ferment mannitol. The organism is unlikely to be *S. aureus*.

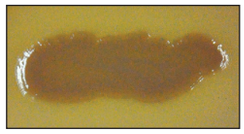
MacConkey (MAC) Agar (From Ex. 23)

Purpose: This media is selective for Gram-negative bacteria and selective based on the fermentation of lactose. MAC is one example of an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the bacterial growth's color.

Results	Interpretations
Poor growth (P) or no growth (N)	Gram-positive bacterium
Good growth (G)	Gram-negative bacterium
Pink to red growth with or without bile precipitate (R) 	The organism produces acid as a byproduct of lactose fermentation; the bacterium may be a coliform bacterium.
Growth is "colorless" (not red or pink) (C) 	The organism does not ferment lactose; it is unlikely to be a coliform.

9

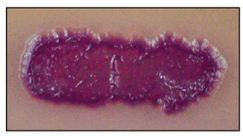
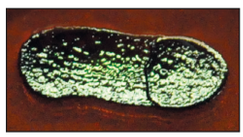
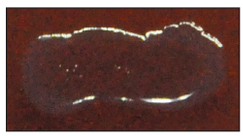
Eosin Methylene Blue (EMB) Agar (From Ex. 24)

Purpose: EMB is an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample. Like MAC agar, EMB is selective for Gram-negative bacteria and differential based on the fermentation of lactose.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the bacterial growth's color.

Results	Interpretations
Poor growth (P) or no growth (N)	Gram-positive bacterium
Good growth (G)	Gram-negative bacterium
Growth is pink and mucoid (Pi) 	The organism produces acid as a byproduct of lactose fermentation and is possibly a coliform bacterium.
Growth is "dark" (purple to black, with or without green metallic sheen) (D) 	The organism produces acid as a byproduct of lactose fermentation and is probably a coliform bacterium.
Growth is "colorless" (not purple, green, red, or pink) (C)  **(*Note:* EMB media has a reddish color so do not mistake the color of the media as being the color of the growth.)**	The organism does not ferment lactose and is unlikely to be a coliform bacterium.

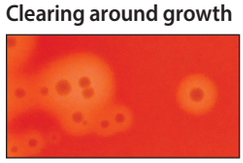
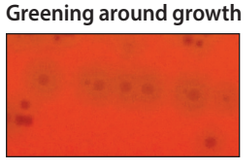
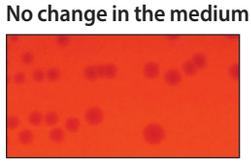
Blood Agar (From Ex. 25)

Purpose: Blood agar detects hemolytic activity and is commonly used to differentiate β-hemolytic, pathogenic *Streptococcus* species from non-pathogenic species of this diverse genus of Gram-positive cocci.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate your sample at 35±2°C for 24 hours.

Interpretations: After incubation, assess the color of the media around the bacterial growth.

Results	Interpretations
Clearing around growth 	The organism hemolyzes RBCs completely (β-hemolysis).
Greening around growth 	The organism partially hemolyzes RBCs; hemoglobin oxidized to methemoglobin (α-hemolysis).
No change in the medium 	The organism does not hemolyze RBCs (γ-hemolysis).

Notecards

A Summarized Guide of Biochemical Tests for the Identification of an Unknown Bacterial Sample

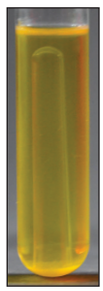
Phenol Red Fermentation Broth: Glucose, Lactose, and Sucrose Fermentation Tests (From Ex. 27)

Purpose: These tests detect the enzymes needed to ferment a given carbohydrate. If a given carbohydrate is fermented, then acids are made that will lower the media's pH. As pH drops, the pH indicator in the media (phenol red) turns from its original red color to yellow. If the bacteria do not ferment the given carbohydrate, then the media remains red. If gas is produced as a by-product of fermentation, then the Durham tube will have a bubble in it.

How to Inoculate: All carbohydrate test media should be inoculated with the transfer loop.

Incubation Parameters: Incubate the sample at 35±2°C for 48 hours.

Interpretations: When making interpretations, make sure you note media coloration as well as note if gas is present or absent in the Durham tube.

Results	Interpretations
 **Yellow broth; bubble in Durham tube**	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. If lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase. Note the result as A/G.
 **Yellow broth; no bubble in Durham tube**	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. If lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase. Note the result as A/–.
 **Red broth; no bubble in Durham tube**	The carbohydrate (insert sugar's name) was not fermented and gas was not produced. Note the result as –/–.
 **Pink broth; no bubble in Durham tube**	Degradation of peptone occurred to form alkaline end products, but the carbohydrate (insert sugar's name) was not fermented; gas was not produced. Note the result as K/–.

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Methyl Red and Voges-Proskauer (MR-VP) Tests (From Ex. 28)

Purpose: The methyl red and Voges-Proskauer tests (MR-VP) help us differentiate bacterial species based on the fermentation pathway used to metabolize glucose. The MR portion of the test detects if mixed acid fermentation occurred. The Voges-Proskauer test reveals if a bacterium metabolized glucose via the butanediol fermentation pathway.

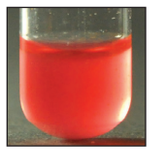
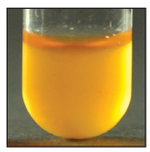
How to Inoculate: Inoculate the MR-VP broth with a transfer loop.

Incubation Parameters: Incubate the sample at 35±2°C for 48 hours.

Test Development Process: For this test, only one tube of media is inoculated. After incubation, 1 mL of the culture is transferred to a clean test tube to serve as the MR portion of the test and another 1 mL of culture is transferred to a tube and analyzed for the VP portion of the test. Follow the development protocols in the following tables for the MR and VP test, respectively.

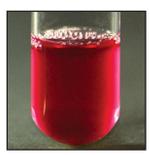
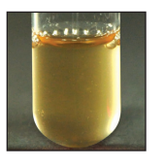
Methyl Red Test

- Add 3 drops of methyl red reagent to the sample and immediately observe for the development of a red color.

Results	Interpretations
 **Red**	Organism performs a mixed acid fermentation. Note this as a positive result (+).
 **No color change**	Organism does not perform a mixed acid fermentation; acidic end products were not made in detectable amounts. Note this as a negative result (–).

Voges-Proskauer Test

- Add 15 drops (0.6 mL) of VP Reagent A. Mix well.
- Add 5 drops (0.2 mL) of VP Reagent B. Mix well.
- Observe for red color formation at 10-minute intervals for up to 1 hour. Periodically mix the sample.

Results	Interpretations
 **Red**	Organism performs a 2,3-butanediol fermentation (acetoin was produced). Note this as a positive result (+).
 **No color change**	Organism does not perform a 2,3-butanediol fermentation (acetoin was not produced or it is not detectable). Note this as a negative result (–).

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Catalase Test (From Ex. 29)

Purpose: This test detects catalase, an enzyme responsible for protecting bacteria from hydrogen peroxide (H_2O_2) accumulation, which can occur during aerobic metabolism. If hydrogen peroxide accumulates, it becomes toxic to the organism. Catalase converts H_2O_2 to water and gaseous oxygen.

Test Development Process: Test fresh (18- to 24-hour) agar slant cultures. Inoculation and incubation are not required. This test is often performed by adding hydrogen peroxide to a bacterial smear on a microscope slide. If using this "slide method," cover the sample after adding hydrogen peroxide so that you reduce workspace contamination from the aerosols a positive test may generate.

Another way to perform the catalase test is to add hydrogen peroxide directly to bacteria growing on an agar slant or agar plate (do not use cultures growing on blood agar or you will likely get a false-positive result). Adding H_2O_2 directly to the culture will render the sample unusable for further tests. If you need the culture for further testing, then use the slide method for this test.

Results	Interpretations
 **Bubbles immediately develop**	Catalase is present; the organism is aerobic. Note this as a positive result (+).
 **No bubbles detected**	Catalase is absent or not detectable. (***Note:*** You *cannot* conclude the bacterium is anaerobic just because it lacks catalase!) Note this as a negative result (–).

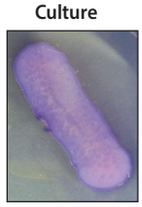
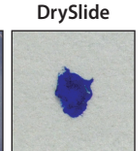
Oxidase Test (From Ex. 30)

Purpose: The oxidase test reveals if a bacterium has cytochrome c oxidase, an enzyme in certain aerobic electron transport chains. Bacteria that have cytochrome c oxidase respire aerobically. However, a negative oxidase test result only means the bacterium lacks cytochrome c oxidase; it does not necessarily mean the bacteria cannot respire aerobically using some other ETC that doesn't require cytochrome c oxidase.

Test Development Process: There are several ways to perform the oxidase test:

1. Add the TMPD reagent directly to bacterial growth on solid media.
2. Smear the test bacterium onto filter paper and then add TMPD to the smear.
3. Smear the test bacterium onto paper infused with the TMPD reagent. (Commercially available DrySlide.)

Regardless of the approach applied, if cytochrome c oxidase is present a color change to purple or blue occurs within seconds as TMPD is oxidized.

Results	Interpretations
Culture  DrySlide  **Dark blue/purple color develops within 20 seconds**	Cytochrome c oxidase is present; the organism can perform aerobic respiration. Note this as a positive result (+).
Culture  DrySlide  **No color change to blue/purple within 20 seconds**	Cytochrome c oxidase is not present or not detectable. (***Note:*** Do *not* assume the tested bacterium is anaerobic; the bacterium may have alternative aerobic ETCs that do not require cytochrome c oxidase.) Note this as a negative result (–).

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Nitrate Reduction Test (From Ex. 31)

Purpose: The nitrate test allows us to differentiate bacterial species from one another based on their ability to reduce nitrate (NO_3^-). An inverted Durham tube in each broth serves to trap a portion of any produced gas.

How to Inoculate: Inoculate nitrate broth with a transfer loop.

Incubation Parameters: Incubate the sample at 35±2°C for 24 to 48 hours.

Test Development Process: Following incubation, analyze the test via a three-step process.

1. **Check for a Bubble in the Durham Tube:** If there is a bubble *and* you are certain the bacterium does *not* conduct fermentation, then you may safely conclude the tested organism reduces nitrate to gaseous end products such as molecular nitrogen (N_2) or nitrous oxide (N_2O). In most cases, you will need to move to Step 2. (See table Development Process 1.)
2. **Add Reagents A and B:** If you are *not* certain as to whether your bacterium conducts fermentation or not, then you must add various reagents to the sample. Add 8 drops (approximately 0.5 mL) of Reagent A and then 8 drops (approximately 0.5 mL) of Reagent B to the tube and mix well. If you see a red color after adding Reagents A and B, then you may conclude the tested bacterium reduces nitrate to nitrite. (See table Development Process 2.)
3. **Add Reagent C if Red Color Does NOT Develop During Step 2 Above:** If you do *not* see a color change to red after adding Reagents A and B in Step 2, then add a small amount of nitrate Reagent C (zinc dust) to determine if the bacterium reduced nitrate beyond nitrite. You can dip a wooden applicator into the zinc dust and transfer the amount that clings to the wood. Let the tubes stand for 10 minutes. If a red color develops, then the organism did *not* reduce nitrate. Lack of a color change after adding Reagent C indicates the organism reduced nitrate to NH_4^+, NO, N_2O, or some other nitrogenous compound other than nitrite. (See Development Process 3 in the following table.)

Development Process	Results	Interpretations
1. Check for a Bubble in the Durham Tube.	Gas in Durham tube (no reagents added). (***Note:*** Only use this measure if you are certain the tested bacterium does *not* conduct fermentation. If you do not know the fermentation status of the bacterium, proceed to Development Process 2.)	If you are certain the test bacterium does *not* perform fermentation, then you may conclude denitrification occurred ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$). Note this as a positive result (+).
2. Add Reagents A and B.	 Red color after adding Reagents A and B. (The test is completed; no further reagents should be added.)	Nitrate reduction to nitrite ($NO_3^- \rightarrow NO_2^-$); nitrate reductase is present. Note this as a positive result (+).
	 No color change after adding Reagents A and B. (Proceed to Development Process 3.)	Incomplete test; requires the addition of zinc dust. (A test result cannot be determined yet. Proceed to adding Reagent C, Development Process 3.)
3. Add Reagent C if Red Color Does NOT Develop During Step 2.	 No color change after adding Reagent C. (The test is completed; no further reagents should be added.)	Nitrate reduction to gaseous (NO, ½N_2O, or ½N_2) or reduction to nongaseous nitrogen compounds such as ammonium (NH_4^+) occurred. Note this as a positive result (+).
	 Red color after adding Reagent C. (The test is completed; no further reagents should be added.)	No nitrate reduction. Note this as a negative result (–).

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Citrate Utilization Test (From Ex. 32)

Purpose: This biochemical test detects the ability of bacteria to use citrate as a sole carbon source. If the bacterium cannot use citrate as its sole carbon source, then it will not grow and the media will remain a greenish color. If the bacterium can use citrate as its sole carbon source, then the bacteria will grow and the media will turn a bright blue as a result of an increase in the pH of the media.

How to Inoculate: Use an inoculating loop to streak the bacteria onto citrate media in a zigzag fashion along the slant surface ("fishtail inoculation"). To avoid confusion between actual growth and a heavy inoculum, inoculate lightly.

Incubation Parameters: Incubate the sample at 35±2°C for 48 hours.

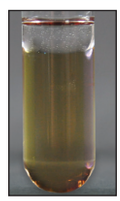
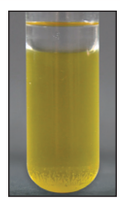
Results		Interpretations
	**Media appears blue (even if only a portion of the media turned blue) and growth is evident on the slant surface.**	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source). Note this as a positive result (+).
	**No color change, but growth is evident on the slant surface.**	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source). Note this as a positive result (+).
	**No color change and no growth on the slant surface.**	Citrate is not utilized (organism lacks one or more of the enzymes needed to use citrate as its sole carbon source). Note this as a negative result (–).

Lysine/Ornithine/Arginine Amino Acid Decarboxylation (Decarboxylase Tests) (From Ex. 33)

Purpose: This biochemical test allows us to examine the ability of bacteria to decarboxylate the amino acid lysine using the enzyme lysine decarboxylase. As optional procedures, you may also test for the presence of ornithine decarboxylase and arginine decarboxylase in the assigned bacteria. (***Note:*** Each decarboxylase is specific to its own amino acid substrate.)

How to Inoculate: Use an inoculating loop to inoculate the broth decarboxylase media. After inoculation, an overlay of mineral oil is added to seal the medium from the air. This ensures the surface of the media's pH is not artificially raised (alkalized) through interactions with the ambient air and it helps to maintain a lower oxygen environment that favors glucose fermentation.

Incubation Parameters: Incubate the sample at 35±2°C for 96 hours (4 days) up to a full week.

Results		Interpretations
	**No color change**	No decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).
	**Yellow**	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).
	**Purple (may be slight)**	Decarboxylation of the amino acid (insert amino acid name)*; organism produces the specific decarboxylase enzyme. Note this as a positive result (+).

* The amino acid included in the media can vary. Therefore, describe all interpretations as they relate to the specific amino acid in the media. Example: If using lysine decarboxylase media, the amino acid in the media is lysine.

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Starch Hydrolysis (Amylase Test) (From Ex. 34)

Purpose: The starch hydrolysis test (or amylase test) reveals if a bacterium can break down the complex polysaccharide starch.

How to Inoculate: Use an inoculating loop to inoculate starch agar with the test bacterium. You may use a streak plate isolation method, or if you have a pure culture, you may use a zigzag inoculation.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 hours.

Test Development Process: After incubation, cover the growth and surrounding areas with Gram's iodine. Do not flood the plate but do add enough iodine so that the agar is completely colorized. *Allow the iodine to soak in.* After the agar has soaked up the iodine, check for a clear zone around the bacterial growth.

Results	Interpretations
 **A clearing around the growth is evident after adding iodine to the plate.**	α-amylase, β-amylase, and/or α-1,6-glucosidase is present (or more simply, amylase is present). Note this as a positive result (+).
 **There is not a clearing around the growth after adding iodine to the plate.**	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name); the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).

Urea Hydrolysis (Urease Test) (From Ex. 35)

Purpose: This test reveals if a bacterium produces the intracellular hydrolytic enzyme urease, which uses water to catabolize (break down) urea to form ammonia and carbon dioxide.

How to Inoculate: Use an inoculating loop to inoculate urea media.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 hours.

Results	Interpretations
 **Orange or yellow**	No urea hydrolysis; urease is absent or not produced in enough quantity to produce results in 24 hours; or the organism cannot live in urease broth. Note this as a negative result (–).
 **Pink**	Rapid urea hydrolysis; urease was produced. Note this as a positive result (+).

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SIM Medium (Sulfur, Indole, and Motility Tests) (From Ex. 36)

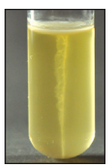
Purpose: This media assesses a bacterium's ability to reduce sulfur, make indole, and move.

How to Inoculate: Use an inoculating needle to stab the SIM media in as straight a line as possible through the middle of the agar deep. Insert the needle into the agar to within 1 cm of the bottom of the tube. Be careful to remove the needle along the original stab line so that you obtain a straight inoculation.

Incubation Parameters: Incubate the sample at 35±2°C for 24 to 48 hours.

Sulfur Reduction (H_2S Production)—The "S" in SIM

- In addition to SIM media, TSI (triple sugar iron) media can also be used to assess sulfur reduction.

Results	Interpretations
 **No black in the medium**	Sulfur is not reduced, or is not reduced in detectable amounts. Note this as a negative result (–).
 **Black in the medium**	Sulfur reduction occurred (H_2S was made); organism either respires anaerobically with sulfate as the final electron acceptor (thiosulfate reductase) or hydrolyzes the amino acid cysteine (cysteine desulfurase). Note this as a positive result (+).

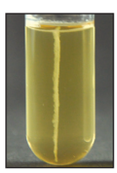
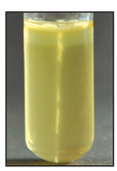
Indole Production—The "I" in SIM

- Add Kovac's reagent to each SIM media tube (including the control) to a depth of 2 mm–3 mm (*not mL*). After several minutes, observe for the formation of red color in the reagent layer.

Results	Interpretations
 **No red layer evident after adding Kovac's reagent**	Tryptophanase is absent and indole is not produced, or it's produced but not in detectable amounts. Note this as a negative result (–).
 **Red layer evident after adding Kovac's reagent**	The organism produces tryptophanase and hydrolyzes tryptophan into indole, NH_3, and pyruvate. Note this as a positive result (+).

Motility—The "M" in SIM

- In addition to SIM media, general motility agar may be used to assess motility. Unlike SIM media, general motility agar only reveals information about a bacterium's motility.

Results	Interpretations
 **No growth radiating away from the original inoculation/ stab line.**	Nonmotile. Note this as a negative result (–).
 **Growth radiating outward from inoculation/stab line.**	Motile. Note this as a positive result (+).
Blackened medium	Motility cannot be determined; use general motility agar to assess motility. See Exercise 36 for details on general motility agar.

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Triple Sugar Iron Agar (TSIA)/Kligler Iron Agar (KIA) (From Ex. 37)

Purpose: Differentiates bacteria based on sulfur reduction and sugar fermentation.

How to Inoculate: Both TSIA and KIA are prepared as a shallow agar slant with a deep butt. This format provides both aerobic and anaerobic growth environments. Both media are double inoculated; first use an inoculating needle to stab the butt of the agar and then use a loop to zigzag inoculate the slant ("fishtail inoculation").

Incubation Parameters: For carbohydrate fermentation assessment, incubate the sample at 35±2°C for 18–24 hours. After interpreting the carbohydrate fermentation results, samples are re-incubated for an additional 24 hours at 35±2°C before making a final H_2S determination.

Results	Interpretations
 **Yellow slant/yellow butt—KIA**	KIA: Glucose **and** lactose fermentation with acid accumulation in slant and butt. Note this test result as A/A.
	TSIA: Glucose and lactose **and/or** sucrose fermentation with acid accumulation in slant and butt. Note this test result as A/A.
 **Red slant/yellow butt**	TSIA and KIA: Glucose fermentation with acid production; proteins catabolized aerobically (in the slant) with alkaline products (reversion). Note this test result as K/A.
 **Red slant/red butt**	TSIA and KIA: No fermentation; peptone catabolized aerobically and anaerobically with alkaline products; isolate is not from *Enterobacteriaceae*. Note this test result as K/K.
Red slant/no change in the butt	TSIA and KIA: No fermentation; peptone catabolized aerobically with alkaline products; isolate is not from *Enterobacteriaceae*. Note this test result as K/NC.
No change in slant/no change in butt	TSIA and KIA: Organism is growing slowly or not at all; isolate is not from *Enterobacteriaceae*. Note this test result as NC/NC.
 **Black precipitate in the agar**	TSIA and KIA: Sulfur reduction (an acidic condition from glucose fermentation exists in the butt even if the yellow color is obscured by the black precipitate). Note this test result as H_2S made.
Cracks in or lifting of agar	TSIA and KIA: Gas production. Note this test result as G.

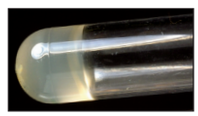
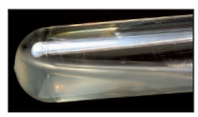
Coagulase Test (From Ex. 38)

Purpose: Differentiates bacteria based on the presence of coagulase, an enzyme that most pathogenic *Staphylococcus aureus* make. The coagulase test differentiates coagulase-positive *Staphylococcus aureus* from other, usually less virulent, coagulase-negative *Staphylococcus* species.

How to Inoculate: Use a sterile wooden applicator or disposable loop to inoculate a tube of rabbit plasma plus EDTA with a pure culture of the test bacterium. Mix the contents by gently rolling the inoculated tube between your hands.

Incubation Parameters: Incubate all tubes at 35±2°C for up to 24 hours, checking for coagulation periodically for the first 4 hours or remainder of your lab. Do not exceed 24 hours of incubation.

Interpretations: Tilt media to see if it solidified.

Results	Interpretations
Medium solidified within 24 hours or there is evident clumping/ coagulation (even if the media did not entirely solidify). 	Free or bound coagulase present; plasma coagulated; probable *Staphylococcus aureus*. Note this as a positive result (+).
Medium remains liquid after 24 hours; no signs of coagulation are evident. 	Plasma did not coagulate; coagulase was not detectable; unlikely to be *S. aureus*. Note this as a negative result (–).

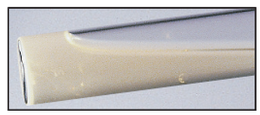
Gelatin Hydrolysis (Gelatinase Test) (From Ex. 39)

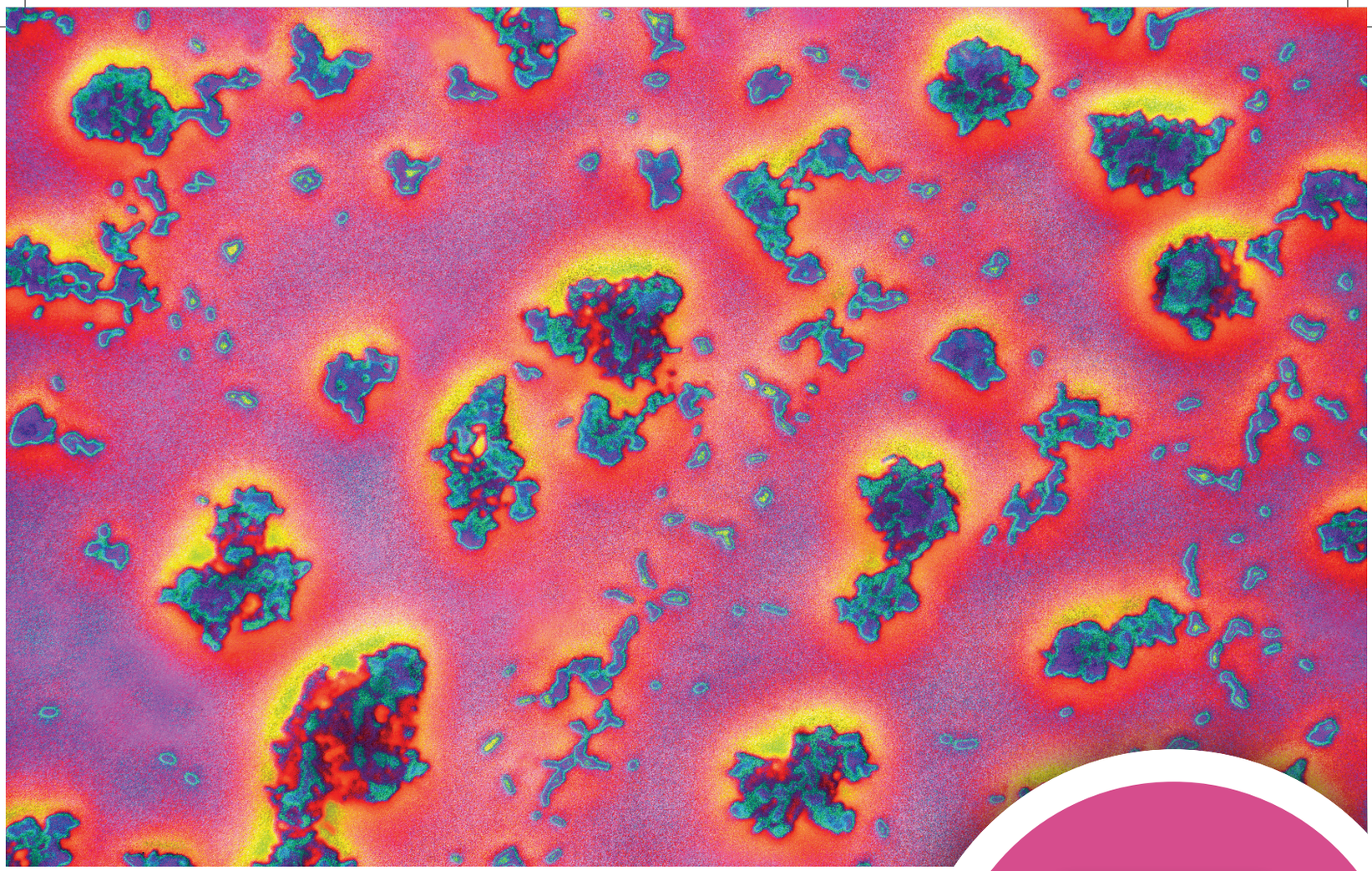
Purpose: To detect the enzyme gelatinase, which breaks down gelatin into amino acids.

How to Inoculate: Use an inoculating needle to stab a tube of nutrient gelatin media. Use a heavy inoculum of 18- to 24-hour-old sample.

Incubation Parameters: Two incubation parameters are acceptable. Either incubate at 25±2°C for up to 2 weeks or at 35±2°C for up to 1 week.

Interpretations: Tilt the media to see if it solidified. ***Note:*** If you incubated the samples at 35°C, then you must place the samples (to include an uninoculated control that was incubated alongside the test sample) in an ice bath or refrigerator for about 15 minutes (or until the uninoculated control solidifies).

Results	Interpretations
Medium remains solid after refrigeration. 	The organism does not make the enzyme gelatinase. Note this as a negative result (–).
Medium remains liquid after refrigeration. 	The organism makes the enzyme gelatinase. Note this as a negative result (+).



SECTION

9

Identifying Unknowns

In This Section

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EXERCISE 48 Bacterial Unknown Exercise and Resources **431**

— Lab Notes —

EXERCISE 47

Case-Based Unknown (Dry Lab)

After doing this exercise, you should be able to:

1. Discuss three misconceptions that you should avoid if using a dichotomous key identification strategy.
2. Explain why a battery of biochemical tests is normally inoculated for identifying an unknown bacterium.
3. State what the API test system is and how it generally works.
4. Apply your knowledge of biochemical tests to determine the identity of a theoretical unknown.

Background

The underlying goal of all clinical microbiology laboratory efforts is to provide accurate information as quickly as possible, delays and inaccurate data cost lives. Data accuracy is in no small part affected by the sample collection process at the point of care; it makes no difference how skilled the microbiology team is if the health-care provider fails to practice proper aseptic techniques and sample collection protocols. During the laboratory part of your microbiology training, you have been practicing aseptic techniques that are essential to saving lives; you'll apply them in patient care as well if you are required to collect a sample for microbiological analysis.

Most of the methods you have learned about in this laboratory course are microscopy- and culture-based. While such methods remain valid assessment tools, they are often accompanied by a wide variety of molecular-based tools (e.g., genetic analysis, serological studies, etc.).

Ultimately, clinical suspicion drives what tests the clinical microbiologist will perform. That is, the clinician collects specific patient samples (e.g., blood, urine, etc.) based on the nature of the patient's signs and symptoms. For example, a urine sample is collected when a urinary tract infection (UTI) is suspected. A throat swab is performed when strep throat (streptococcal pharyngitis) needs to be ruled out. A sample of cerebrospinal fluid is often collected if the patient is experiencing signs and symptoms of meningitis. Because clinical suspicion is really the driving force for clinical microbiological analysis, it's worthwhile for you to perform an "unknown" analysis with some infusion of that context. In this lab, you'll follow a patient from signs and symptoms to definitive diagnosis using microscopy and biochemical test data to inform clinical decisions.

Dichotomous Key Strategies: Avoiding Misconceptions

Dichotomous keys, like that shown in Figure 47.1, are a common approach to teaching students about identifying a bacterial unknown.[1] These branching-style maps are useful because they provide a visual representation of how one species differs from another. In that spirit, some of you may be asked to develop a dichotomous key to show that you understand how certain bacteria differ from one another. While dichotomous keys have their place in bacterial identification, they have the potential to perpetuate several misconceptions about the bacterial identification process:

1. Students may see such keys and conclude that one biochemical test is performed at a time until the bacterium's identity is determined;
2. Dichotomous keys imply a rank-and-file order/hierarchy for performing biochemical tests (always start with test A, then do B, followed by C, etc.); and
3. Students may perceive that tests earlier in the key are more accurate/reliable than those situated lower in the key.

The reality is that once a pure culture is obtained, a battery of biochemical tests is normally inoculated all at once and interpreted as soon as possible. There is not a stepwise, test-by-test inoculation. There are good reasons for this:

1. If clinical lab workers embraced a stepwise approach (inoculated one or two biochemical tests per day based on the data of a previous test), it may be more economical in terms of resources used, but it could *easily* triple the time required to determine the bacterium's identity.

[1] In addition to the dichotomous key in this exercise, there is a collection of dichotomous keys provided in the appendix of this book. Your instructor may have you use them for other lab exercises.

2. The types of tests performed hinge largely on clinical suspicion. For example, the clinical lab team will not analyze a urine sample with the intention of differentiating pathogens that cause UTIs from those that cause strep throat. That is, not all bacteria are reasonable suspects, the analysis is almost always narrowed based on the clinical presentation. In such cases, there is usually an empiric battery of tests—tests that experience has repeatedly shown are likely to lead to the correct identification of the culprit. Of course, if those empiric tests are inconclusive (which *can* happen), then further testing is necessary. That said, such a dead end would be detected in 24–48 hours by the battery approach versus a week of "if/then" testing that occurs in stages; time saved is lives saved.
3. No test is 100% accurate 100% of the time. Based on your experiences in the microbiology lab, this point may seem obvious; after all, you've learned there are always sources of error that can range from media formulations to strain variations to everything in between. Based on this fact alone, it doesn't make sense to hinge your testing workflow on a one-test-at-a-time strategy. What if the first test you performed yielded inaccurate results? You would probably waste a lot of time before figuring out the error, if you figured that out at all. Thus, you are *far* more likely to correctly identify your unknown if you perform a *collection* of tests and see where the closest match lies based on the various pieces of data.
4. Even if a particular bacterial species is noted as being positive for a particular test, that doesn't mean all isolates of the species are positive; the room for variation is often 10%. As many as 10% of the strains of a species listed as positive or negative on a test give the opposite result. That is, 90% of strains (genetic variants of the same species) may have a positive test result for a specific biochemical test while 10% of isolates may generate a negative test result. Indeed, perfect alignment is exceedingly rare. So, embarking on a

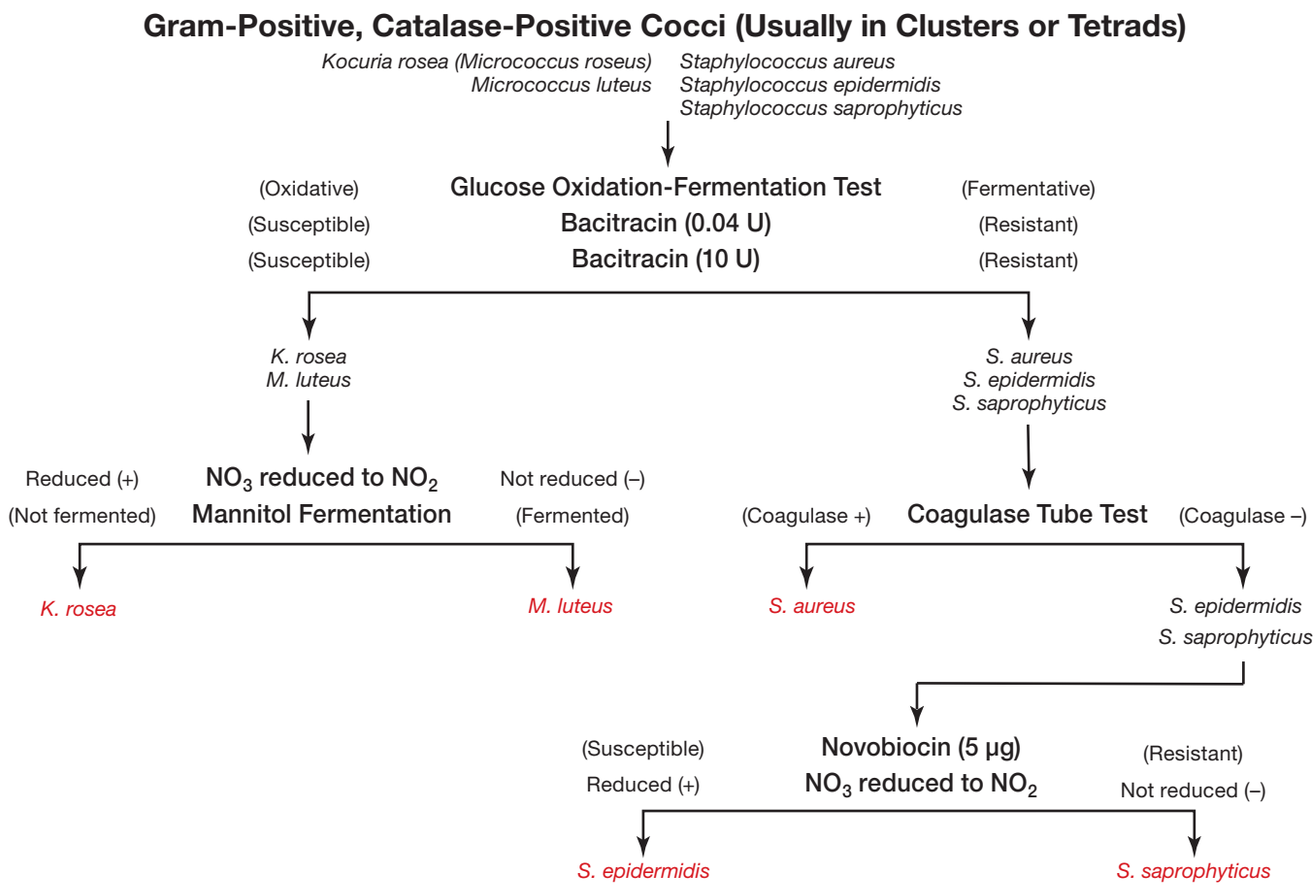


FIGURE **47.1** **Dichotomous Key Flowchart for Gram-Positive, Catalase-Positive Cocci (Usually in Tetrads or Clusters)** • A dichotomous key approach can be useful for bacterial identification. For example, this dichotomous key could be useful for discovering the identity of a pure culture of Gram-positive, catalase-positive, cocci arranged in clusters (or tetrads).

one-test-at-a-time strategy increases the risk of inaccurately identifying the isolate. It's much safer to consider all the data together rather than taking a piecemeal approach.

Based on these four points, when analyzing biochemical test results many clinical labs lean toward a "closest fit" approach rather than a traditional dichotomous key approach. In the closest fit approach, the data from a battery of biochemical tests are entered into a computer program, which then compares the reported data to one or more databases to determine the statistically most likely candidate (or candidates). One of the more widely used examples of this technology is the API system (Analytical Profile Index system manufactured by bioMérieux) (Fig. 47.2).

To more closely simulate the clinical lab bacterial identification strategy, this lab exercise will not use a dichotomous key strategy. You will instead analyze a clinical case, decide which battery of tests are most appropriate to isolate and identify the potential pathogen, and proceed from there with your analysis to determine the specimen's identity.

Clinical Applications

This lab provides a way for you to walk through the clinical specimen identification process. The process of identifying a pathogen in a sample typically begins with a health-care provider making the decision to collect a sample for microbiological analysis. That decision is usually made based on the signs and symptoms the patient is experiencing. Once the sample is collected, it is conveyed to a clinical microbiology lab. There the specimen is typically inoculated onto various media for isolation. Once an isolated candidate is grown, it is usually subjected to staining and microscopy to ascertain features like Gram property, cell shape, and cellular arrangement. Then, if the sample requires further analysis to make a definitive identification, it may be subjected to further biochemical testing.

MATERIALS

Because this is a "dry lab" (meaning the exercise doesn't require reagents or other lab materials), it can be an excellent way to gain a better grasp of the process before you are required to perform a bacterial identification lab with a real sample. Dry labs are also a great way to practice your problem-solving skills before you are called to use those skills in higher-stake settings with possibly dangerous biological and chemical substances.

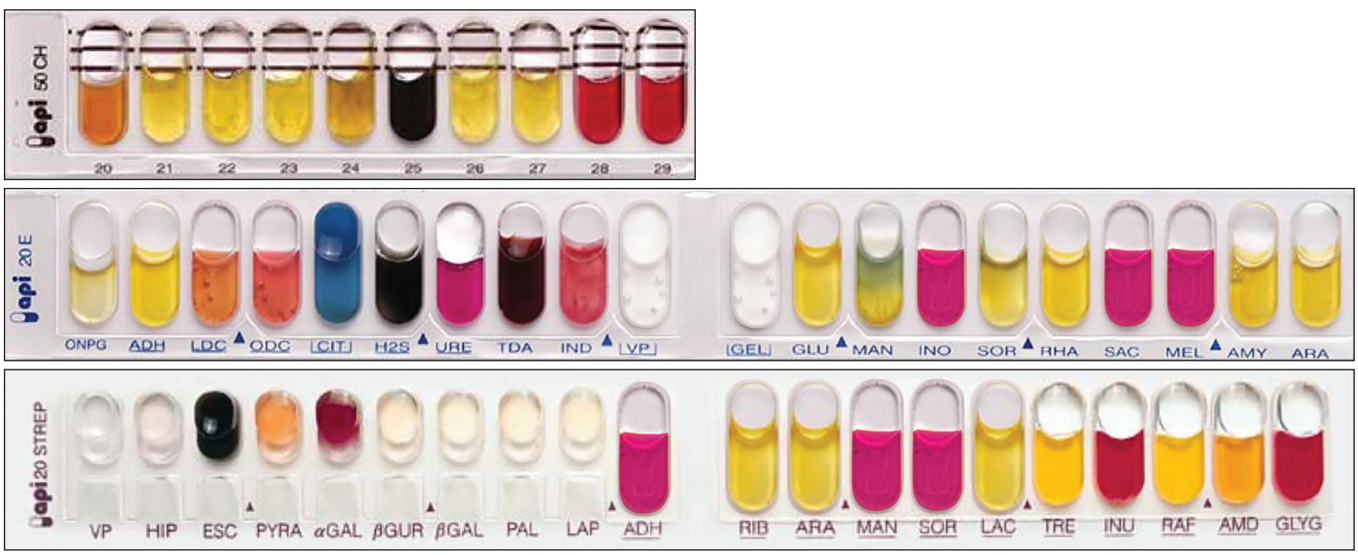


FIGURE **47.2** **API Test Strips** • Although only three examples of API test strips are shown, many other API test strips are available; most deliver test results in fewer than 24 hours. Before reading the test results, the microbiologist processing the samples would add specific reagents to certain cupules on the strip. If you look closely, you may notice that under each cupule there is an abbreviation that represents the test name. You may even recognize some of them. For example, CIT is the citrate test and GLU stands for the glucose fermentation test.

PROCEDURE

Read the case sections and perform the analyses as instructed.

At the Patient's Bedside

A 68-year-old man is brought to the emergency department (ED) of the hospital where you work. He reports a severe stabbing pain in his lower back, nausea without vomiting, and blood in his urine. CT scan imaging revealed two kidney stones (renal calculi) in the left kidney, as well as one stone lodged in the urethra and another stuck in the distal portion of the left ureter. He is running a fever and has underlying health conditions such as diabetes and heart disease. The ED doctor orders urine and blood samples to be collected for microbiological analysis as well as for other tests. Once the samples are collected, the doctor starts the patient on a broad spectrum intravenous antibacterial drug and admits him for inpatient hospital care.

In the Clinical Microbiology Lab

Day_0: Same Day as Sample Collection

You are responsible for analyzing the patient's urine sample and detecting the pathogen of interest. You start with a Gram stain of the centrifuged urine sample. You observe the following via light microscopy (Fig. 47.3):



FIGURE **47.3** **Gram Stain of Centrifuged Urine Sample**

1. Gram property = _____
2. Cell shape = _____
3. Cellular arrangement = _____
4. Assuming oil immersion microscopy with a 10× ocular and 100× objective, what would the final magnification be for the image? _____
5. Based on the Gram-stain and morphological information, you can make an informed decision about your next steps. For each of the following, state if you would or would not perform the procedure and explain the reasoning behind your decision. Remember, performing unnecessary tests takes time and costs your lab money, so you would only perform tests that would help you further narrow down the bacterium's identity.

a Inoculate the sample onto mannitol salt agar (MSA).

b Test the bacteria for coagulase.

c Test the bacteria for oxidase.

d Perform an acid-fast stain.

e Inoculate the sample onto eosin methylene blue agar (EMB).

At the Patient's Bedside

The infectious disease specialist familiarizes themselves with their newly admitted patient's case. In the meantime, the assigned urologist also visits the patient. They consult on the care plan for their shared patient. Their plan primarily centers on removing the lodged kidney stone within the next 12 hours while simultaneously managing the infection that has developed. The infectious disease doctor states that the Gram-stain data suggests a narrower-spectrum drug might be a more desirable option as compared to the broad-spectrum drug started in the ED.

However, until they have more data on the identity of the bacterium causing the infection and its antimicrobial drug susceptibility profile, they will keep the patient on the broad-spectrum antibacterial drug initiated in the ED.

6 Look up information on narrow-spectrum versus broad-spectrum drugs. Explain why a narrow-spectrum drug could be preferable to a broad-spectrum drug?

7 Based on this information, how would you likely describe the coloration of the growth on MacConkey agar? Explain the reasoning behind your response. (***Note:*** To answer this question, assume you are only describing the color of a single colony of the suspected pathogen. Do not concern yourself with colony morphology.)

In the Clinical Microbiology Lab

Day$_1$: Approximately 24 hours after Sample Collection

Upon arriving at the lab today, you check your various cultures from yesterday. You observe that you have successfully isolated a bacterium that could be the pathogen responsible for the patient's urinary tract infection. The suspected bacterium appears to ferment lactose when grown on MacConkey agar.

Today your work will focus on fine tuning the bacterium's identity and performing drug susceptibility testing. Provided all goes well, you should know the bacterium's identity and drug susceptibility data by tomorrow, at which point you can send the microbiology report to the attending health-care team.

The lab where you work has an API system. This helps to reduce the time for identification because the tests are miniaturized and require shorter incubation periods. Some of the API test strip panels can return biochemical test results in as few as 4 hours. To help you decide which API test strip you should use, you refer to the following chart (Fig. 47.4):

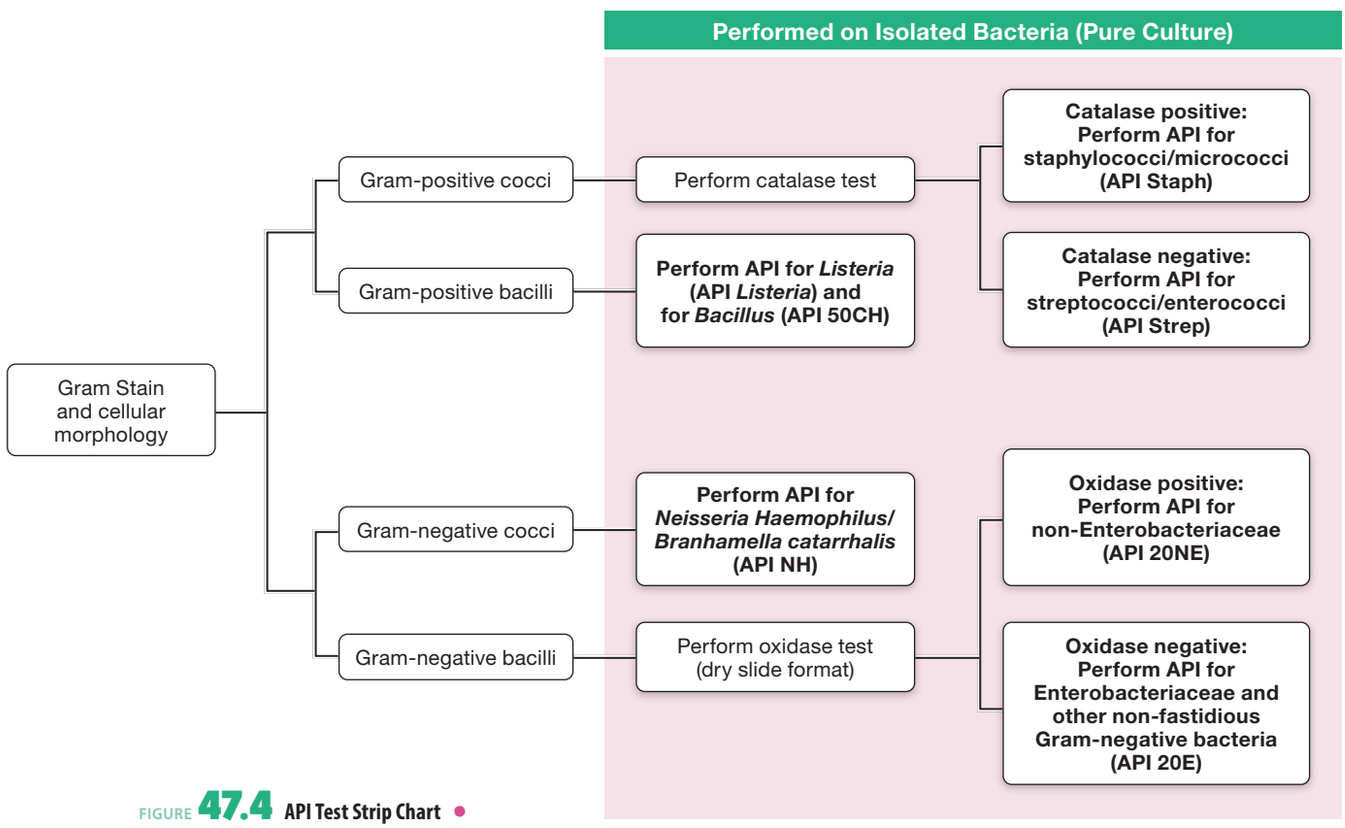


FIGURE **47.4** API Test Strip Chart

8 Based on the data you have thus far and considering the chart information, what is your next step (e.g., what API strip and/or test should you conduct)?

At the Patient's Bedside

The kidney stone was successfully removed. A temporary stent was placed in the ureter to keep it open as it heals from the trauma inflicted by the lodged stone. The urologist and infectious disease specialist are now concerned about the patient's infection and hope that by tomorrow the microbiological data will be ready.

In the Clinical Microbiology Lab

Day_2: Approximately 48 hours after Sample Collection

Today you are ready to analyze the biochemical test data to hopefully arrive at a definitive bacterial identification. You also will have some drug susceptibility data to send to the health-care team.

You retrieve the API test strip from the incubator, add reagents to the cupules per the manufacturer's specification, and observe the results in Figure 47.5.

Table 47.1 presents details for each test on the API strip you used to analyze the isolated bacterium. Unfortunately, your intern just dripped some reagents on the document and blurred a few areas. The good news is you have your lab manual from your undergraduate microbiology course available and your experience with biochemical tests, so you can fill in the few spaces where the information was blurred (numbers 9 through 13 in the table).

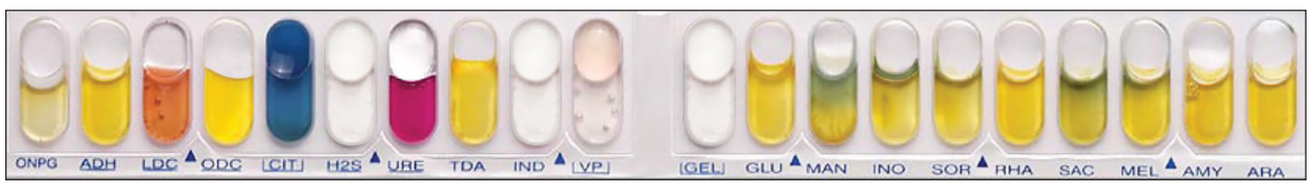


FIGURE **47.5** **API Test Strip Results for Bacterium Isolated from Case Study Patient's Urine**

TABLE **47.1** **Results for Various Biochemical Tests**

Test (Abbreviation)	Detects	Negative Result	Positive Result
ONPG	β-galactosidase (lactose hydrolysis)	Colorless	Yellow (pale color is possible)
ADH	Arginine dihydrolase	Yellow	Red or orange
LDC	Lysine decarboxylase	Yellow	Red or orange
ODC	Ornithine decarboxylase	Yellow	Red or orange
CIT	Citrate utilization	**9.**	**10.**
H_2S	Hydrogen sulfide (H_2S) production	Colorless	**11.**
URE	Urea hydrolysis	**12.**	**13.**
TDA	Tryptophan deamination	Yellow	Brown
IND	Indole production	Colorless/light yellow	Pink or red
VP	Acetoin production (Voges-Proskauer test)	Colorless	Pink or red (pale color is possible)
GEL	Gelatin hydrolysis	Colorless	Black (pigment diffuses)

(continues)

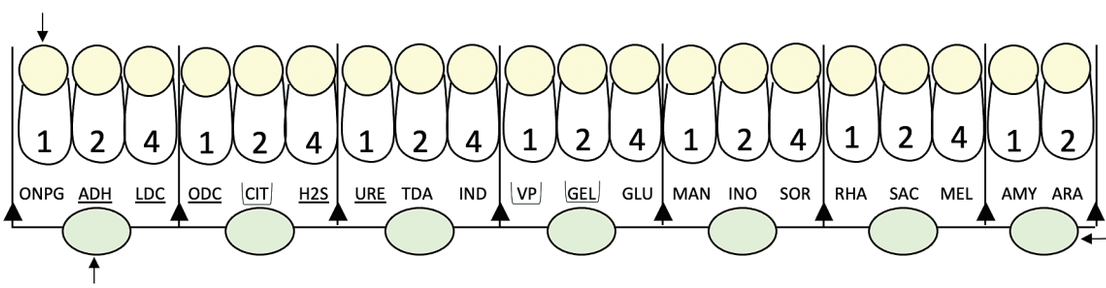
TABLE **47.1** *(continued)* **Results for Various Biochemical Tests**

Test (Abbreviation)	Detects	Negative Result	Positive Result
GLU	Glucose fermentation/oxidation	Blue	Yellow
MAN	Mannitol fermentation/oxidation	Blue	Yellow
INO	Inositol fermentation/oxidation	Blue	Yellow
SOR	Sorbitol fermentation/oxidation	Blue	Yellow
RHA	Rhamnose fermentation/oxidation	Blue	Yellow
SAC	Sucrose fermentation/oxidation	Blue	Yellow
MEL	Melibiose fermentation/oxidation	Blue	Yellow
AMY	Amagdalin fermentation/oxidation	Blue	Yellow
ARA	Arabinose fermentation/oxidation	Blue	Yellow

14 Finally, you're ready to analyze the test results and determine the bacterium's identity. Observe the API test results (Fig. 47.5) and use your completed table (Table 47.1) to fill out the following data recording sheet.

API RESULTS Sample Origin: _____ Date Inoculated: _____

Step 1: In each yellow circle, write a "+" for each positive result and a "–" for each negative result.



If the oxidase test was positive, add 4 to this last number. Note, the oxidase test is not on the test strip, but counts toward the final ID number.

Step 2: Add the numbers for the *positive* (+) test results for each section and write the sum in each green oval. For example, if all three tests in the first section (ONPG, ADH, and LDC) were positive, you'd write a "7" in the first green oval. If only ODC and H2S were positive for the second section, you'd write a "5" in the second green oval. Do this until each green oval has a numerical value. If none of the tests in a section are positive, enter a "0" for the section.

Step 3: To determine the bacterium's identity, look at Table 47.2 and find the 7-digit numerical sequence generated by the green ovals.

Step 4: Bacterium's most likely identity: _____

TABLE **47.2** API Identification Table*

Bacterium	ID number	Bacterium	ID number
Xanthomonas axonopodis	0001000	*Pantoea ananatis*	1245573
Erwinia tracheiphila	0004520	*Pasteurella haemolytica*	1422000
Erwinia amylovora	0005020	*Stenotrophomonas maltophilia*	1422351
Morganella morganii	0174000	*Citrobacter freundii*	1604573
Pseudomonas solanacearum	0201004	*Pseudomonas aeruginosa*	2214006
Erwinia salicis	0204731	*Pasteurella multocida*	3000004
Pseudomonas rubrilineans	0211004	*Vibrio hollisae*	3042140
Abiotrophia adiacens	0402000	*Enterobacter cloacae*	3305773
Streptococcus acidominimus	0470000	*Enterobacter agglomerans*	3307173
Proteus mirabilis	0736000	*Aeromonas hydrophila /caviae*	3577754
Xanthomonas albilineans	1001000	*Hafnia alvei*	4105112
Escherichia adecarboxylata	1004173	*Edwardsiella tarda*	4744000
Erwinia stewartii	1005162	*Streptococcus mutans*	5040710
Corynebacterium fascians	1010000	*Escherichia coli*	5144572
Agrobacterium tumefaciens	1010007	*Klebsiella pneumoniae*	5215773
Agrobacterium radiobacter	1015046	*Serratia marcescens*	5306563
Pasteurella pneumotropica	1020300	*Chryseomonas luteola*	5477761
Bordetella bronchiseptica	1200063	*Aerococcus viridans*	6112711
Serratia rubidaea	1207360	*Salmonella choleraesuis*	6704510
Erwinia chrysantherni	1244173	*Vibrio parahaemolyticus*	7467746

* These API codes are simply examples, they are for teaching purposes only and do not necessarily represent the most recent API code data maintained in the subscription-based API database.

At the Patient's Bedside

The infectious disease doctor reported the microbiology findings to the rest of the health-care team. Based on the microbiology data, the bacterium proved susceptible to several common oral antibiotics. The care providers agreed that the patient could be discharged from the hospital and sent home with a three-day course of ciprofloxacin. The patient fully recovered, although he did experience two more kidney infections over the next 5 years.

15 Look up the bacterium that you identified as the causative agent and describe some of the features that can make it a challenging pathogen to combat.



EXERCISE 48

Bacterial Unknown Exercise and Resources

After doing the unknown exercise students typically are able to:

1. Perform various staining protocols and biochemical tests on a bacterial unknown sample.
2. Use a light microscope to make morphological and staining observations.
3. Record and analyze observations for staining and biochemical tests to discover the identity of an unknown bacterial sample.
4. Report observations and conclusions for a variety of staining procedures and biochemical tests.

Background

A capstone laboratory experience in most undergraduate microbiology courses is the identification of a bacterial unknown. While the approach your instructor uses for this sort of exercise may vary, the general principle is the same: the student applies their newly developed microbiology lab skills to determine the identity of a bacterial sample. The sample may be provided to you as a pure culture or as a mixed culture.

Most instructors have students perform this lab once the class has some experience with staining methods and students have learned how to perform and interpret a variety of biochemical tests. In some courses, you may complete an unknown project at a midpoint in the course and then a more sophisticated unknown project closer to the end of the course. In other courses, you may simply have one unknown project that serves as a laboratory capstone experience.

What to Expect During an Unknown Exercise

You can expect to perform a variety of biochemical tests and stains on a bacterial specimen issued to you and then use the data from those procedures to decide your bacterium's identity. Typically, this exercise is done over two or more lab sessions. You should not expect much guidance from your instructor; rather this is *your* opportunity to solve a real-world problem with your own acquired skills. You've got great new skills and now is your chance to show them off!

In most cases, the better you are at implementing the lab techniques you explored in your course, the more likely you are to correctly identify your bacterial specimen's identity. However, correctly identifying your bacterium's identity is often just one part of your score. Usually your instructor will be looking at your lab writeup or report to see if you truly know how to perform and interpret a variety of biochemical tests and staining protocols. They will also be checking that you are detail-oriented and thorough in terms of how you report your observations and formulate conclusions. And of course, you are always expected to use the proper language and writing conventions for your documentation.

Useful Resources

Refer to the notecards at the end of the book, you'll likely find them useful as you undertake this exercise. These notecards can also be cut out and used as a study resource! Additional resources that you'll likely find useful for this exercise include the Instructional Staining Atlas (pp. 131–135) along with the identification flowcharts in Appendices F–I (pp. 487–500).

MATERIALS

- ❑ Lab coat
- ❑ Gloves
- ❑ Safety glasses
- ❑ Unknown mixture with number (1, your instructor will advise you whether the sample is pure or mixed)
- ❑ Appropriate stains
- ❑ Appropriate biochemical test media and reagents
- ❑ Recommended organisms (samples may be mixed or pure as your instructor decides)

Gram-Positive Candidates

- □ *Bacillus cereus*
- □ *Bacillus coagulans*
- □ *Bacillus subtilis*
- □ *Corynebacterium xerosis*
- □ *Enterococcus faecalis* (BSL-2)
- □ *Kocuria rosea*
- □ *Lactobacillus plantarum*
- □ *Micrococcus luteus*
- □ *Mycobacterium smegmatis* (BSL-2)
- □ *Staphylococcus aureus* (BSL-2)
- □ *Staphylococcus epidermidis*
- □ *Streptococcus salivarius*

(continues)

MATERIALS *(continued)*

Gram-Negative Candidates

- ☐ *Citrobacter freundii*
- ☐ *Enterobacter aerogenes* (BSL-2)
- ☐ *Enterobacter cloacae*
- ☐ *Escherichia coli*
- ☐ *Hafnia alvei*
- ☐ *Klebsiella pneumoniae* (BSL-2)
- ☐ *Morganella morganii* (BSL-2)
- ☐ *Proteus mirabilis* (BSL-2)
- ☐ *Proteus vulgaris* (BSL-2)
- ☐ *Providencia stuartii*
- ☐ *Serratia liquefaciens*
- ☐ *Serratia marcescens*

Other bacteria as decided by your instructor

- ☐ _____
- ☐ _____
- ☐ _____

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____



EXERCISE 48

DATA SHEET



PROCEDURE

Unknown Report

Unknown Sample # _____

My instructor wants us to work in a group of: _____ (You'll usually work either alone or in a group of two, but this is at your instructor's discretion.)



CAUTION!

Unless your instructor has specified otherwise, assume your sample could be a BSL-2 bacterium and follow all BSL-2 precautions.

STEP 1: OBTAIN A PURE CULTURE AND NOTE COLONY FEATURES

The provided unknown culture is: mixed/pure (select one).
If you are given a mixed culture, your instructor will *likely* tell you how many bacteria are in the sample, you should take note of that information. If you have a mixed culture, then you will need to perform a streak plate isolation to detect the constituents.

Observe the culture characteristics of your pure culture.
Once you have a pure culture with isolated colonies, report features such as the color, shape, elevation, shine or no shine, and borders of the colonies.

Write your observations here (refer to Ex. 6 for help).

Size: _____

Shape (form): _____

Margin (edge): _____

Elevation: _____

Consistency: _____

Surface Texture: _____

Other useful features (e.g., color, optical properties): _____

STEP 2: PERFORM STAINS ON YOUR PURE CULTURE AND MAKE OBSERVATIONS

Place a checkmark next to the stains your instructor wants you to perform and write your observations and conclusions in the following table for each stain you perform.

Stain	Observations
Simple (See Ex. 11)	
Negative (See Ex. 12)	
Gram (See Ex. 13)	
Acid-Fast (See Ex. 14)	
Capsule Stain (See Ex. 15)	
Endospore Stain (See Ex. 16)	

Based on your observations, write a final description of the cellular morphology and specific structures and/or cell wall properties of your bacterium:

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

STEP 3: PERFORM BIOCHEMICAL TESTS ON YOUR PURE CULTURE AND MAKE OBSERVATIONS

Place a checkmark next to the plated media and various biochemical tests your instructor will have available for you to assess your sample.

Differential/Selective Media

On pages 441–443, you will find four summary notecards of differential and selective media. These can be cut out and mounted onto 4 × 6 or 5 × 8 notecards as a reference tool during the lab.

Media	Observations	Conclusions
Mannitol Salt Agar (MSA) (See Ex. 22)		
MacConkey (MAC) Agar (See Ex. 23)		
Eosin Methylene Blue (EMB) Agar (See Ex. 24)		
Blood Agar (See Ex. 25)		

Biochemical Tests

On pages 445–461, you will find 13 summary notecards of biochemical tests. These can be cut out and mounted onto 4 × 6 or 5 × 8 notecards, or other cardstock as a reference tool during the lab.

Test	Observations	Conclusions
Glucose (Dextrose) Fermentation Phenol Red Broth (See Ex. 27)		
Lactose Fermentation Phenol Red Broth (See Ex. 27)		
Sucrose Fermentation Phenol Red Broth (See Ex. 27)		
Methyl Red and Voges-Proskauer (MR-VP) Tests (See Ex. 28)	**MR:** **VP:**	**MR:** **VP:**
Catalase Test (See Ex. 29)		
Oxidase Test (See Ex. 30)		
Nitrate Reduction Test (See Ex. 31)		
Citrate Utilization (See Ex. 32)		
Lysine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		
Ornithine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		
Arginine Amino Acid Decarboxylation (Decarboxylase Tests) (See Ex. 33)		

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

Test	Observations	Conclusions
Starch Hydrolysis (Amylase Test) (See Ex. 34)		
Urea Hydrolysis (Urease Test) (See Ex. 35)		
SIM Medium: Sulfur, Indole, and Motility Tests (See Ex. 36)	**H_2S:** **Indole:** **Motility:**	**H_2S:** **Indole:** **Motility:**
Triple Sugar Iron Agar/Kligler Iron Agar (See Ex. 37)		
Coagulase Test (See Ex. 38)		
Gelatin Hydrolysis (Gelatinase Test) (See Ex. 39)		

OTHER TESTS

Test name	Observations	Conclusions

STEP 4: USE YOUR DATA TO DETERMINE THE BACTERIUM'S IDENTITY

Use the flow charts in Appendices F–I (pp. 487–500) to determine which bacterium you most likely have. Note that you are unlikely to have a perfect match, your task is to find the best match.

Bacterium's Identity: _____ (include genus and species)

STEP 5: REFLECTIVE ANALYSIS

1 What test results for this exercise did *not* align with your conclusion about the bacterium's identity?

2 Regarding the data you stated were incongruent with your conclusion, explain what may have gone wrong.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 48
DATA SHEET
(continued)

3 Provide some background about the bacterium you identified. Include the following in your discussion:

a. Morphological features

b. Gram property

c. Places the bacterium can naturally be found

d. Clinical, environmental, and/or industrial significance of the bacterium

e. Three additional interesting facts about the bacterium

— Lab Notes —

A Summarized Guide of Differential/ Selective Media for the Identification of an Unknown Bacterial Sample

Notecards

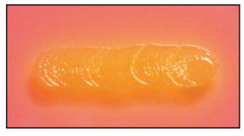
Mannitol Salt Agar (MSA) (From Ex. 22)

Purpose: This media is selective for salt-tolerant organisms such as *Staphylococcus*, *Bacillus*, *Enterococcus*, *Micrococcus*, and others. MSA also contains the sugar mannitol, so those bacteria that grow on the media can be differentiated based on their ability to ferment mannitol.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the media's color around the colonies.

Results	Interpretations
Poor growth (P) or no growth (N)	The organism's growth is inhibited by NaCl; the bacterium is unlikely to be a *Staphylococcus* species.
Good growth (G)	The organism's growth is not inhibited by NaCl. It's possible the bacterium is a *Staphylococcus* species, or some other bacterium known to grow well on MSA (e.g., a *Bacillus*, *Enterococcus*, *Micrococcus* species).
Yellow growth or halo (Y) 	The organism fermented mannitol; the pH was lowered by acids generated during mannitol fermentation. The bacterium may be pathogenic *Staphylococcus aureus* (further testing needed to make this conclusion; many *Staphylococcus* species ferment mannitol and some ferment none; *Staphylococcus* bacteria may ferment mannitol).
Red growth (no halo) (R) 	The organism does not ferment mannitol. The organism is unlikely to be *S. aureus*.

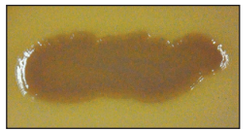
MacConkey (MAC) Agar (From Ex. 23)

Purpose: This media is selective for Gram-negative bacteria and selective based on the fermentation of lactose. MAC is one example of an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the bacterial growth's color.

Results	Interpretations
Poor growth (P) or no growth (N)	Gram-positive bacterium
Good growth (G)	Gram-negative bacterium
Pink to red growth with or without bile precipitate (R) 	The organism produces acid as a byproduct of lactose fermentation; the bacterium may be a coliform bacterium.
Growth is "colorless" (not red or pink) (C) 	The organism does not ferment lactose; it is unlikely to be a coliform.

9

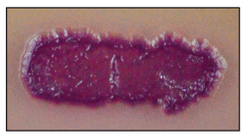
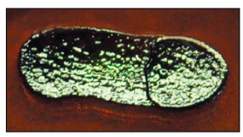
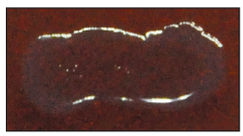
Eosin Methylene Blue (EMB) Agar (From Ex. 24)

Purpose: EMB is an enriched selective and differential growth media used for detecting coliforms (to include *E. coli*) in a sample. Like MAC agar, EMB is selective for Gram-negative bacteria and differential based on the fermentation of lactose.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate all the samples at 35±2°C for 48 hours.

Interpretations: After incubation, note if the bacterium grew or not. If you observe growth, then assess the bacterial growth's color.

Results	Interpretations
Poor growth (P) or no growth (N)	Gram-positive bacterium
Good growth (G)	Gram-negative bacterium
Growth is pink and mucoid (Pi) 	The organism produces acid as a byproduct of lactose fermentation and is possibly a coliform bacterium.
Growth is "dark" (purple to black, with or without green metallic sheen) (D) 	The organism produces acid as a byproduct of lactose fermentation and is probably a coliform bacterium.
Growth is "colorless" (not purple, green, red, or pink) (C)  **(*Note:* EMB media has a reddish color so do not mistake the color of the media as being the color of the growth.)**	The organism does not ferment lactose and is unlikely to be a coliform bacterium.

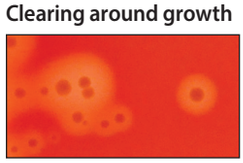
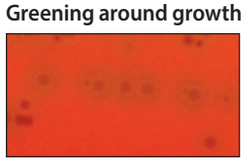
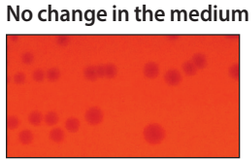
Blood Agar (From Ex. 25)

Purpose: Blood agar detects hemolytic activity and is commonly used to differentiate β-hemolytic, pathogenic *Streptococcus* species from non-pathogenic species of this diverse genus of Gram-positive cocci.

How to Inoculate: Use an inoculating loop to perform a streak plate isolation or T-streak of the plated media.

Incubation Parameters: Invert the plate and incubate your sample at 35±2°C for 24 hours.

Interpretations: After incubation, assess the color of the media around the bacterial growth.

Results	Interpretations
Clearing around growth 	The organism hemolyzes RBCs completely (β-hemolysis).
Greening around growth 	The organism partially hemolyzes RBCs; hemoglobin oxidized to methemoglobin (α-hemolysis).
No change in the medium 	The organism does not hemolyze RBCs (γ-hemolysis).

Notecards

A Summarized Guide of Biochemical Tests for the Identification of an Unknown Bacterial Sample

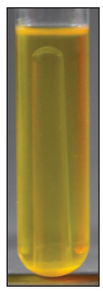
Phenol Red Fermentation Broth: Glucose, Lactose, and Sucrose Fermentation Tests (From Ex. 27)

Purpose: These tests detect the enzymes needed to ferment a given carbohydrate. If a given carbohydrate is fermented, then acids are made that will lower the media's pH. As pH drops, the pH indicator in the media (phenol red) turns from its original red color to yellow. If the bacteria do not ferment the given carbohydrate, then the media remains red. If gas is produced as a by-product of fermentation, then the Durham tube will have a bubble in it.

How to Inoculate: All carbohydrate test media should be inoculated with the transfer loop.

Incubation Parameters: Incubate the sample at 35±2°C for 48 hours.

Interpretations: When making interpretations, make sure you note media coloration as well as note if gas is present or absent in the Durham tube.

Results	Interpretations
 **Yellow broth; bubble in Durham tube**	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. If lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase. Note the result as A/G.
 **Yellow broth; no bubble in Durham tube**	The carbohydrate (insert sugar's name) was fermented and acidic end products were formed; gas was also produced as a byproduct. If lactose was fermented, you can note that your test bacterium makes the enzyme β-galactosidase; if sucrose was fermented, you can state your test bacterium makes sucrase. Note the result as A/–.
 **Red broth; no bubble in Durham tube**	The carbohydrate (insert sugar's name) was not fermented and gas was not produced. Note the result as –/–.
 **Pink broth; no bubble in Durham tube**	Degradation of peptone occurred to form alkaline end products, but the carbohydrate (insert sugar's name) was not fermented; gas was not produced. Note the result as K/–.

9

Methyl Red and Voges-Proskauer (MR-VP) Tests (From Ex. 28)

Purpose: The methyl red and Voges-Proskauer tests (MR-VP) help us differentiate bacterial species based on the fermentation pathway used to metabolize glucose. The MR portion of the test detects if mixed acid fermentation occurred. The Voges-Proskauer test reveals if a bacterium metabolized glucose via the butanediol fermentation pathway.

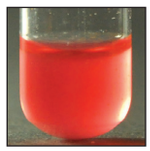
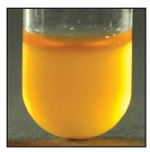
How to Inoculate: Inoculate the MR-VP broth with a transfer loop.

Incubation Parameters: Incubate the sample at 35±2°C for 48 hours.

Test Development Process: For this test, only one tube of media is inoculated. After incubation, 1 mL of the culture is transferred to a clean test tube to serve as the MR portion of the test and another 1 mL of culture is transferred to a tube and analyzed for the VP portion of the test. Follow the development protocols in the following tables for the MR and VP test, respectively.

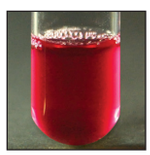
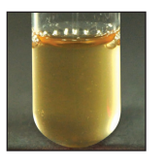
Methyl Red Test

- Add 3 drops of methyl red reagent to the sample and immediately observe for the development of a red color.

Results	Interpretations
 **Red**	Organism performs a mixed acid fermentation. Note this as a positive result (+).
 **No color change**	Organism does not perform a mixed acid fermentation; acidic end products were not made in detectable amounts. Note this as a negative result (–).

Voges-Proskauer Test

- Add 15 drops (0.6 mL) of VP Reagent A. Mix well.
- Add 5 drops (0.2 mL) of VP Reagent B. Mix well.
- Observe for red color formation at 10-minute intervals for up to 1 hour. Periodically mix the sample.

Results	Interpretations
 **Red**	Organism performs a 2,3-butanediol fermentation (acetoin was produced). Note this as a positive result (+).
 **No color change**	Organism does not perform a 2,3-butanediol fermentation (acetoin was not produced or it is not detectable). Note this as a negative result (–).

Notecards

Catalase Test (From Ex. 29)

Purpose: This test detects catalase, an enzyme responsible for protecting bacteria from hydrogen peroxide (H_2O_2) accumulation, which can occur during aerobic metabolism. If hydrogen peroxide accumulates, it becomes toxic to the organism. Catalase converts H_2O_2 to water and gaseous oxygen.

Test Development Process: Test fresh (18- to 24-hour) agar slant cultures. Inoculation and incubation are not required. This test is often performed by adding hydrogen peroxide to a bacterial smear on a microscope slide. If using this "slide method," cover the sample after adding hydrogen peroxide so that you reduce workspace contamination from the aerosols a positive test may generate.

Another way to perform the catalase test is to add hydrogen peroxide directly to bacteria growing on an agar slant or agar plate (do not use cultures growing on blood agar or you will likely get a false-positive result). Adding H_2O_2 directly to the culture will render the sample unusable for further tests. If you need the culture for further testing, then use the slide method for this test.

Results	Interpretations
 **Bubbles immediately develop**	Catalase is present; the organism is aerobic. Note this as a positive result (+).
 **No bubbles detected**	Catalase is absent or not detectable. (***Note:*** You *cannot* conclude the bacterium is anaerobic just because it lacks catalase!) Note this as a negative result (–).

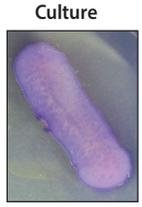
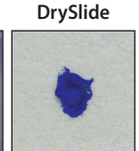
Oxidase Test (From Ex. 30)

Purpose: The oxidase test reveals if a bacterium has cytochrome c oxidase, an enzyme in certain aerobic electron transport chains. Bacteria that have cytochrome c oxidase respire aerobically. However, a negative oxidase test result only means the bacterium lacks cytochrome c oxidase; it does not necessarily mean the bacteria cannot respire aerobically using some other ETC that doesn't require cytochrome c oxidase.

Test Development Process: There are several ways to perform the oxidase test:

1. Add the TMPD reagent directly to bacterial growth on solid media.
2. Smear the test bacterium onto filter paper and then add TMPD to the smear.
3. Smear the test bacterium onto paper infused with the TMPD reagent. (Commercially available DrySlide.)

Regardless of the approach applied, if cytochrome c oxidase is present a color change to purple or blue occurs within seconds as TMPD is oxidized.

Results	Interpretations
Culture  DrySlide  **Dark blue/purple color develops within 20 seconds**	Cytochrome c oxidase is present; the organism can perform aerobic respiration. Note this as a positive result (+).
Culture  DrySlide  **No color change to blue/purple within 20 seconds**	Cytochrome c oxidase is not present or not detectable. (***Note:*** Do *not* assume the tested bacterium is anaerobic; the bacterium may have alternative aerobic ETCs that do not require cytochrome c oxidase.) Note this as a negative result (–).

9

Notecards

Nitrate Reduction Test (From Ex. 31)

Purpose: The nitrate test allows us to differentiate bacterial species from one another based on their ability to reduce nitrate (NO_3^-). An inverted Durham tube in each broth serves to trap a portion of any produced gas.

How to Inoculate: Inoculate nitrate broth with a transfer loop.

Incubation Parameters: Incubate the sample at 35±2°C for 24 to 48 hours.

Test Development Process: Following incubation, analyze the test via a three-step process.

1. **Check for a Bubble in the Durham Tube:** If there is a bubble *and* you are certain the bacterium does *not* conduct fermentation, then you may safely conclude the tested organism reduces nitrate to gaseous end products such as molecular nitrogen (N_2) or nitrous oxide (N_2O). In most cases, you will need to move to Step 2. (See table Development Process 1.)
2. **Add Reagents A and B:** If you are *not* certain as to whether your bacterium conducts fermentation or not, then you must add various reagents to the sample. Add 8 drops (approximately 0.5 mL) of Reagent A and then 8 drops (approximately 0.5 mL) of Reagent B to the tube and mix well. If you see a red color after adding Reagents A and B, then you may conclude the tested bacterium reduces nitrate to nitrite. (See table Development Process 2.)
3. **Add Reagent C if Red Color Does NOT Develop During Step 2 Above:** If you do *not* see a color change to red after adding Reagents A and B in Step 2, then add a small amount of nitrate Reagent C (zinc dust) to determine if the bacterium reduced nitrate beyond nitrite. You can dip a wooden applicator into the zinc dust and transfer the amount that clings to the wood. Let the tubes stand for 10 minutes. If a red color develops, then the organism did *not* reduce nitrate. Lack of a color change after adding Reagent C indicates the organism reduced nitrate to NH_4^+, NO, N_2O, or some other nitrogenous compound other than nitrite. (See Development Process 3 in the following table.)

Development Process	Results	Interpretations
1. Check for a Bubble in the Durham Tube.	Gas in Durham tube (no reagents added). (***Note:*** Only use this measure if you are certain the tested bacterium does *not* conduct fermentation. If you do not know the fermentation status of the bacterium, proceed to Development Process 2.)	If you are certain the test bacterium does *not* perform fermentation, then you may conclude denitrification occurred ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$). Note this as a positive result (+).
2. Add Reagents A and B.	 Red color after adding Reagents A and B. (The test is completed; no further reagents should be added.)	Nitrate reduction to nitrite ($NO_3^- \rightarrow NO_2^-$); nitrate reductase is present. Note this as a positive result (+).
	 No color change after adding Reagents A and B. (Proceed to Development Process 3.)	Incomplete test; requires the addition of zinc dust. (A test result cannot be determined yet. Proceed to adding Reagent C, Development Process 3.)
3. Add Reagent C if Red Color Does NOT Develop During Step 2.	 No color change after adding Reagent C. (The test is completed; no further reagents should be added.)	Nitrate reduction to gaseous (NO, ½N_2O, or ½N_2) or reduction to nongaseous nitrogen compounds such as ammonium (NH_4^+) occurred. Note this as a positive result (+).
	 Red color after adding Reagent C. (The test is completed; no further reagents should be added.)	No nitrate reduction. Note this as a negative result (–).

9

Citrate Utilization Test (From Ex. 32)

Purpose: This biochemical test detects the ability of bacteria to use citrate as a sole carbon source. If the bacterium cannot use citrate as its sole carbon source, then it will not grow and the media will remain a greenish color. If the bacterium can use citrate as its sole carbon source, then the bacteria will grow and the media will turn a bright blue as a result of an increase in the pH of the media.

How to Inoculate: Use an inoculating loop to streak the bacteria onto citrate media in a zigzag fashion along the slant surface ("fishtail inoculation"). To avoid confusion between actual growth and a heavy inoculum, inoculate lightly.

Incubation Parameters: Incubate the sample at 35±2°C for 48 hours.

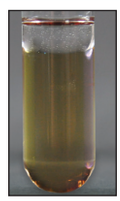
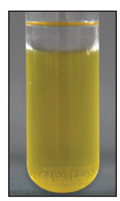
Results		Interpretations
	**Media appears blue (even if only a portion of the media turned blue) and growth is evident on the slant surface.**	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source). Note this as a positive result (+).
	**No color change, but growth is evident on the slant surface.**	Citrate is utilized (organism has the enzymes needed to use citrate as its sole carbon source). Note this as a positive result (+).
	**No color change and no growth on the slant surface.**	Citrate is not utilized (organism lacks one or more of the enzymes needed to use citrate as its sole carbon source). Note this as a negative result (–).

Lysine/Ornithine/Arginine Amino Acid Decarboxylation (Decarboxylase Tests) (From Ex. 33)

Purpose: This biochemical test allows us to examine the ability of bacteria to decarboxylate the amino acid lysine using the enzyme lysine decarboxylase. As optional procedures, you may also test for the presence of ornithine decarboxylase and arginine decarboxylase in the assigned bacteria. (***Note:*** Each decarboxylase is specific to its own amino acid substrate.)

How to Inoculate: Use an inoculating loop to inoculate the broth decarboxylase media. After inoculation, an overlay of mineral oil is added to seal the medium from the air. This ensures the surface of the media's pH is not artificially raised (alkalized) through interactions with the ambient air and it helps to maintain a lower oxygen environment that favors glucose fermentation.

Incubation Parameters: Incubate the sample at 35±2°C for 96 hours (4 days) up to a full week.

Results		Interpretations
	**No color change**	No decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).
	**Yellow**	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name)*; the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).
	**Purple (may be slight)**	Decarboxylation of the amino acid (insert amino acid name)*; organism produces the specific decarboxylase enzyme. Note this as a positive result (+).

* The amino acid included in the media can vary. Therefore, describe all interpretations as they relate to the specific amino acid in the media. Example: If using lysine decarboxylase media, the amino acid in the media is lysine.

9

Starch Hydrolysis (Amylase Test) (From Ex. 34)

Purpose: The starch hydrolysis test (or amylase test) reveals if a bacterium can break down the complex polysaccharide starch.

How to Inoculate: Use an inoculating loop to inoculate starch agar with the test bacterium. You may use a streak plate isolation method, or if you have a pure culture, you may use a zigzag inoculation.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 hours.

Test Development Process: After incubation, cover the growth and surrounding areas with Gram's iodine. Do not flood the plate but do add enough iodine so that the agar is completely colorized. *Allow the iodine to soak in.* After the agar has soaked up the iodine, check for a clear zone around the bacterial growth.

Results	Interpretations
 **A clearing around the growth is evident after adding iodine to the plate.**	α-amylase, β-amylase, and/or α-1,6-glucosidase is present (or more simply, amylase is present). Note this as a positive result (+).
 **There is not a clearing around the growth after adding iodine to the plate.**	Fermentation of glucose; no decarboxylation of the amino acid (insert amino acid name); the specific decarboxylase is absent or products are not detectable. Note this as a negative result (–).

Urea Hydrolysis (Urease Test) (From Ex. 35)

Purpose: This test reveals if a bacterium produces the intracellular hydrolytic enzyme urease, which uses water to catabolize (break down) urea to form ammonia and carbon dioxide.

How to Inoculate: Use an inoculating loop to inoculate urea media.

Incubation Parameters: Incubate the sample at 35 ± 2°C for 24 hours.

Results	Interpretations
 **Orange or yellow**	No urea hydrolysis; urease is absent or not produced in enough quantity to produce results in 24 hours; or the organism cannot live in urease broth. Note this as a negative result (–).
 **Pink**	Rapid urea hydrolysis; urease was produced. Note this as a positive result (+).

Notecards

SIM Medium (Sulfur, Indole, and Motility Tests) (From Ex. 36)

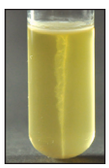
Purpose: This media assesses a bacterium's ability to reduce sulfur, make indole, and move.

How to Inoculate: Use an inoculating needle to stab the SIM media in as straight a line as possible through the middle of the agar deep. Insert the needle into the agar to within 1 cm of the bottom of the tube. Be careful to remove the needle along the original stab line so that you obtain a straight inoculation.

Incubation Parameters: Incubate the sample at 35±2°C for 24 to 48 hours.

Sulfur Reduction (H_2S Production)—The "S" in SIM

- In addition to SIM media, TSI (triple sugar iron) media can also be used to assess sulfur reduction.

Results	Interpretations
 **No black in the medium**	Sulfur is not reduced, or is not reduced in detectable amounts. Note this as a negative result (–).
 **Black in the medium**	Sulfur reduction occurred (H_2S was made); organism either respires anaerobically with sulfate as the final electron acceptor (thiosulfate reductase) or hydrolyzes the amino acid cysteine (cysteine desulfurase). Note this as a positive result (+).

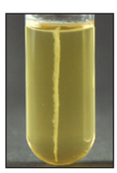
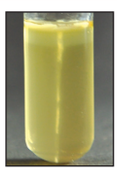
Indole Production—The "I" in SIM

- Add Kovac's reagent to each SIM media tube (including the control) to a depth of 2 mm–3 mm (*not mL*). After several minutes, observe for the formation of red color in the reagent layer.

Results	Interpretations
 **No red layer evident after adding Kovac's reagent**	Tryptophanase is absent and indole is not produced, or it's produced but not in detectable amounts. Note this as a negative result (–).
 **Red layer evident after adding Kovac's reagent**	The organism produces tryptophanase and hydrolyzes tryptophan into indole, NH_3, and pyruvate. Note this as a positive result (+).

Motility—The "M" in SIM

- In addition to SIM media, general motility agar may be used to assess motility. Unlike SIM media, general motility agar only reveals information about a bacterium's motility.

Results	Interpretations
 **No growth radiating away from the original inoculation/ stab line.**	Nonmotile. Note this as a negative result (–).
 **Growth radiating outward from inoculation/stab line.**	Motile. Note this as a positive result (+).
Blackened medium	Motility cannot be determined; use general motility agar to assess motility. See Exercise 36 for details on general motility agar.

Notecards

Triple Sugar Iron Agar (TSIA)/Kligler Iron Agar (KIA) (From Ex. 37)

Purpose: Differentiates bacteria based on sulfur reduction and sugar fermentation.

How to Inoculate: Both TSIA and KIA are prepared as a shallow agar slant with a deep butt. This format provides both aerobic and anaerobic growth environments. Both media are double inoculated; first use an inoculating needle to stab the butt of the agar and then use a loop to zigzag inoculate the slant ("fishtail inoculation").

Incubation Parameters: For carbohydrate fermentation assessment, incubate the sample at 35±2°C for 18–24 hours. After interpreting the carbohydrate fermentation results, samples are re-incubated for an additional 24 hours at 35±2°C before making a final H_2S determination.

Results	Interpretations
 **Yellow slant/yellow butt—KIA**	KIA: Glucose **and** lactose fermentation with acid accumulation in slant and butt. Note this test result as A/A.
	TSIA: Glucose and lactose **and/or** sucrose fermentation with acid accumulation in slant and butt. Note this test result as A/A.
 **Red slant/yellow butt**	TSIA and KIA: Glucose fermentation with acid production; proteins catabolized aerobically (in the slant) with alkaline products (reversion). Note this test result as K/A.
 **Red slant/red butt**	TSIA and KIA: No fermentation; peptone catabolized aerobically and anaerobically with alkaline products; isolate is not from *Enterobacteriaceae*. Note this test result as K/K.
Red slant/no change in the butt	TSIA and KIA: No fermentation; peptone catabolized aerobically with alkaline products; isolate is not from *Enterobacteriaceae*. Note this test result as K/NC.
No change in slant/no change in butt	TSIA and KIA: Organism is growing slowly or not at all; isolate is not from *Enterobacteriaceae*. Note this test result as NC/NC.
 **Black precipitate in the agar**	TSIA and KIA: Sulfur reduction (an acidic condition from glucose fermentation exists in the butt even if the yellow color is obscured by the black precipitate). Note this test result as H_2S made.
Cracks in or lifting of agar	TSIA and KIA: Gas production. Note this test result as G.

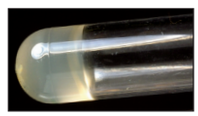
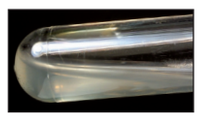
Coagulase Test (From Ex. 38)

Purpose: Differentiates bacteria based on the presence of coagulase, an enzyme that most pathogenic *Staphylococcus aureus* make. The coagulase test differentiates coagulase-positive *Staphylococcus aureus* from other, usually less virulent, coagulase-negative *Staphylococcus* species.

How to Inoculate: Use a sterile wooden applicator or disposable loop to inoculate a tube of rabbit plasma plus EDTA with a pure culture of the test bacterium. Mix the contents by gently rolling the inoculated tube between your hands.

Incubation Parameters: Incubate all tubes at 35±2°C for up to 24 hours, checking for coagulation periodically for the first 4 hours or remainder of your lab. Do not exceed 24 hours of incubation.

Interpretations: Tilt media to see if it solidified.

Results	Interpretations
Medium solidified within 24 hours or there is evident clumping/ coagulation (even if the media did not entirely solidify). 	Free or bound coagulase present; plasma coagulated; probable *Staphylococcus aureus*. Note this as a positive result (+).
Medium remains liquid after 24 hours; no signs of coagulation are evident. 	Plasma did not coagulate; coagulase was not detectable; unlikely to be *S. aureus*. Note this as a negative result (–).

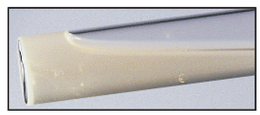
Gelatin Hydrolysis (Gelatinase Test) (From Ex. 39)

Purpose: To detect the enzyme gelatinase, which breaks down gelatin into amino acids.

How to Inoculate: Use an inoculating needle to stab a tube of nutrient gelatin media. Use a heavy inoculum of 18- to 24-hour-old sample.

Incubation Parameters: Two incubation parameters are acceptable. Either incubate at 25±2°C for up to 2 weeks or at 35±2°C for up to 1 week.

Interpretations: Tilt the media to see if it solidified. ***Note:*** If you incubated the samples at 35°C, then you must place the samples (to include an uninoculated control that was incubated alongside the test sample) in an ice bath or refrigerator for about 15 minutes (or until the uninoculated control solidifies).

Results	Interpretations
Medium remains solid after refrigeration. 	The organism does not make the enzyme gelatinase. Note this as a negative result (–).
Medium remains liquid after refrigeration. 	The organism makes the enzyme gelatinase. Note this as a negative result (+).



Appendices

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— Lab Notes —



APPENDIX A
Biochemical Pathways

A lot of what we do in microbiology relates back to basic biochemical pathways (Fig. A.1). The discussion and figures in this appendix illustrate how the various biochemical tests presented in this manual fit into the overall biochemical scheme.

Oxidation of Glucose: Glycolysis, Entner-Doudoroff, and Pentose-Phosphate Pathways

Most organisms perform glycolysis (Embden-Meyerhof-Parnas pathway) (Fig. A.2). Glycolysis breaks glucose into two molecules of pyruvate and, in the process, releases some energy. The energy-releasing (exergonic) reactions that generate ATP do so via a process called **substrate phosphorylation**. Although glycolysis yields a total of four ATP, two ATP are invested early in the pathway, leaving the cell with a net production (profit) of two ATP for each glucose molecule that enters glycolysis.

Glycolysis also generates reducing power—namely NADH—that is important for a variety of reactions to include the production of ATP in electron transport chains by **oxidative phosphorylation**. If environmental conditions do not favor the operation of electron transport chains (e.g., a necessary final electron acceptor is absent), then many cells can funnel NADH into fermentation pathways. Although fermentation pathways do not usually make ATP[1], they do help to regenerate NAD^+ (a necessary cofactor to oxidize glucose) so that glycolysis can continue to make some ATP for cell survival. In summary, each glucose that enters glycolysis will generate two pyruvate molecules, two NADH, and two net molecules of ATP (Table A.1).

In addition to metabolizing sugars, glycolysis is a sort of "metabolic crossroad" that supports anabolism (building) and catabolism (break down) of other biomolecules. Glycolytic intermediates serve as building blocks of certain amino acids, lipids, and nucleotides. The carbon skeletons of these same biomolecules can also be funneled into glycolysis and broken down. Some of these anabolism/catabolism linkages are shown in Figure A.1.

[1] The acetate fermentation pathway does generate some ATP.

The Entner-Doudoroff pathway is an alternative way to break down glucose into two pyruvate molecules (Fig. A.3). Because it generates only one ATP and one NADH (Table A.2), it is less efficient than glycolysis. This pathway is almost exclusively found in bacteria (e.g., *Pseudomonas* and *E. coli*, as well as other Gram negatives) and certain archaea. Some obligate aerobes use this pathway because they cannot convert glucose to glyceraldehyde 3-phosphate, a necessary intermediate in glycolysis. The pathway also helps cells metabolize a different category of sugars (aldonic acids) than glycolysis, enhancing the chances for survival by broadening the organism's range of nutrients.

The pentose-phosphate pathway is a complex set of cyclic reactions that provides a mechanism for producing 5-carbon sugars (pentoses) from 6-carbon sugars (hexoses) (Fig. A.4). The resulting pentose sugars can enter pathways that make ribonucleotides and deoxyribonucleotides, as well as aromatic amino acids. Further, this pathway produces NADPH, an important electron donor in anabolic pathways.

TABLE A.1 Summary of Key Glycolytic Reactants and Products per Glucose (Theoretical Maximum Yield)

Reactant	Product
Glucose ($C_6H_{12}O_6$)	2 Pyruvate ($C_3H_3O_3$)
2 ATP + 4 ADP + 2P_i	4 ATP + 2 ADP (***Note:*** The *profit* is 2 ATP when we consider the initial 2 ATP investment)
2 NAD^+	2 NADH

TABLE A.2 Summary of Entner-Doudoroff Reactants and Products per Glucose (Theoretical Maximum Yield)

Reactant	Product
Glucose ($C_6H_{12}O_6$)	2 Pyruvate ($C_3H_3O_3$)
1 ATP + 2 ADP + P_i	1 ADP + 2 ATP (***Note:*** The *profit* is 1 ATP when we consider the initial ATP investment)
1 NAD^+	1 NADH
1 $NADP^+$	1 NADPH

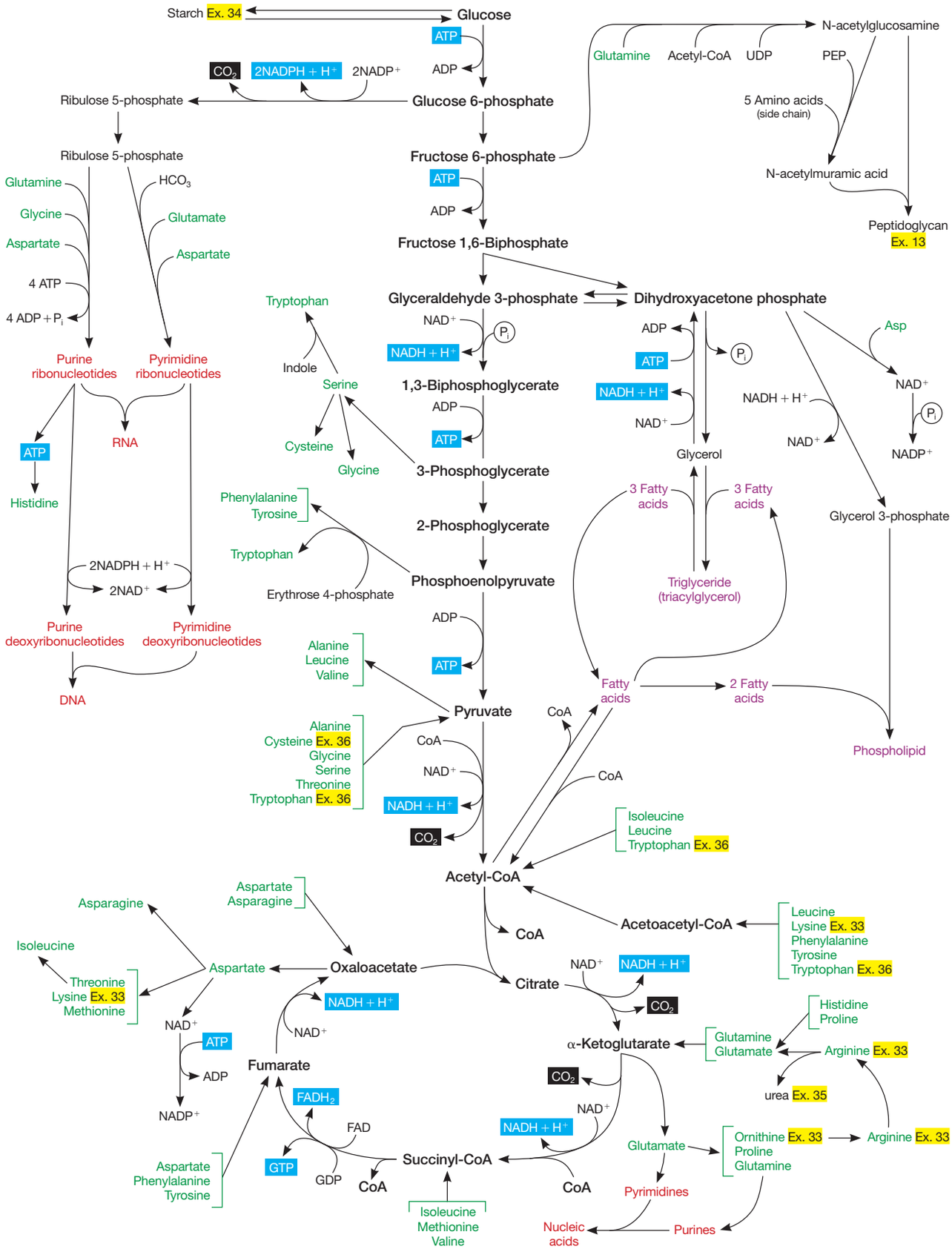


FIGURE **A.1** **Integrated Metabolism** • This diagram shows select bacterial anabolic and catabolic pathways and examples of where lab exercises touch on these metabolic pathways. **Abbreviations: CoA**: coenzyme A; **P_i**: inorganic phosphate; **GTP**: guanosine triphosphate; **GDP**: guanosine diphosphate; **UDP**: uridine diphosphate; **NAD^+/NADH**: nicotinamide adenine dinucleotide (oxidized and reduced forms); **$NADP^+$/NADPH**: nicotinamide adenine dinucleotide phosphate (oxidized and reduced forms); **FAD/$FADH_2$**: flavin adenine dinucleotide (oxidized/reduced).

The pentose-phosphate reactants and products are listed in Table A.3. To completely oxidize one hexose to 6 CO_2, a total of six hexoses must enter the cycle as glucose 6-phosphate and follow one of three different routes (notice the symmetry of pathways as drawn). Notice in Figure A.4 that each hexose loses a CO_2 upon entering the cycle, but at the end five hexoses are produced. Thus, the net reaction is one hexose being oxidized to 6 CO_2. Notice also the reactions that transfer 2-carbon and 3-carbon fragments between the 5-carbon intermediates. Transketolase catalyzes the 2-carbon transfer, whereas transaldolase catalyzes the 3-carbon transfer. Alternatively, the 5-carbon intermediates can be redirected into pathways for synthesis of aromatic amino acids and nucleotides (not shown).

The decarboxylation of glucose 6-phospate in the pentose phosphate pathway is not as simple as shown in Figure A.4. The preliminary steps include production of 6-phosphogluconate (an Entner-Doudoroff pathway intermediate), to accommodate the passage of carbon skeletons between the two pathways. The pentose phosphate pathway also intersects with glycolysis at glyceraldehyde 3-phosphate and fructose 6-phosphate.

TABLE **A.3** Summary of Pentose-Phosphate Reactants and Products per Glucose 6-phosphate (Theoretical Maximum Yield)

Reactant	Product
Glucose 6-phosphate (C_6)	6 CO_2 + 1 P_i
12 $NADP^+$	12 NADPH

Oxidation of Pyruvate: The Citric Acid Cycle and Fermentation

Pyruvate represents a major crossroad in metabolism, and all three pathways discussed so far have the potential to make it, though not in equal quantities. Pyruvate has a variety of possible fates. It can be funneled toward the citric acid cycle, which produces ATP and NADH. Or, in some cases, it can enter fermentation pathways, which generally do not produce ATP.

Organisms that carry out aerobic and/or anaerobic respiration tend to rely on the citric acid cycle as a major metabolic pathway (Fig. A.5). Before the pyruvate enters the citric acid cycle, an intermediate (or gateway) step converts it to acetyl-coenzyme A (acetyl-CoA). Then, acetyl-CoA enters the citric acid cycle through a condensation reaction with oxaloacetate.

For each pyruvate that enters the cycle via the intermediate (or entry) step, the following products are made: 3 CO_2, 4 $NADH^+$, 1 $FADH_2$, and 1 GTP. (Because two pyruvate molecules are made per glucose, these numbers are doubled in Table A.4, which shows the theoretical maximum yield per glucose.) Electron transport chains then oxidize NADH and $FADH_2$, thereby fueling ATP production. Table A.5 shows theoretical maximum ATP yields from the complete oxidation of glucose.

Like glycolysis, many of the citric acid cycle's intermediates are entry points for amino acid, nucleotide, and lipid catabolism, as well as a source of carbon skeletons for synthesis of the same compounds. These pathways are shown in Figure A.1. Single arrows may represent several reactions, and other carbon compounds, not illustrated, may be required to complete a given reaction.

Figure A.6 illustrates some major microbial fermentation pathways (though no single organism performs all of them). Pyruvate (shown in the blue box) is typically the starting point for each. Fermentation end products are shown in red. Fermentation allows some cells living under anaerobic conditions to replenish the supply of oxidized coenzymes (e.g., convert NADH back to NAD^+, shown in blue) generated during glycolysis or other pathways. Some bacteria (aerotolerant anaerobes) exclusively rely on fermentation, even if oxygen is available. Table A.6 summarizes major fermentation pathways and representative organisms that perform each.

Notice that fermentation end products typically fall into three categories: acid, gas, or an organic solvent (an alcohol or a ketone). The specific fermentation performed is useful for bacterial classification.

TABLE **A.4** Summary of Reactants and Products per Glucose for the Intermediate Step and Citric Acid Cycle (Theoretical Maximum Yield)

Intermediate (or entry) Step		Citric Acid Cycle	
Reactant	**Product**	**Reactant**	**Product**
2 Pyruvate + 2 Coenzyme A	2 Acetyl-CoA + 2 CO_2	2 Acetyl-CoA	4 CO_2 + 2 Coenzyme A
2 NAD^+	2 NADH	6 NAD^+	6 NADH
		2 GDP + 2 P_i (= 2 ADP + 2 P_i)	2 GTP (= 2 ATP)
		2 FAD	2 $FADH_2$

TABLE **A.5** Prokaryotic Aerobic Cellular Respiration Theoretical Maximum ATP Yields

Compound	Number Produced	ATP Value[1]	Total ATP per Glucose
NADH (from glycolysis, intermediate step, and citric acid cycle)	10	3	30
$FADH_2$ (from citric acid cycle)	2	2	4
ATP (from substrate phosphorylation in glycolysis)	4		4

[1] Experimental evidence indicates that the ATP values of 3 per NADH and 2 per $FADH_2$ in the aerobic electron transport chain are probably too high. For mitochondria, values of 2.5 and 1.5, respectively, are closer to reality. However, because multiple electron transport chains have been identified within the domain *Bacteria* (and even within single species!) no single, universal number for ATP yields per NADH and $FADH_2$ can be assigned. So, we fall back on the accepted theoretical maximum of 3 and 2, respectively.

TABLE **A.6** Major Fermentations, Their End Products, and Some Organisms That Perform Them

Fermentation	Major End Products	Representative Organisms
Alcoholic fermentation	Ethanol and CO_2	*Saccharomyces cerevisiae*
Homolactic fermentation	Lactate	*Streptococcus* and some *Lactobacillus*
Heterolactic fermentation	Lactate, ethanol, and acetate	*Streptococcus*, *Leuconostoc*, and *Lactobacillus*
Mixed acid fermentation	Acetate, formate, succinate, CO_2, H_2, and ethanol	*Escherichia*, *Salmonella*, *Klebsiella*, and *Shigella*
2,3-butanediol fermentation	2,3-butanediol	*Enterobacter*, *Serratia*, and *Erwinia*
Butyrate/butanol fermentation	Butanol, butyrate, acetone, and isopropanol	*Clostridium*, *Butyrivibrio*, and some *Bacillus*
Propionic acid fermentation	Propionate, acetate, and CO_2	*Propionibacterium*, *Veillonella*, and some *Clostridium*

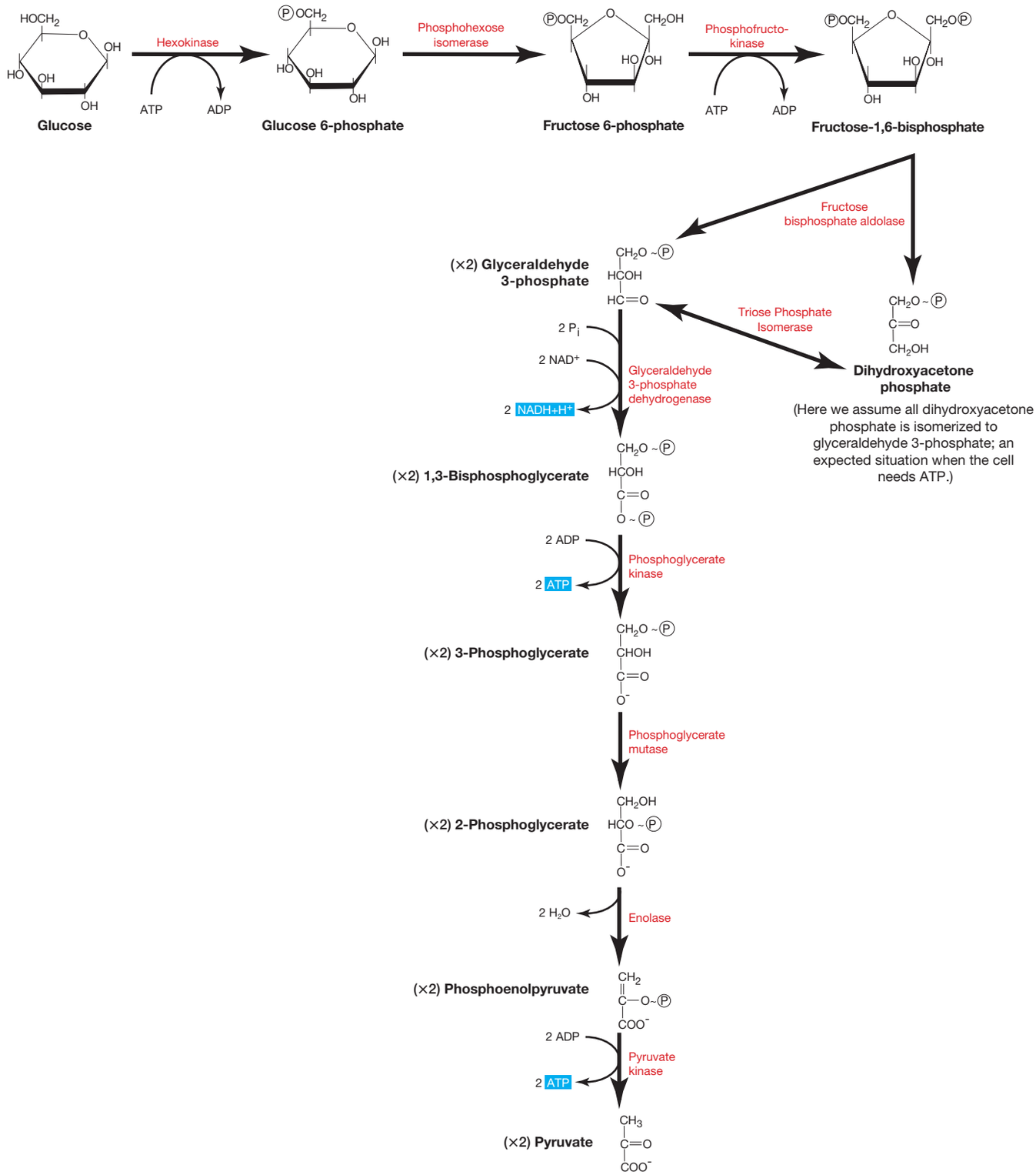


FIGURE **A.2** **Glycolysis** • Glycolytic intermediates are in black; the enzyme names are in red. Reducing power (NADH) and ATP are highlighted in blue. The pathway shown here assumes that all dihydroxyacetone phosphate is isomerized to glyceraldehyde 3-phosphate, the expected situation when a cell needs ATP. Table A.1 summarizes the products and reactants of glycolysis.

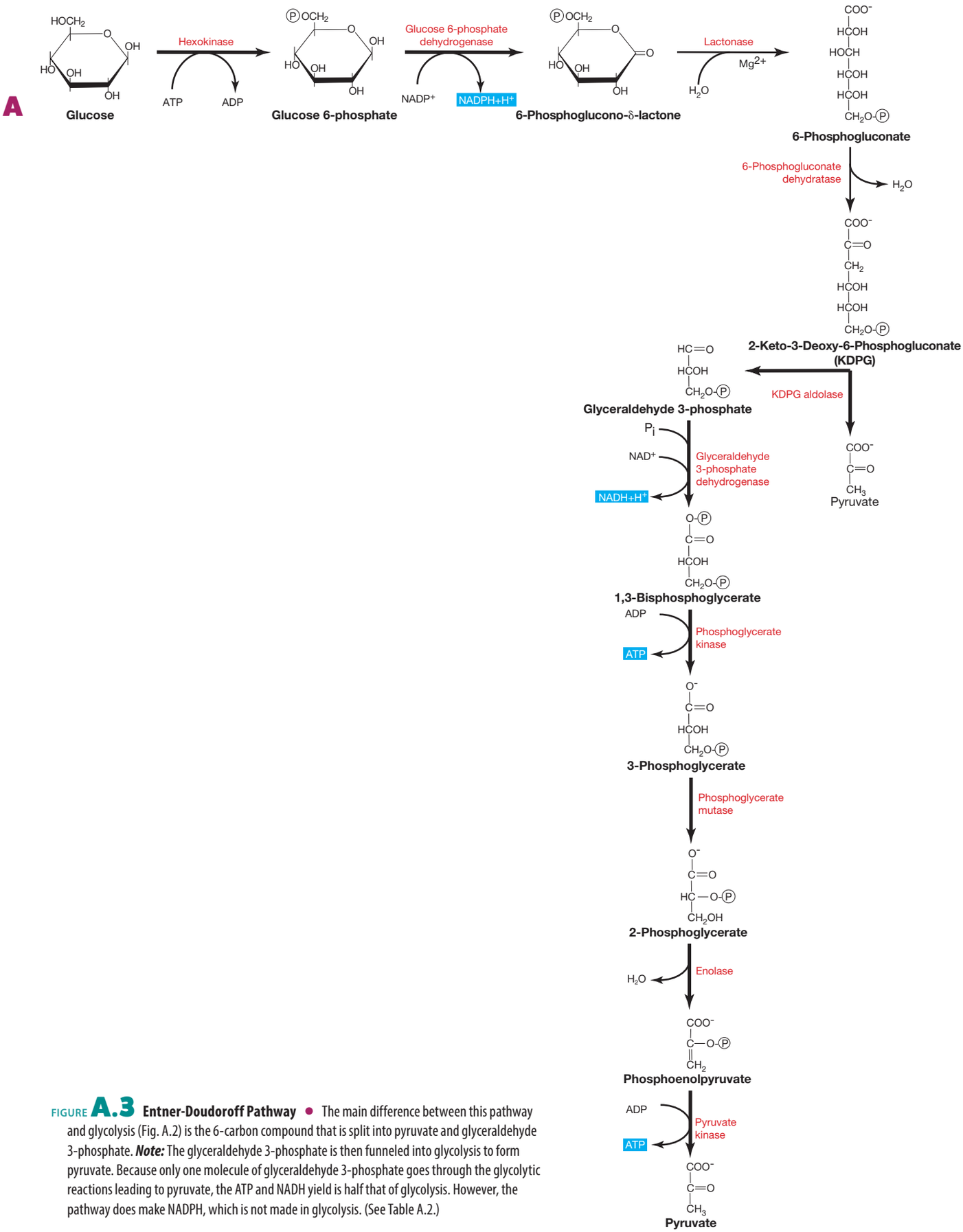


FIGURE **A.3** **Entner-Doudoroff Pathway** • The main difference between this pathway and glycolysis (Fig. A.2) is the 6-carbon compound that is split into pyruvate and glyceraldehyde 3-phosphate. ***Note:*** The glyceraldehyde 3-phosphate is then funneled into glycolysis to form pyruvate. Because only one molecule of glyceraldehyde 3-phosphate goes through the glycolytic reactions leading to pyruvate, the ATP and NADH yield is half that of glycolysis. However, the pathway does make NADPH, which is not made in glycolysis. (See Table A.2.)

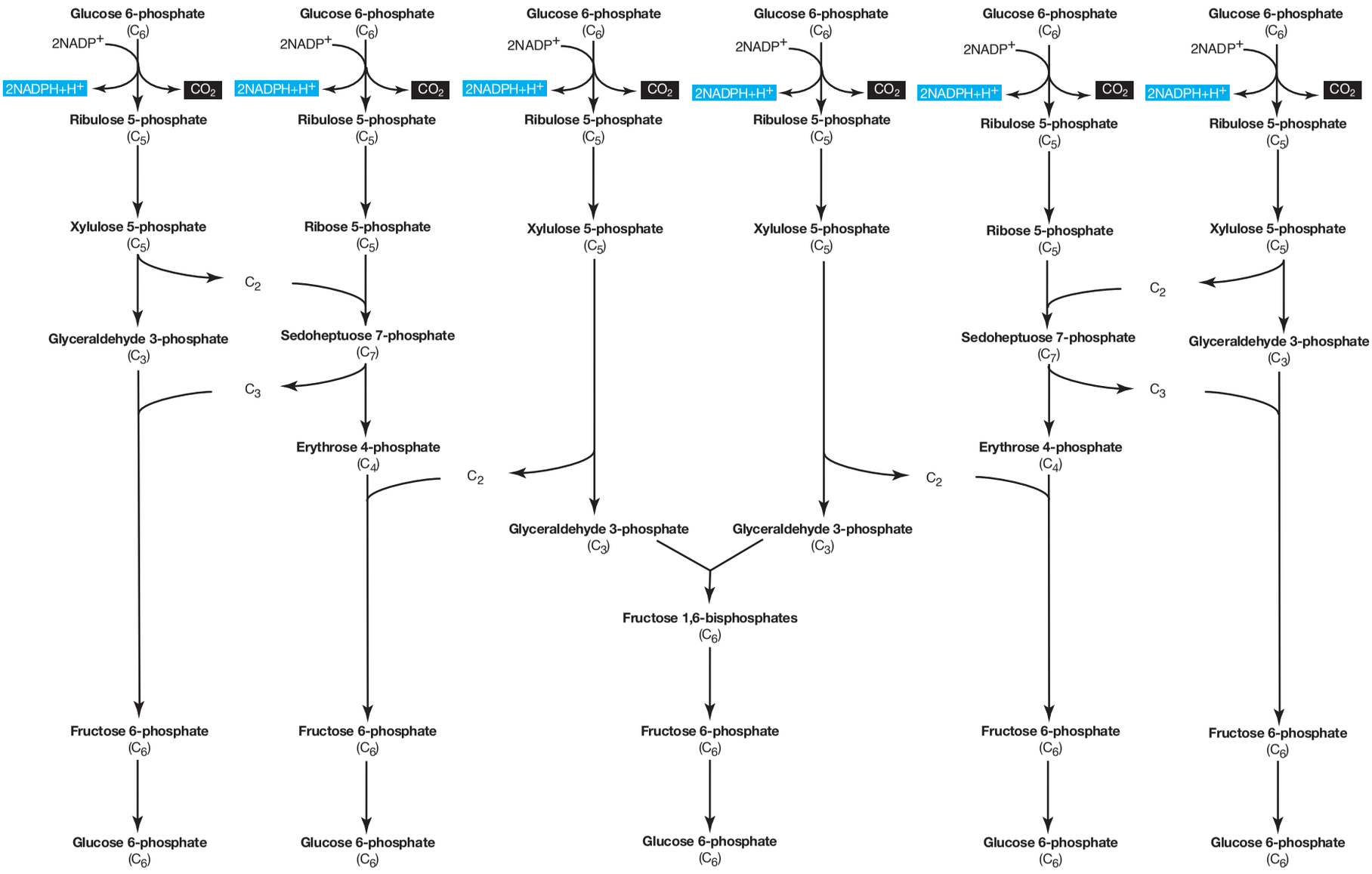


FIGURE **A.4** **Pentose-Phosphate Cycle** • For every six glucose 6-phosphates that enter and complete the cycle, 6 CO_2 and 12 NADPH are produced. Some of the 5-carbon intermediates, however, may be used to build aromatic amino acids and nucleotides. If the cycle is performed as shown, 36 carbons enter as six glucose 6-phosphates ($6 \times C_6 = 36C$). Six CO_2 are immediately lost, leaving a total of 30C to get shuffled around by the remaining reactions to form five glucose 6-phosphates ($5 \times C_6 = 30C$). (See Table A.3.)

A

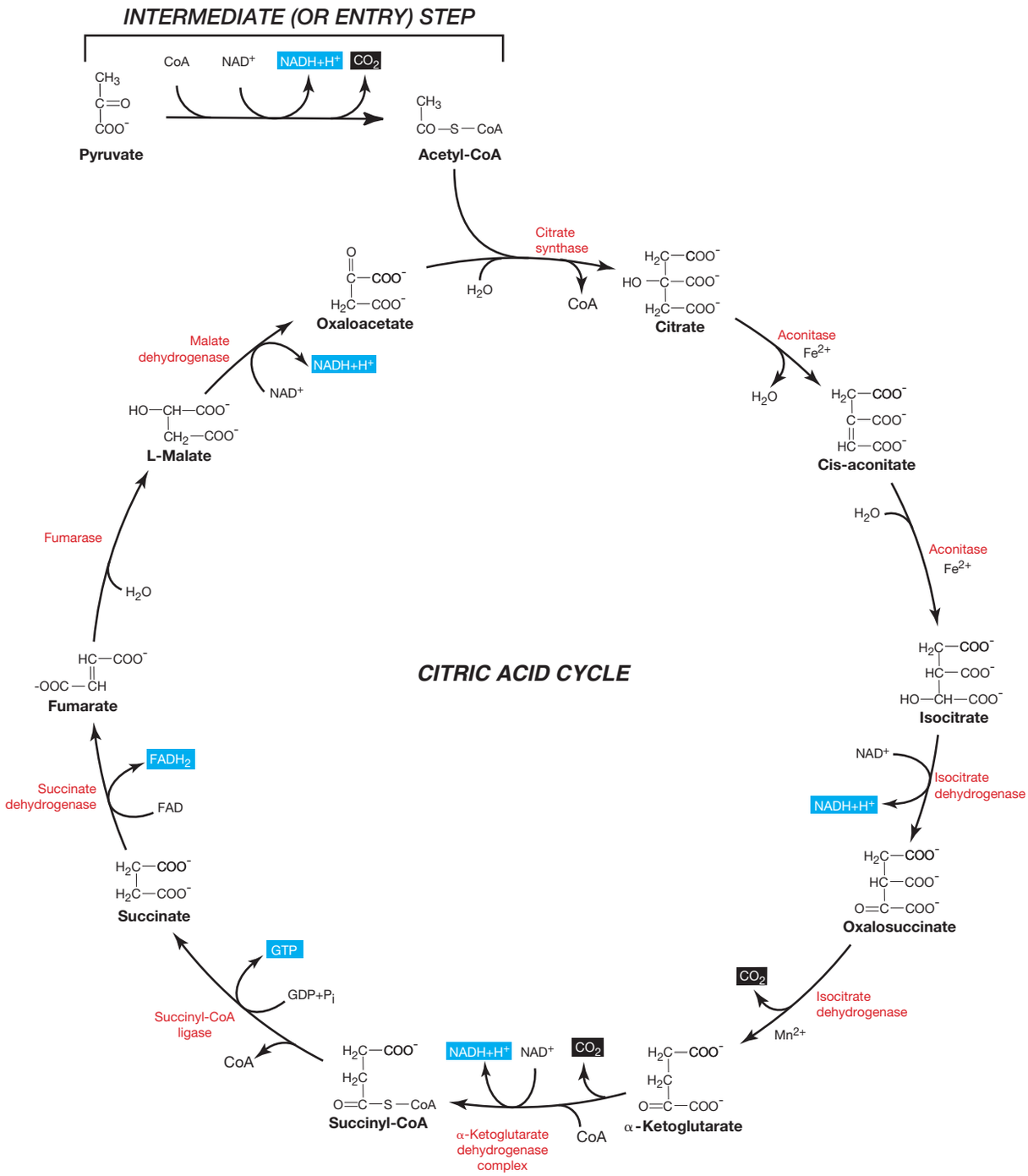


FIGURE **A.5** **Intermediate Step and Citric Acid Cycle** ● Intermediates are printed in black; enzymes are in red. Reducing power (in the form of NADH and $FADH_2$) and energy (in the form of GTP) are highlighted in blue. CO_2 produced from the oxidation of carbon is highlighted in black. (See Table A.4.)

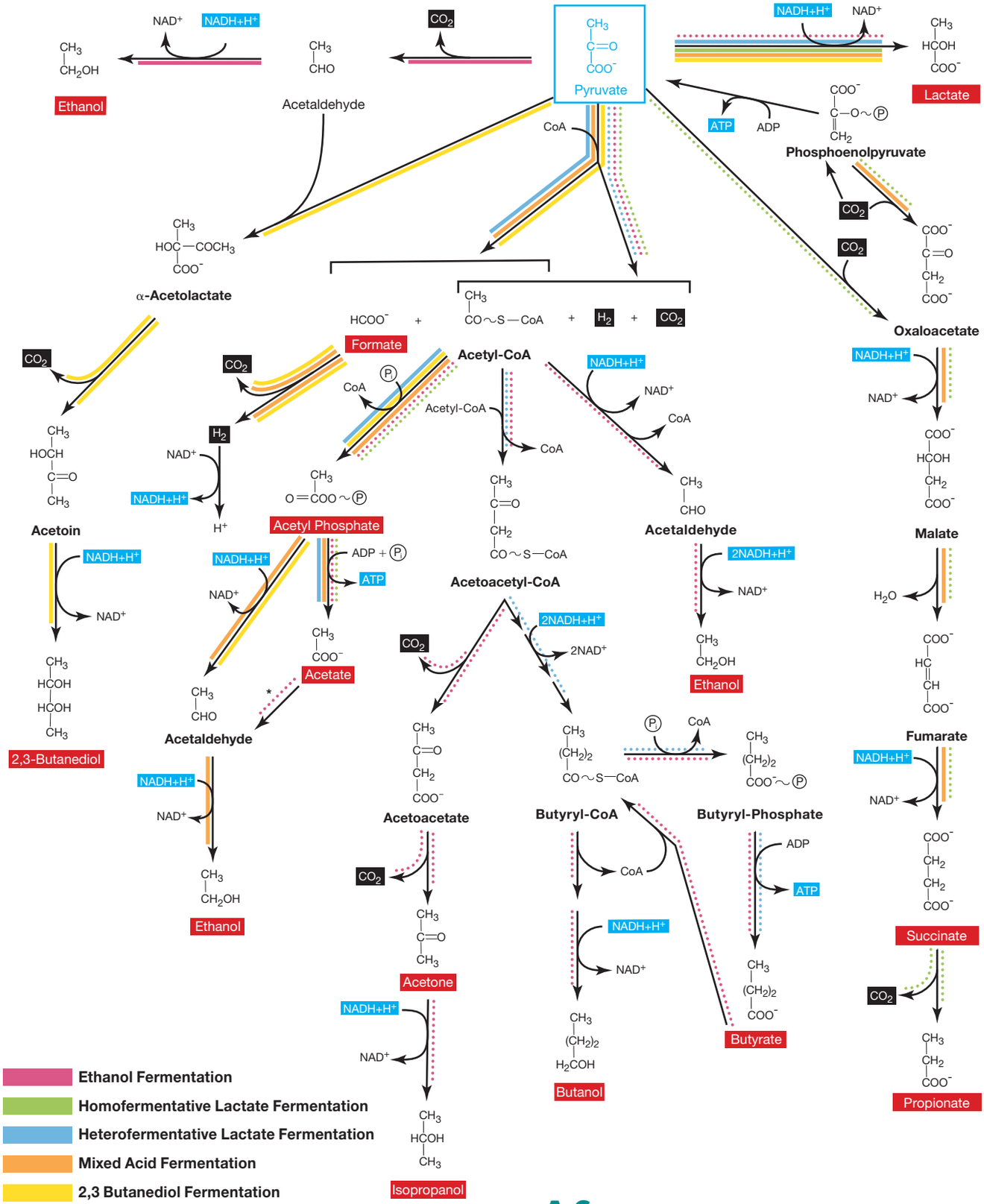


FIGURE **A.6** **Sampling of Fermentation Pathways** • Note that all pathways start with pyruvate and, at some point, NADH (in blue) is oxidized to NAD^+. The resulting end products are acids, gases, or alcohols. Major fermentation pathways are highlighted by different colors and are summarized in Table A.5.

— Lab Notes —

APPENDIX B
Miscellaneous Transfer Methods

Following are instructions for transfer methods that are performed less routinely than those in Exercises 3 and 4.

Transfers Using a Sterile Cotton Swab

A sterile swab generally is used to obtain a sample from a primary source, either a patient or an environmental site. Occasionally, swabs are used to transfer pure cultures (e.g., Kirby-Bauer test, Ex. 43, pp. 389–395). Sterile swabs may be dry, or they may be in sterile water, depending on the nature of the sample to be taken (wet or dry). In either case, care must be taken not to contaminate the swab by unintentionally touching other surfaces with it. Your instructor may provide specific instructions on collection of samples from sources other than the ones listed here.

Obtaining a Sample from a Patient's Throat with a Swab

1. **Use BSL-2 precautions throughout.** Wear a lab coat, disposable gloves, and eye protection.
2. Use a sterile tongue depressor and swab prepared in sterile water to obtain a sample from the throat. Have the patient open their mouth, and then gently press down on the tongue (Fig. B.1).
3. With the swab in your dominant hand, carefully sample the patient's throat with a swirling motion. Touching other parts of the oral cavity is likely to cause contamination. Also, avoid touching the soft palate or it may initiate a gag reflex!
4. Transfer the sample to an appropriate plated medium as quickly as possible. If plating is to be done at a later time, place the swab in an appropriate sterile container (such as a sterile, capped test tube with sterile water deep enough to immerse the cotton). Store in the lab refrigerator if necessary.
5. Use the swab to begin a quadrant streak (Ex. 4, Fig. 4.3, p. 70). Properly dispose of the swab according to your lab's protocol, and then complete the streaking with your loop. Incinerate the loop or dispose of it in the appropriate autoclave container.
6. Label the plate with your name, the sample source, date, and medium.



FIGURE **B.1** **Taking a Throat Sample** • Use a sterile tongue depressor and swab to obtain a sample from a patient's throat. Be careful not to touch other parts of the oral cavity or the sample will get contaminated. (The communities of microorganisms differ in different parts of the oral cavity and throat!) Transfer the sample to a sterile medium as soon as possible.

7. Incubate the plate in an inverted position at the appropriate temperature for the designated time.

Obtaining an Environmental Sample with a Cotton Swab

1. **Use BSL-2 precautions throughout.** Wear a lab coat, disposable gloves, and eye protection.
2. Use a sterile swab prepared in sterile water to obtain a sample from an environmental source.
3. Rotate the swab to collect from the area to be sampled (Fig. B.2).
4. Transfer the sample to an appropriate plated medium as quickly as possible. If plating is to be done at a later time, place the swab in an appropriate sterile container (such as a sterile, capped test tube with sterile water deep enough to immerse the cotton). Store in the lab refrigerator if necessary.
5. Depending on the anticipated cell density, use the swab to perform a zigzag streak pattern (Ex. 4, Fig. 4.6, p. 72) or to begin a quadrant streak with a disposable loop (Ex. 4, Fig. 4.3, p. 70). Properly dispose of the swab and loop (if used) in the appropriate autoclave container.

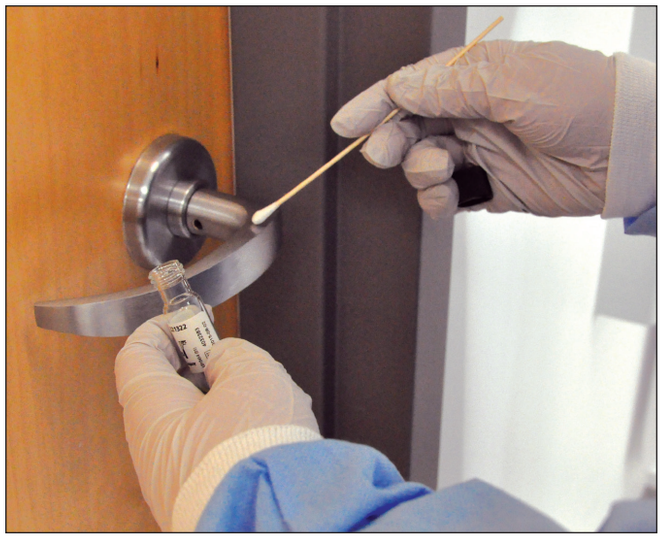


FIGURE **B.2** **Taking an Environmental Sample** • Use a spinning motion of a sterile swab to sample inanimate objects in the environment. The swab may be placed in a sterile test tube until it is convenient to transfer the sample to a growth medium. Notice the tube's cap being held in the pinky of the swab hand.

6. Label the plate with your name, the sample source, date, and medium.
7. Incubate the plate in an inverted position at the appropriate temperature for the designated time.

Spot Inoculation of an Agar Plate

Sometimes an agar plate may be used to grow several different specimens at once. This is a typical practice with plated *differential* media (i.e., media designed to differentiate organisms based on growth characteristics).

Prior to beginning the transfer, divide the plate into as many as four sectors using a marking pen. (Some plates already have marks on the base for this purpose.) Then each may be inoculated with a different organism.

Inoculation involves touching the loop to the agar surface once so growth is restricted to a single spot, hence the name "spot inoculation."

1. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.
2. Label the plate's base with your name, date, medium, and organism(s) inoculated. Write these toward the periphery, not across the center, which makes results difficult to read.
3. With a marking pen, divide the base of the plate into the desired number of sectors. Figure B.3 shows a plate divided into four sectors.
4. Aseptically get growth from the source culture on the inoculating loop.
5. Lift the lid of the sterile agar plate and use it as a shield to prevent airborne contamination.
6. Touch the agar surface toward the periphery of the sector (Fig. B.3).
7. Remove the loop and replace the lid.
8. Incinerate your loop as before. It is especially important to flame it from base to tip now because the loop has bacteria on it.
9. Repeat Steps 4 through 8 for the remaining cultures.
10. Incubate the plate in an inverted position for the assigned time at the appropriate temperature.
11. Save or dispose of the original cultures as directed by your instructor.

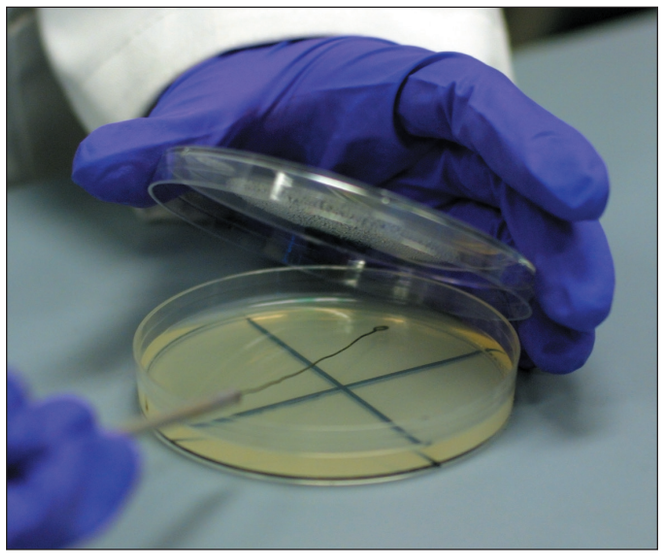


FIGURE **B.3** **Spot Inoculation of a Plate** • Each of four sectors is spot inoculated with a different organism by touching the loop to the surface and making a mark about 1 cm in length. Generally, spot inoculations are done toward the edge (rather than the crowded middle) to prevent overlapping growth and/or test results.

APPENDIX C

Using a Glass Pipette

Glass pipettes are used to transfer a known volume of liquid diluent, media, or culture. Originally pipettes were filled by sucking on them like a drinking straw, but mouth pipetting is dangerous and has been replaced by mechanical pipettes. Three examples are shown in Figure C.1, each with its own method of operation. Your instructor will show you how to properly use the type of pipette available in your lab.

To use a pipette correctly, you must be able to correctly read the calibration. Examine Figure C.2. The numbers at the top indicate the pipette's *total volume* and its *smallest calibrated increments*. This is a 10.0 mL pipette divided into 0.1 mL increments.

When reading volumes, use the base of the meniscus (Fig. C.3). The volume in the center pipette is read as exactly 3.0 mL because the meniscus is resting on the line. The right pipette is read as 2.9 mL and the left pipette is read as 3.1 mL (0 is always at the top of the pipette). Although the difference in volume between these three pipettes may seem negligible (1 part in 70[1] is about a 1.4% error), it introduces avoidable error into your work.

Two pipette styles are used in microbiology (Fig. C.4): the serological pipette and the Mohr pipette. A serological pipette is calibrated to deliver (TD) its volume by completely draining it and blowing out the last drop. The tip of a Mohr pipette is not graduated, so fluid flow must be stopped at a calibration line. Stopping the fluid beyond the last line on a Mohr pipette results in an unknown volume being dispensed. In either case, volumes are read at the bottom of the fluid meniscus.

[1] This number presumes the pipette shown has a total volume of 10 mL. If the meniscus is on the 3, the pipette will dispense 7 mL. If emptied completely, an error of 0.1 mL above (delivering 7.1 mL) or below the 3 (delivering 6.9 mL) would represent 0.1 mL in 7 mL, or 1 part in 70, a 1.4% error that is avoidable.

CAUTION!

It's important if you are pipetting a bacterial culture to work over a disinfectant-soaked towel in case any culture drips from the pipette before disposing of it in the autoclave container. This is especially important if transferring a BSL-2 organism. Clean up any spills with additional disinfectant. Also, it is recommended that you use a Mohr pipette to transfer BSL-2 organisms. These are not "blown-out," so aerosol production is reduced.



FIGURE C.1 **Mechanical Pipettes** • Three examples of mechanical pipettes are shown here, each with its own method of operation. Your instructor will show you how to properly use the style of pipette available in your lab. From left to right: A semi-automated piette, a pipette bulb, and a plastic pump.

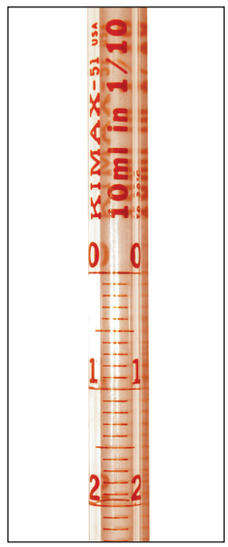


FIGURE C.2 **Pipette Calibration** • Prior to using a pipette, read its calibration. The numbers indicate the pipette's total volume and its smallest calibrated increments. This is a 10.0 mL pipette divided into 1/10 (0.1) mL increments.

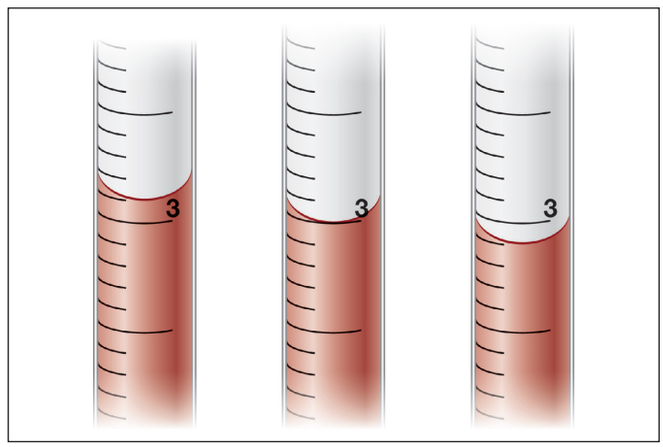


FIGURE C.3 **Read the Base of the Meniscus** • When reading volumes, use the base of the meniscus. The volume in the center pipette is read at exactly 3.0 mL because the meniscus is resting on the line. The right pipette is read as 2.9 mL and the left pipette is read as 3.1 mL (0 is always at the top of the pipette). ***Note:*** The number read usually isn't the volume delivered. If these are all 10.0 mL pipettes, then completely emptying the center one would deliver 10.0 mL – 3.0 mL = 7.0 mL. The pipette on the right would deliver 7.1 mL and the one on the left 6.9 mL.

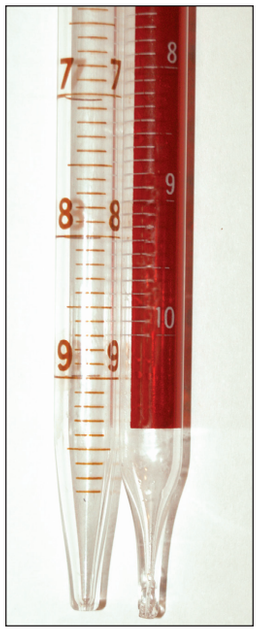


FIGURE **C.4** **Two Types of Pipettes** • Two pipette styles—the serological pipette (left) and the Mohr pipette (right)—are used in microbiology. A serological pipette is calibrated to deliver (TD) its volume by completely draining it and blowing out the last drop. The tip of a Mohr pipette is not graduated, so fluid flow must be stopped at a calibration line. Stopping the fluid beyond the last line on a Mohr pipette results in an unknown volume being dispensed.

C

Filling a Glass Pipette

1. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.
2. Bacteria should be suspended in the broth with a vortex mixer or by agitating with your fingers (Ex. 3, Fig. 3.6, p. 54). Be careful not to splash the broth into the cap or lose control of the tube. If transferring a sterile liquid, mixing is not necessary.
3. Pipettes are sterilized in metal canisters, individually in sleeves, or as multiples in packages (if disposable). They typically are stored in groups of a single size (Fig. C.5). *Be sure you know what volume your pipette will deliver.* Set the canister at the table edge and remove its lid. (Canisters—even cylindrical ones—should not be stored in an upright position, because they may fall over and break the pipettes or become contaminated.) If using pipettes in a package, open the end *opposite the tips*. Grasp *one pipette only* and remove it.
4. Carefully insert the pipette into the mechanical pipette (Fig. C.6). Grasp the pipette near the end with your fingertips. This gives you more control and reduces the chance that you will break the pipette and cut your hand or jam the broken pipette into your palm. **Notice how the microbiologist is holding it in Figure C.6.** *Do not touch* any part of the pipette that will contact the specimen or the medium or you risk introducing a contaminant. Also, do not lay the pipette on the tabletop while you continue.
5. While keeping your pipette hand still, bring the culture tube toward it. Use your little finger to remove and hold its cap.
6. Flame the open end of the tube by passing it through a Bunsen burner flame two or three times.
7. Hold the tube at an angle to prevent contamination from above.
8. Insert the pipette and withdraw the appropriate volume (Fig. C.7). Bring the pipette to a vertical position briefly to read the meniscus accurately. (**Remember:** The volumes in the pipette are correct only if the meniscus of the fluid inside is resting *on* the line, not above or below it.) Then carefully remove the pipette from the tube.



FIGURE **C.5** **Getting the Sterile Pipette** • Pipettes of the same size are autoclaved in canisters, which then are opened and placed flat on the table for stability. Pipettes are removed as needed by only touching the end opposite the tip.



FIGURE **C.6** **Assembling the Pipette** • Carefully insert the pipette into a mechanical pipette. Notice that the pipette is held near the end with the fingertips. For safety, the hand is out of the way in case the pipette breaks.



FIGURE **C.7** **Filling the Pipette** • Carefully draw the fluid into the pipette. Briefly bring it to a vertical position and read the volume. Notice how the tube's cap is being held by the pinky finger of the pipette hand.

9. Flame the tube lip as before. Keep your pipette hand still.
10. Keeping the pipette hand still (remember, it may contain fluid with microbes in it), move the tube to replace its cap.
11. What you do next depends on the medium to which you are transferring the liquid. Please continue with the appropriate inoculation section.

Inoculation of Broth Tubes with a Pipette

Pipettes are often used to inoculate a known volume of culture into a tube or bottle containing a known volume of diluent during serial dilutions. They also would have been used to deliver the volume of diluent waiting in the tube or bottle.

1. While keeping the pipette hand still, bring the dilution tube/bottle toward it. Use your little finger to remove and hold its cap.
2. Flame the lip of the tube/bottle by quickly passing it through the Bunsen burner flame two or three times. Keep your pipette hand still.
3. Hold the open tube/bottle on an angle to minimize airborne contamination. Keep your pipette hand still.
4. Insert the pipette tip and gently dispense the correct volume of inoculum. Aerosols can be produced if you dispense too quickly.
5. Withdraw the tube/bottle from over the pipette. If using a serological pipette, touch its tip to the glass to remove any excess broth before completely removing it.
6. Completely remove the pipette, but avoid waving it around. This can create aerosols.
7. Flame the tube/bottle lip as before. Keep your pipette hand still.
8. Keeping the pipette hand still, move the tube/bottle to replace its cap.
9. The pipette may be contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Glass pipettes typically are placed in a pipette disposal receptacle containing a small amount of disinfectant until they are autoclaved and reused (Fig. C.8). Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipette. There is danger of culture dripping from the pipette or of breaking the glass or plastic.



FIGURE **C.8** **Dispose of the Pipette** • The pipette must be disposed of correctly, especially if it is contaminated with microbes. Each lab has its own specific procedures, and your instructor will advise you what to do. Shown here is a jar used for reusable glass pipettes. Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipette. There is danger of culture dripping from the pipette or of breaking the glass or plastic.

Inoculation of Agar Plates with a Pipette

Pipettes also can be used to dispense a known volume of inoculum to an agar plate.

1. Lift the lid of the plate and use it as a shield to protect it from airborne contamination. It also will help in containing any aerosols you inadvertently produce delivering the inoculum.
2. Hold the pipette over the agar and dispense the correct volume (often 0.1 mL) onto the agar surface between the middle and one edge of the dish (Fig. C.9). From this point, the remaining steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
3. The pipette is contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Glass pipettes typically are placed in a pipette disposal receptacle containing a small amount of disinfectant until they are autoclaved and reused (Fig. C.8). Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipette. There is danger of culture dripping from the pipette or of breaking the glass or plastic.
4. Continue with the spread plate technique (Ex. 5, pp. 81–84).



FIGURE **C.9** **Dispensing to an Agar Plate** • Open the lid and dispense the inoculum onto the surface of the agar between the middle and edge. This provides open agar on which to cool the spreading rod. Notice how the plate's lid is held to prevent aerial contamination and to minimize aerosols. The plate may be placed on a disinfectant-soaked towel in case the pipette drips (not shown).

APPENDIX D
Using a Micropipette

Modern molecular biology procedures often involve transferring extremely small volumes of liquid with great precision and accuracy. This has led to the development of micropipettes (sometimes called a digital adjustable pipette) (Fig. D.1) that can be set to dispense microliter volumes through milliliter volumes (recall that 1 mL equals 1,000 µL). Common micropipette calibrations include volumes of 1 to 10 µL, 10 to 100 µL, 100 to 1,000 µL, or 1,000 to 5,000 µL.

Filling a Micropipette

Many manufacturers make micropipettes, but they all work basically the same way. The following instructions are for Eppendorf Series 2100 models. ***Caution!*** **Use BSL-2 precautions, if appropriate.** Before you begin, label the destination tube(s) or plate(s) with your name, the date, source of inoculum, and medium.

1. Wear a lab coat, gloves, and chemical eye protection when performing this procedure.
2. Growth may be suspended in the broth with a vortex mixer or by agitating with your fingers (Ex. 3, Fig. 3.6, p. 54). Be careful not to splash the broth into the cap or lose control of the tube. If transferring a sterile liquid, mixing is not necessary.
3. Determine which micropipette should be used to dispense the desired volume.
4. Turn the setting ring to set the desired volume (Fig. D.2). Do not turn the dial past either extreme of the pipette's volume range. Doing so will damage it.
5. Hold the micropipette in your dominant hand.
6. Open the case of appropriate pipette tips for your pipette (these are often color-coded and match the pipette) and push the pipette into a sterile tip (Fig. D.3). Close the case. Do not touch the pipette tip with your hands or leave the case open. **Never use a micropipette without a tip.**
7. Remove the cap from the culture tube with the little finger of your dominant hand, and flame the tube. If transferring from a microtube, simply open the cap, but don't flame it!
8. Press down the control button with your thumb to the first stop. This is the measuring stroke.
9. Insert the tip into the broth approximately 3 mm while holding it vertically (Fig. D.4). Make sure you put the tip in deep enough—but not too deep—so it doesn't come out as the liquid level drops while filling.



FIGURE **D.2** **Setting the Volume** • Rotate the adjustment knob (setting ring) to set the volume on a micropipette. This pipette has been set at 90.0 µL (the line between the last two zeroes is a decimal point). Never rotate the adjustment knob beyond the volume limits of the pipette as it may become damaged.



FIGURE **D.1** **Micropipette** • This is a micropipette that dispenses volumes between 10 µL and 100 µL. Also, always use a micropipette with the appropriate tip. As shown here, tips and pipettes are often color-coded. If the tips have been sterilized, always close the tip case when not picking up a tip.

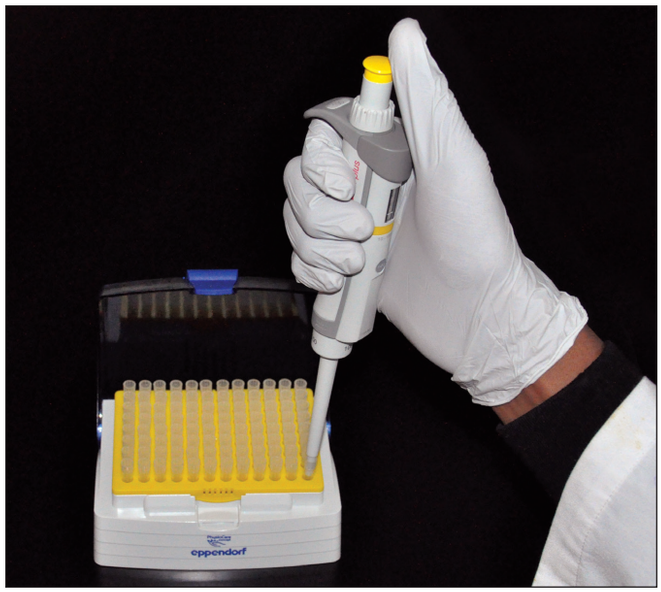


FIGURE **D.3** **Micropipette Tips** • Micropipettes must be fitted with a (sterile) tip of appropriate size (often color-coded to match the pipette). Open the case and press the pipette into a tip, then close the case to maintain sterility. Do not touch the pipette tip.



FIGURE **D.4** **Filling the Micropipette** • Depress the control button with your thumb to the first stop (measuring stroke). Holding the tube and pipette in a vertical position, insert the tip deep enough into the fluid (about 3 mm, see inset) that the tip won't be exposed as the liquid level drops during filling. Slowly release pressure with your thumb to fill the pipette.

10 *Slowly* release pressure with your thumb to draw fluid into the tip. Be careful not to pull any air into the tip.

11 Remove the pipette and hold it still as you flame the tube as before or close the microtube's cap (without flaming).

12 Keeping the pipette hand still, move the tube to replace its cap.

13 What you do next depends on the medium to which you are transferring the growth. Please continue with the appropriate inoculation section.

Using a Micropipette to Inoculate Broth Tubes

Micropipettes are often used to inoculate a known volume of culture into a tube or bottle containing a known volume of diluent during serial dilutions. They also would have been used to deliver the volume of diluent waiting in the tube or bottle.

1 Remove the cap of the sterile medium with the little finger of your pipette hand, and hold it there. If using a microtube, simply open the cap and continue with Step 3.

2 Flame the tube by quickly passing it through the Bunsen burner flame a couple of times. Keep your pipette hand still.

3 Insert the pipette tip into the tube. Hold it at an angle against the inside of the tube (Fig. D.5).

4 Depress the control button slowly with your thumb to the first stop and pause when no more liquid is dispensed. Then continue pressing gently to the second stop to deliver the remaining volume. This is the blow-out stroke, but don't do it so forcefully that you splash liquid.

5 While keeping pressure on the control button, carefully remove the pipette from the tube by sliding it along the inside of the tube. Once it is out, slowly release pressure on the control button.

6 Keeping your pipette hand still, flame the tube lip as before. If using a microtube, close the cap and continue with Step 8.

7 Keeping the pipette hand still, move the tube to replace its cap.

8 The pipette tip is contaminated with microbes, and must be disposed of correctly. Use the ejector button to remove the tip into an appropriate

biohazard container (Fig. D.6). Each lab has its own specific procedures and your instructor will advise you what to do.

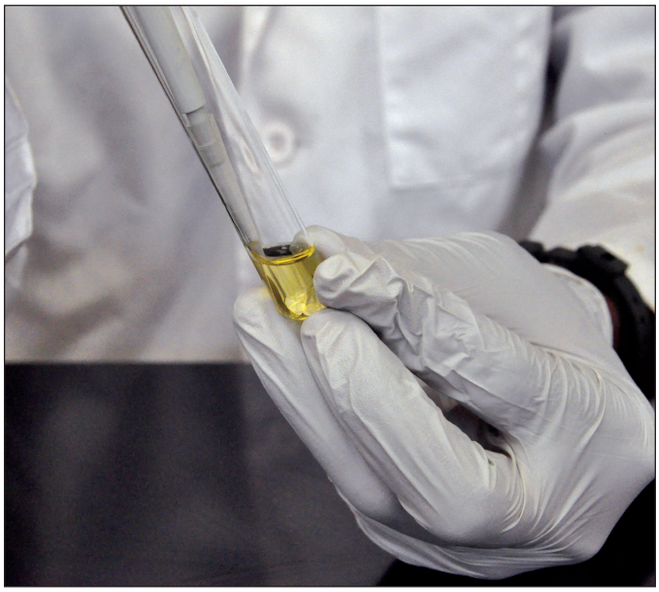


FIGURE **D.5** **Dispensing to a Liquid** • Hold the pipette on a slight angle with the tip immersed in the liquid and held against the glass of the test tube (or plastic of a microtube). Press the control button gently to the first stop (don't shoot out the liquid!). When no more fluid comes out, continue pressing to the second stop to completely empty the pipette. Remove the pipette along the glass (plastic), then slowly release pressure on the control button and replace the cap on the test tube. Eject the tip into a biohazard container if transferring a culture (Fig. D.6).

Using a Micropipette to Inoculate Agar Plates

1. Lift the lid of the plate and use it as a shield to protect from airborne contamination as well as limiting the spread of any aerosols as you deliver the inoculum.
2. Place the pipette tip over, but not touching, the agar surface. Hold the pipette in a vertical position between the middle and one edge of the plate (Fig. D.7).
3. Depress the control button slowly with your thumb to the first stop, and pause when no more liquid is dispensed. Then continue gently pressing to the second stop to deliver the appropriate volume. This is the blow-out stroke. From this point, the remaining steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
4. Because the pipette tip is contaminated with microbes, you must dispose of it correctly. Use the ejector button to remove the tip into an appropriate biohazard container (Fig. D.6). Each lab has its own specific procedures, and your instructor will advise you what to do.
5. Continue with the spread plate technique (Ex. 5, pp. 81–84).



FIGURE **D.6** **Ejecting the Tip** • Use the tip ejector button to eject the tip into an appropriate biohazard container. Be sure the tip goes into the container and doesn't hit an edge and bounce back. (If this happens, treat it as a spill and wipe down all areas the tip contacted with disinfectant. Manually put the tip in the biohazard container while wearing gloves, then properly remove and dispose of them.) Your instructor will advise you as to the proper procedure in your laboratory. If transferring nonbiohazardous liquids (e.g., sterile water or saline), tips can be ejected into a cup or other suitable container for disposal.



FIGURE **D.7** **Dispensing to a Plate** • Notice how the lid is held as a shield to prevent aerial contamination of the plate and to contain aerosols. Hold the pipette in a vertical position and press the control button to the first stop. When no more fluid is released, continue pressing to the second stop. If transferring a BSL-2 organism, try to deliver the inoculum as far under the lid as is practical. Then, slowly release pressure on the control button and eject the tip into a proper biohazard container (Fig. D.6). Notice the work area is covered with a disinfectant-soaked towel. This is always a good idea in case of drips, but it is a necessity when transferring a BSL-2 organism.

Modifications for Transferring BSL-2 Microorganisms

Several modifications to the instructions above should be made to minimize aerosol production when using a micropipette. They may also be employed for other transfers, but are especially important when transferring BSL-2 organisms.

In all cases, transfers should be made over a disinfectant-soaked towel in case inoculum leaks from the pipette tip. Put more disinfectant on any spills. Also, keep the culture and destination media as close together as is safe in order to minimize hand movements.

Following are suggested modifications for transferring BSL-2 organisms with a micropipette.

- The pipette tip containing inoculum may be inserted into the broth. Then, gently deliver the inoculum into the broth. Thus, the "blow-out" stroke (pressing to the second stop) occurs within the broth and does not produce aerosols. However, if you use too much force you can cause bubbling of the broth with concurrent aerosol production, so be sure to *gently* dispense the inoculum. Also be sure to keep pressure on the control button as you withdraw the pipette from the broth or you risk changing an intended concentration as you pull back some broth. Use the ejector button to dispose of the tip according to your lab's conventions with a minimum amount of pipette movement.
- If you are delivering the inoculum to a plate, keep the lid down low and *gently* dispense as far under the lid as possible while still holding the pipette more or less vertically. This will help to contain aerosols. Use the ejector button to dispose of the tip according to your lab's conventions with a minimum amount of pipette movement.
- Another method involves using the pipette "in reverse." Instead of pressing to the first stop to fill, filling can be done by pressing to the *second* stop. Then, instead of dispensing by pressing to the second stop, delivery of the inoculum is done by pressing to the *first* stop to avoid the inevitable spraying that occurs when completely emptying the pipette tip. Use the ejector button to dispose of the tip and the remaining inoculum within it according to your lab's conventions with a minimum amount of pipette movement.

Reference

Brinkmann Instruments, *Eppendorf Series 2100 Pipette Instruction Manual.* Westbury, New York.

APPENDIX E

Alternative Procedure for Exercise 10: Standard Plate Count

What follows is an alternative procedure for Exercise 10, using traditional glass pipettes and mL volumes versus micropipettes and microliter (µL) volumes.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Sterile 0.1 mL, 1.0 mL, and 10.0 mL pipettes
- ❑ Mechanical pipette
- ❑ Sterile dilution tubes with caps (5)
- ❑ Flask of 100 mL sterile normal saline or sterile water
- ❑ Nutrient agar (NA) plates (8)
- ❑ Jar (and its lid) containing ethanol, cotton in the bottom, and a bent glass rod
- ❑ Hand-tally counter
- ❑ Colony counter (optional)
- ❑ 24-hour broth culture of *Escherichia coli* (this culture will have between 2×10^7 and 2×10^{10} CFU/mL)

PROCEDURE

Wear a lab coat, gloves, and eye protection when performing this procedure. Refer to the procedural diagram in Figure E.1 as needed.

1. **Label Your Plates and Tubes:** Obtain eight plates, organize them into four pairs, and label them A_1, A_2, B_1, B_2, etc. Obtain five dilution tubes, and label them 1–5, respectively. Make sure they remain covered until needed.
2. **Add Diluent (Water or Saline) to Each Tube:** Aseptically add 9.9 mL sterile water/saline to Dilution Tubes 1 and 2. Cover when finished. Aseptically add 9.0 mL sterile water/saline to Dilution Tubes 3, 4, and 5. Cover when finished.
3. **Make a 10^{-2} Dilution:** Carefully, but thoroughly, mix the broth culture, and aseptically transfer 0.1 mL to Dilution Tube 1. Mix well.
4. **Make a 10^{-4} Dilution:** Aseptically transfer 0.1 mL from Dilution Tube 1 to Dilution Tube 2; mix well.
5. **Make a 10^{-5} Dilution:** Aseptically transfer 1.0 mL from Dilution Tube 2 to Dilution Tube 3; mix well.
6. **Make a 10^{-6} Dilution:** Aseptically transfer 1.0 mL from Dilution Tube 3 to Dilution Tube 4; mix well.
7. **Make a 10^{-7} Dilution:** Aseptically transfer 1.0 mL from Dilution Tube 4 to Dilution Tube 5; mix well.
8. **Inoculate Your Plates:** Aseptically transfer 0.1 mL from Dilution Tube 2 to Plate A_1. Using the spread plate technique (Ex. 5, pp. 81–84), disperse the sample evenly over the entire surface of the agar. Repeat the procedure with Plate A_2, and label both plates "10^{-5} mL original sample," or simply "10^{-5}." Following the same procedure, transfer 0.1 mL volumes from Dilution Tubes 3, 4, and 5 to Plates B, C, and D, respectively. Label the plates accordingly. Allow the inocula to soak into the agar for a few minutes before continuing.
9. **Incubate the Plates:** Invert the plates and incubate at 35°C for 24 to 48 hours.
10. **Dispose of Waste:** Save or dispose of the original *E. coli* culture as directed by your instructor. Tightly cap the dilution tubes, and dispose of them in the appropriate autoclave container.

➜ Next Lab Session

1. **Examine Your Samples:** After incubation, examine the plates and determine the countable pair—plates with 30 to 300 colonies. Only one pair of plates should be countable. The remainder should be "too few to count" (TFTC) or "too numerous to count" (TNTC).
2. **Record Your Result:** Count the colonies on both of the "countable plates" and then calculate the average number of colonies. Various methods are available for counting. A simple way is to use a hand-tally counter and a felt-tip marker. Mark each colony on the plastic base and simultaneously click the counter.[1] Record the tallies in the appropriate table of the data sheet, page 125. ***Note:*** You may have more than one pair of countable plates. For the practice, count all plates that have between

[1] Other, more sophisticated counting methods are available. One uses an electronic pen that, when touched to the plastic petri plate below a colony, records a tally. There are also software systems that capture an image of the plate and then colonies are counted by the computer.

30–300 colonies, and then identify the pair in which you have the most confidence. If no plates are in the 30–300 colony range, count the pair that is closest.

3 **Calculate Original Cell Density (OCD):** Using the formula provided on the data sheet, calculate the sample's OCD and record it in the space provided.

4 **Dispose of Your Plates:** Dispose of the plates in an appropriate autoclave container when finished.

E

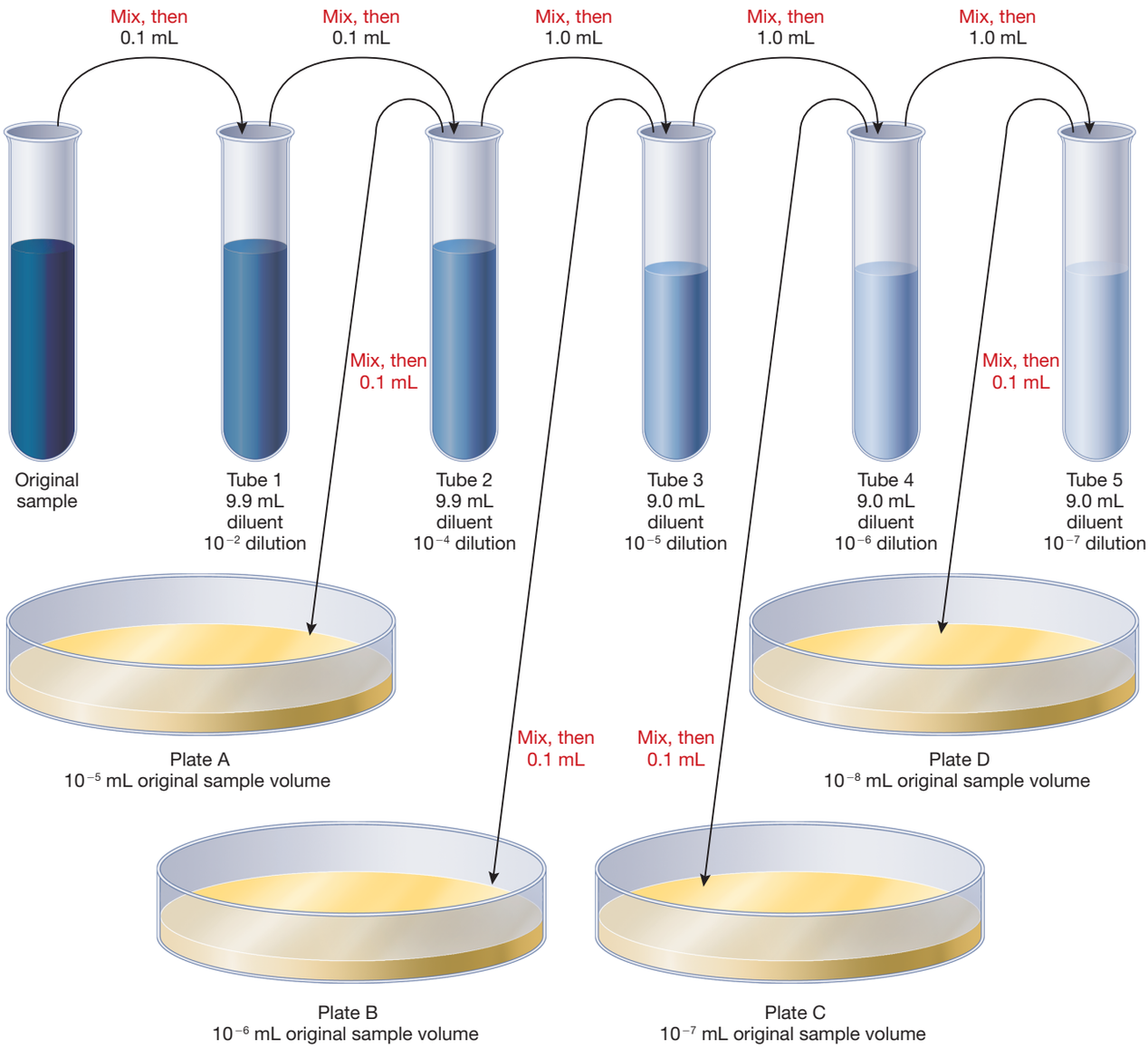


FIGURE **E.1** **Serial Dilution Procedural Diagram** • This is an illustration of the dilution scheme outlined in the procedure. The dilution assigned to each tube (written below the tube) represents the proportion of original sample inside that tube. For example, if the dilution is 10^{-4}, the proportion of original sample inside the tube would be 1/10,000th of the total volume inside. When 0.1 mL of that solution is transferred to a plate, the volume of sample in the plate is 0.1 mL $\times$ 10^{-4} = 10^{-5} mL.

APPENDIX F

Morphological Features and Staining Results for Select Bacteria

These results are typical for the species listed. Specific strains may vary because of their genetics, their age, or the environment in which they are grown. Always record your observations in case strain variability leads to misidentification.

Organism	Gram Stain	Cell Morphology	Cell Arrangement[1]	Acid-Fast Stain	Motility (wet mount)[2]	Capsule[3]	Spore Stain
Bacillus subtilis	+	rod	usually single cells	–	+	Varies	+
Corynebacterium xerosis	+	irregular rods	single cells or multiples in angular or palisade arrangement	–	–	–	–
Lactococcus lactis	+	ovoid cocci (appearing stretched in the chain or pair)	chains and some pairs	–	–	+	–
Micrococcus luteus	+	cocci	tetrads (some singles and pairs)	–	–	–	–
Mycobacterium phlei (BSL-2) or *Mycobacterium smegmatis* (BSL-2)	weak +	thin rods	single cells or branched; sometimes in dense clusters	+	–	–	–
Neisseria sicca	–	cocci	pairs and/or tetrads with adjacent sides flattened	–	–	+	–
Pseudomonas putida	–	straight to slightly curved rods	single cells	–	+	–	–
Rhizobium leguminosarum	–	short rods	usually single rods	–	–	+	–
Rhodospirillum rubrum	–	thick spirilla or bent rods	single	–	+	–	–
Shigella flexneri (BSL-2)	–	rods	single	–	–	–	–
Staphylococcus epidermidis	+	cocci	irregular clusters (some singles, pairs, and tetrads)	–	–	–	–

[1] It may be necessary to grow your unknown in broth culture to see the arrangement clearly.

[2] It may be useful to transfer some culture to a small amount of sterile broth (in a microtube, for instance) and incubate the sample for about an hour at 35°C. Then make a wet mount from that culture. See Figure 2.15, page 40, for instructions on making a wet mount. Do not stain your slide.

[3] To be considered a capsule, the white halo needs to be at least the thickness of the cell. If you suspect cells have shrunk, measure them and compare their size and the size of the "capsule" to the cell dimensions as measured in your Gram stain.

— Lab Notes —

APPENDIX G

Gram Stain and Cellular Morphology Flowchart

This sample flowchart may be helpful as you conduct the bacterial unknown lab in Exercise 48.

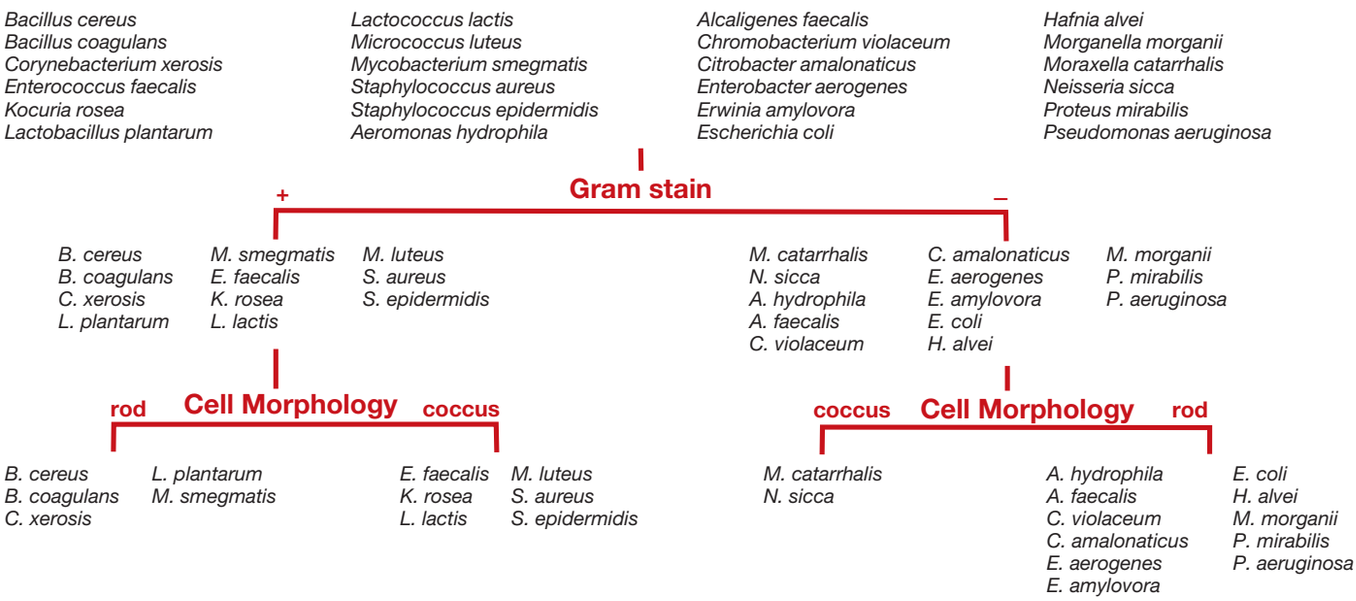


FIGURE **G.1** **Gram Stain and Cellular Morphology Flowchart** • This chart may be useful to you as you work on discovering the identity of your unknown specimen.

G

— Lab Notes —

APPENDIX H

Identification Flowcharts for Gram-Positive Cocci and Rods

The sample icon keys and flowcharts provided here may be helpful as you conduct the labs in Exercises 47 and 48.

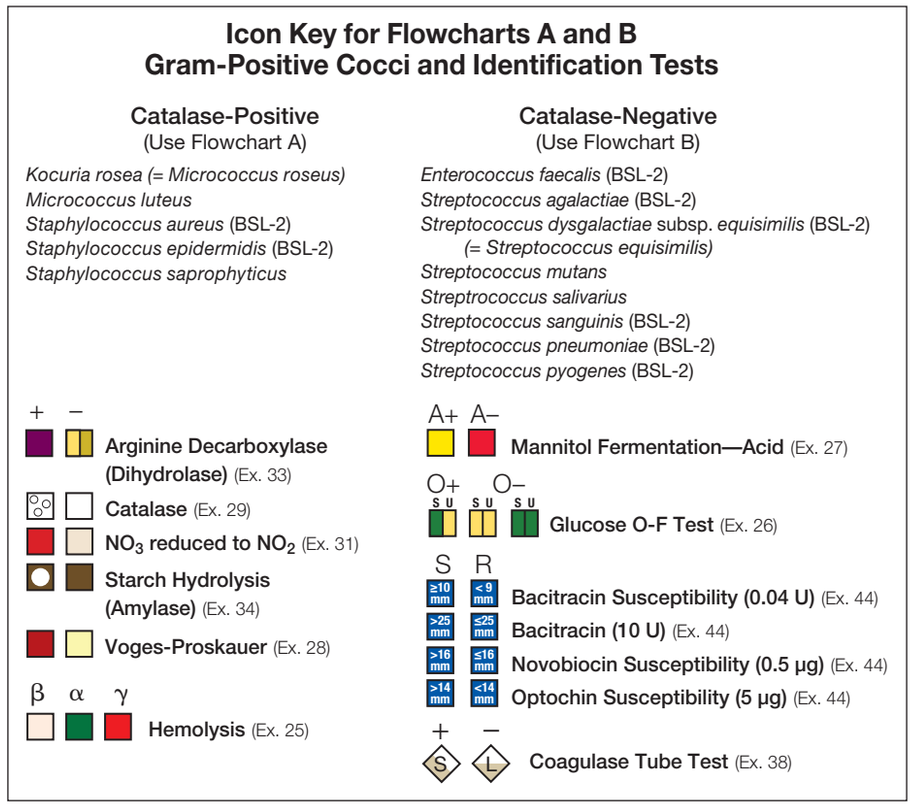


FIGURE H.1 Icon Key for Identification Flowcharts A and B for Gram-Positive Cocci • The bacteria listed can be identified using flowcharts A and B. Your instructor may or may not give you an unknown bacterium from this list. If you do have a bacterium from this list, the tests noted may be useful for identifying your unknown. Be aware, the list is not exhaustive, but rather is to provide you with some direction as you try to identify your unknown bacterium. Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A−" means acid is not made.

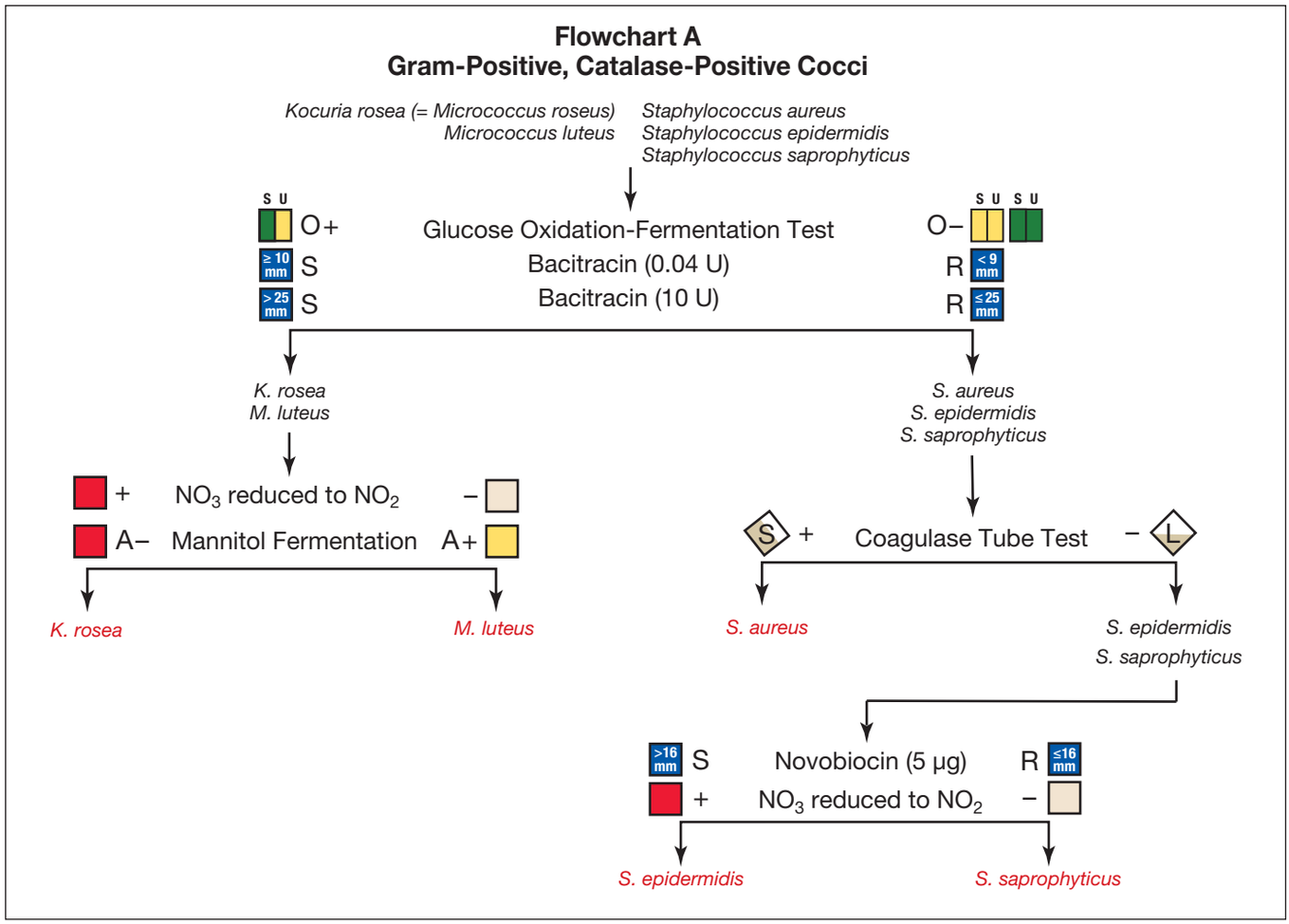


FIGURE **H.2** **Identification Flowchart A (Gram-Positive, Catalase-Positive Cocci)** • This chart can help with identifying Gram-positive, catalase-positive cocci arranged mainly in tetrads or clusters. See the exercise(s) as listed in Figure H.1 for instructions on how to run and interpret each test. In some cases, final identification to species is difficult. Try to get that far, but you may reach a point where your lab strains cannot be differentiated any further.

H

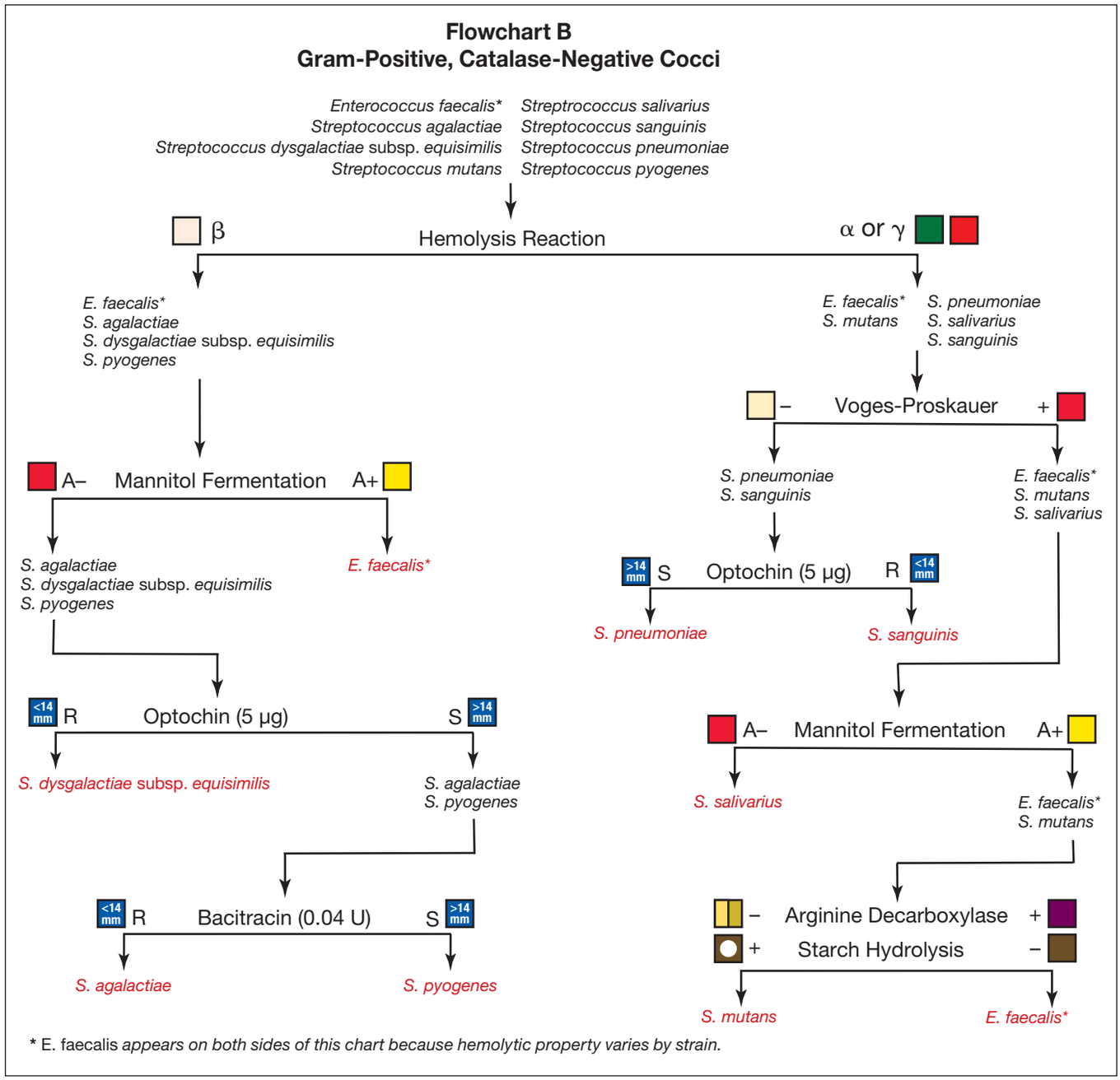


FIGURE **H.3** **Identification Flowchart B (Gram-Positive, Catalase-Negative Cocci)** • This chart can help with identifying Gram-positive, catalase-negative cocci that are mainly arranged as pairs or in chains. Refer to the exercise(s) as listed in Figure H.1 for details on how to perform and interpret each test.

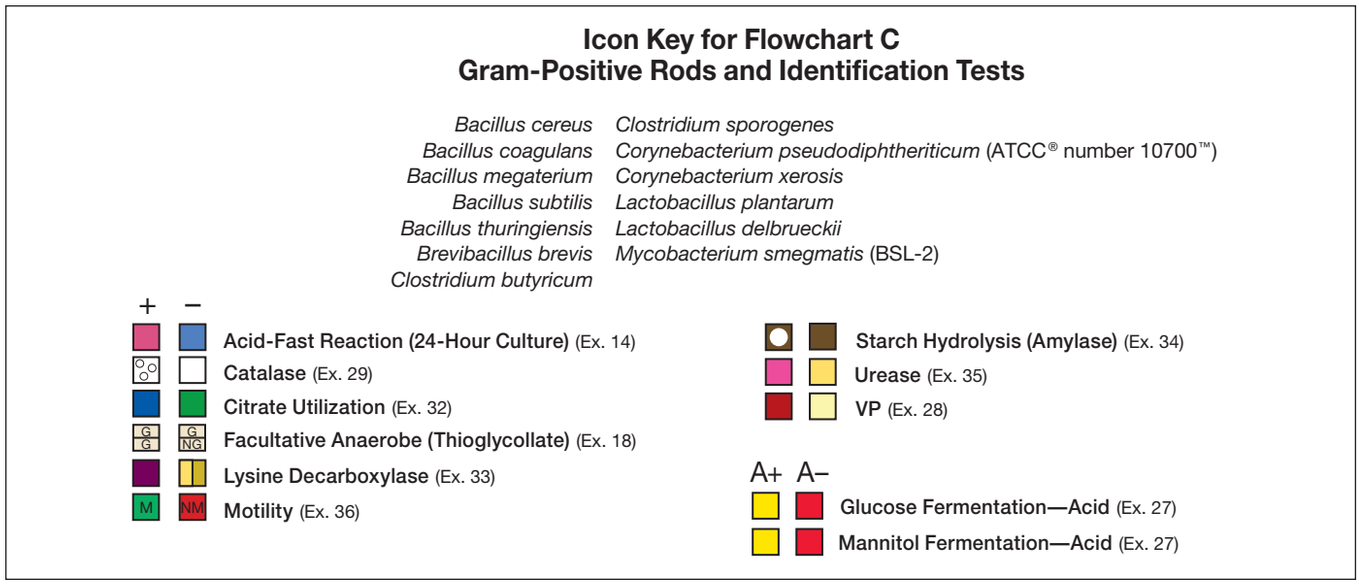


FIGURE **H.4** **Icon Key for Identification Flowchart C for Gram-Positive Rods** • The bacteria listed can be identified using flowchart C. Your instructor may or may not give you an unknown bacterium from this list. If you do have a bacterium from this list, the tests noted may be useful for identifying your unknown. Be aware, the list is not exhaustive, but rather is to provide you with some direction as you try to identify your unknown bacterium. Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A–" means acid is not made.

H

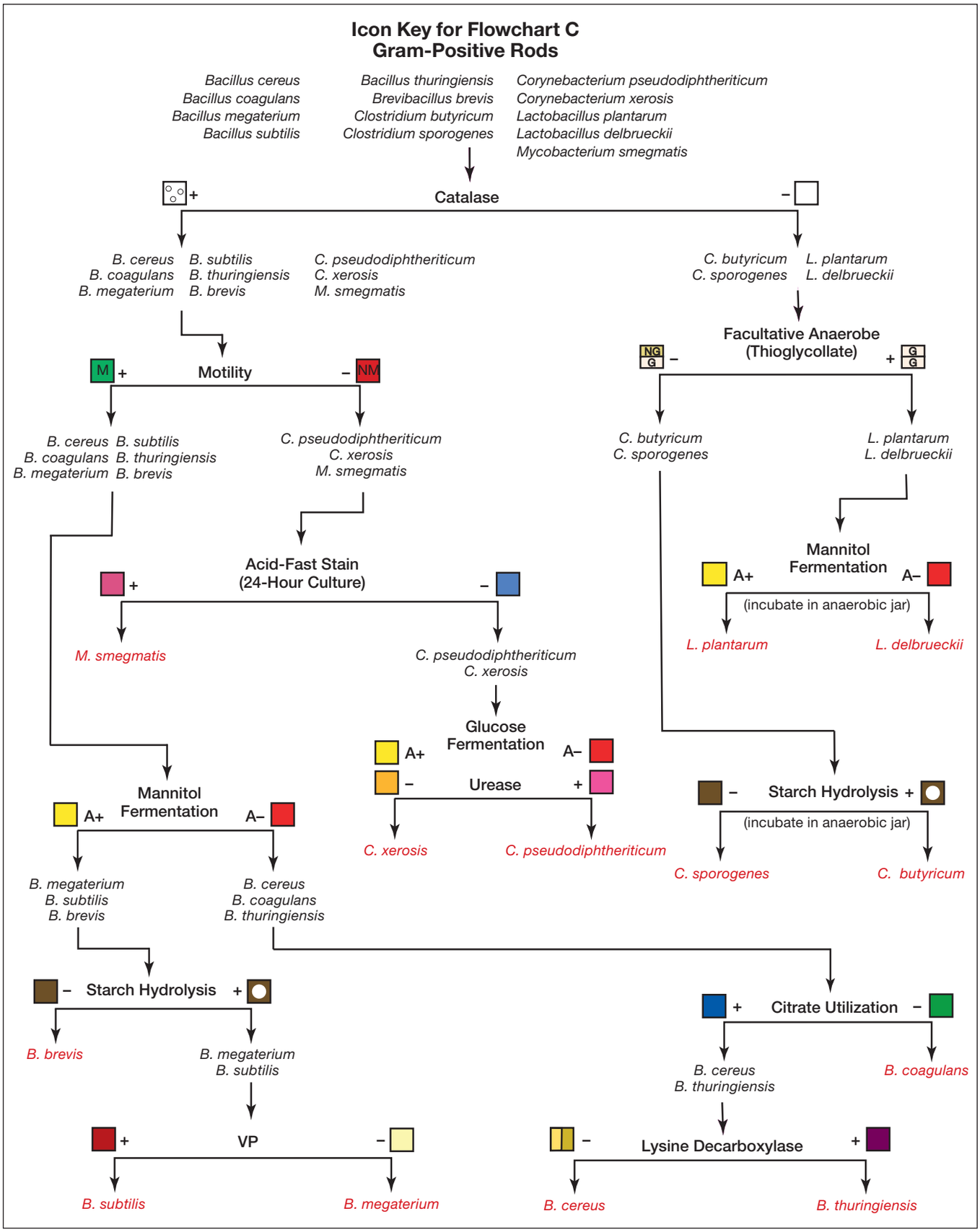


FIGURE **H.5** **Identification Flowchart C (Gram-Positive Rods)** • This flowchart can help with identifying Gram-positive rods. See the exercise(s) as listed in Figure H.4 for instructions on how to run and interpret each test.

— Lab Notes —

APPENDIX I

Identification Flowcharts for *Enterobacteriaceae*

These sample icon keys and flowcharts may be helpful as you conduct the bacterial unknown lab in Exercise 48.

Key for Selecting *Enterobacteriaceae* Identification Flowchart Based on IMViC Results

Citrobacter freundii *
Citrobacter koseri
Enterobacter aerogenes
Enterobacter cloacae
Escherichia coli
*Hafnia alvei**
*Klebsiella pneumoniae**+ (BSL-2)
Morganella morganii (BSL-2)
*Proteus mirabilis** (BSL-2)
Proteus vulgaris (BSL-2)
Providencia alcalifaciens
Providencia stuartii
Salmonella enterica++ (BSL-2)
Serratia liquefaciens
*Serratia marcescens**
*Shigella flexneri (Group B)** (BSL-2)
Shigella sonnei (Group D) (BSL-2)

*Organism shows up in more than one figure due to variable results of one or more IMViC tests

+*K. pneumoniae* is *K. pneumonie* subsp. *K. pneumoniae*

++*Salmonella enterica* is *Salmonella enterica* subsp. *enterica* and includes *S. enteritidis* and *S. typhimurium*

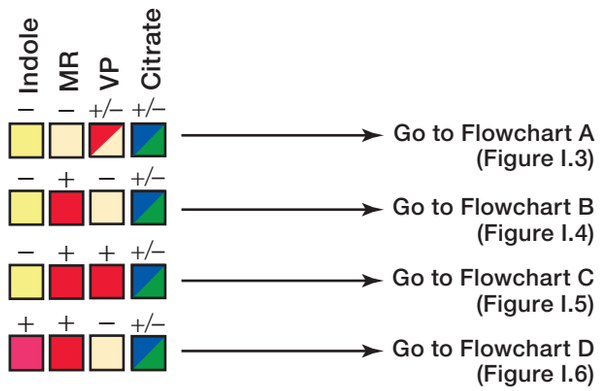


FIGURE I.1 Key for Selecting *Enterobacteriaceae* Identification Flowchart Based on IMViC Results ● The bacteria listed can be identified using the noted flowcharts. Your instructor may or may not give you an unknown bacterium from this list. To decide which flowchart would be the most useful, match the IMViC results of your isolate with one of the four shown, and then proceed with the flowchart in the figure indicated. Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. Some organisms are listed in more than one flowchart because of variable results for a particular test. A box divided diagonally with the symbol "+/–" means either result for that test is a match.

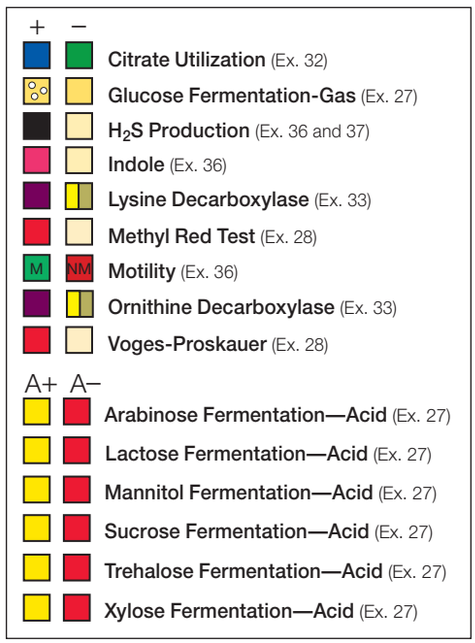


FIGURE I.2 Key to Tests and Result Icons Used in Flowcharts A–D to Identify *Enterobacteriaceae* ● Icon colors are to remind you what the results look like. The icons should not be used to compare with your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A–" means acid is not made.

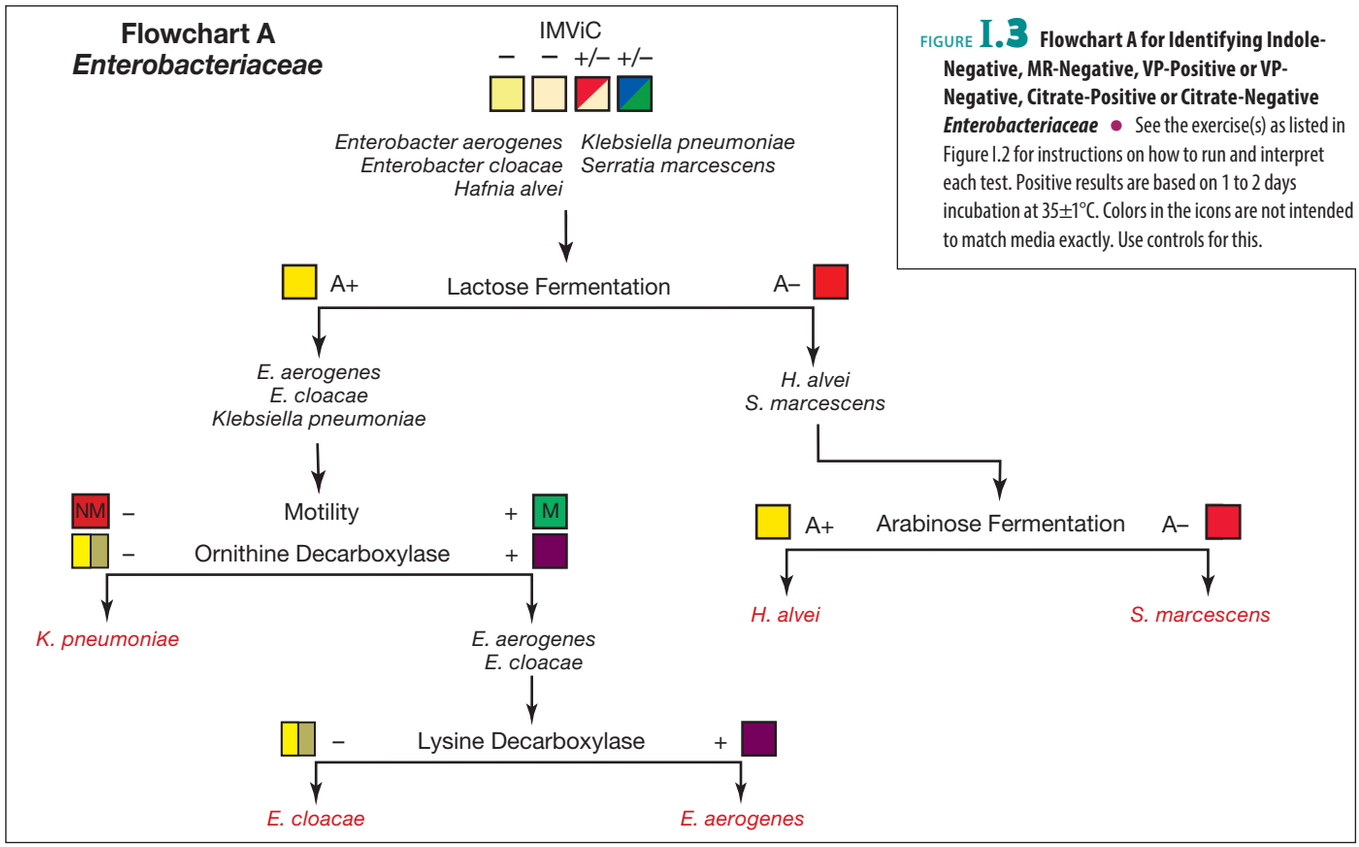


FIGURE I.3 Flowchart A for Identifying Indole-Negative, MR-Negative, VP-Positive or VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae* ● See the exercise(s) as listed in Figure I.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

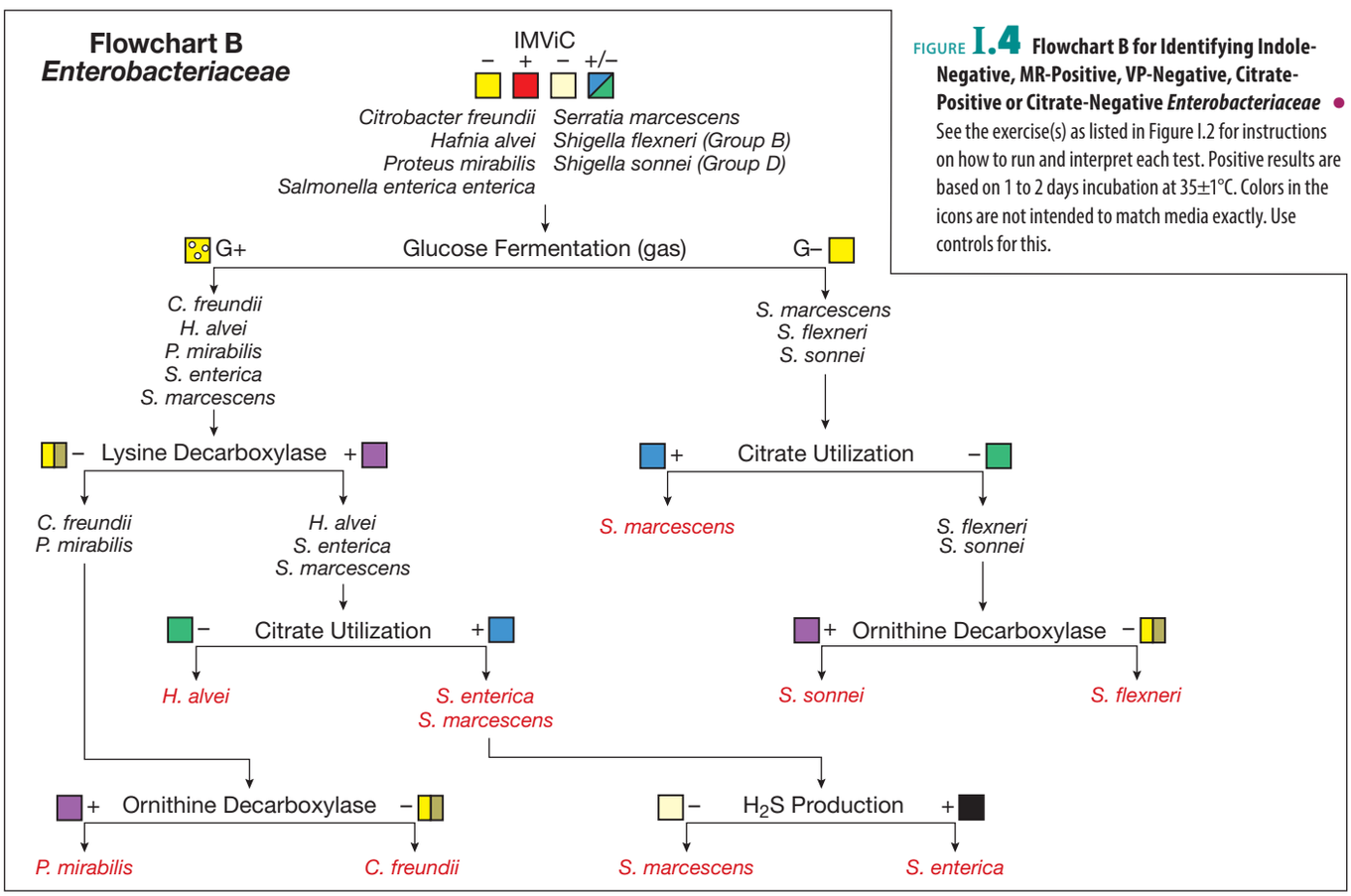


FIGURE I.4 Flowchart B for Identifying Indole-Negative, MR-Positive, VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae* ● See the exercise(s) as listed in Figure I.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

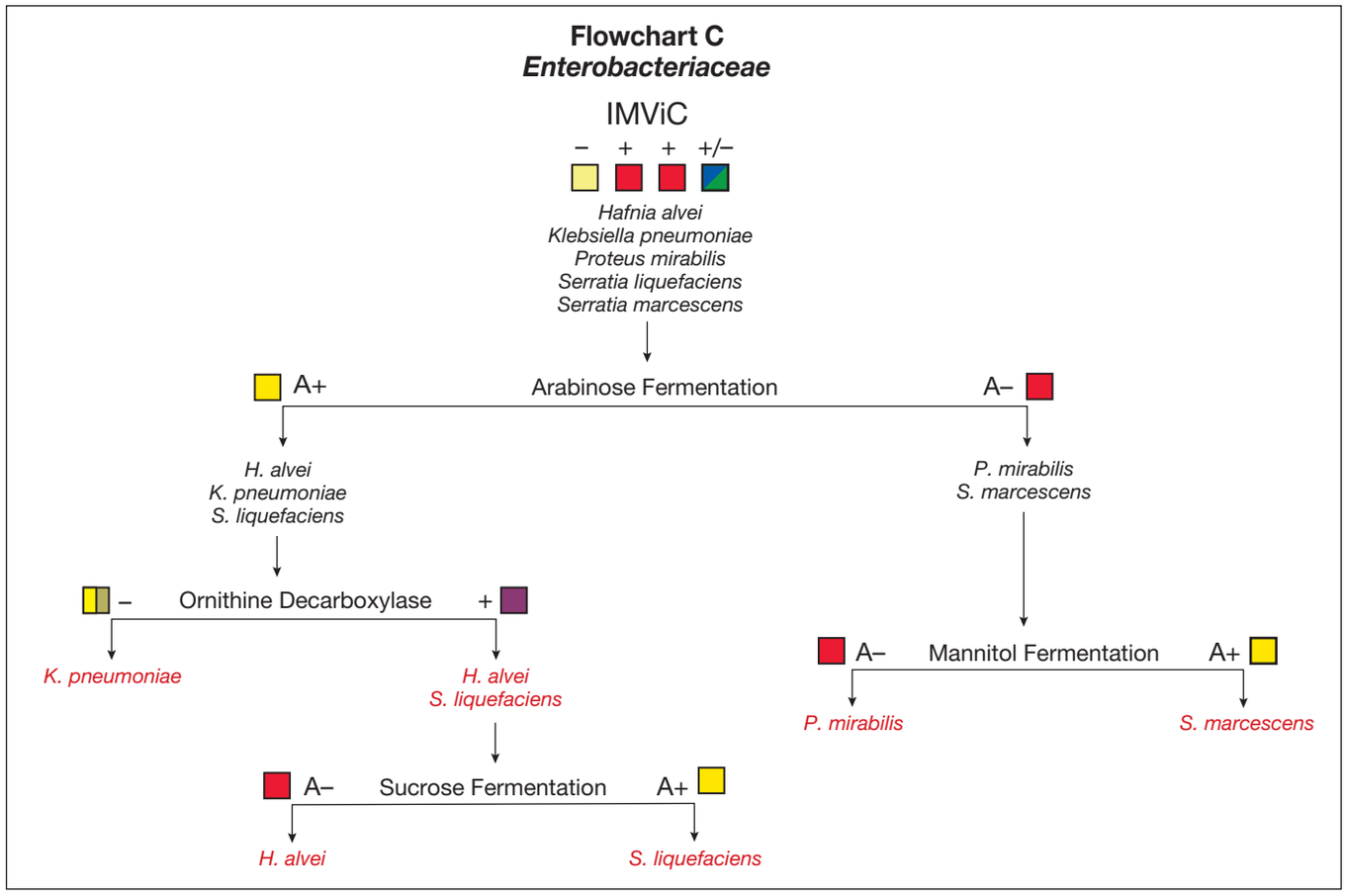


FIGURE **I.5** **Flowchart C for Identifying Indole-Negative, MR-Positive, VP-Positive, Citrate-Positive or Citrate-Negative *Enterobacteriaceae*** ● See the exercise(s) as listed in Figure 1.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

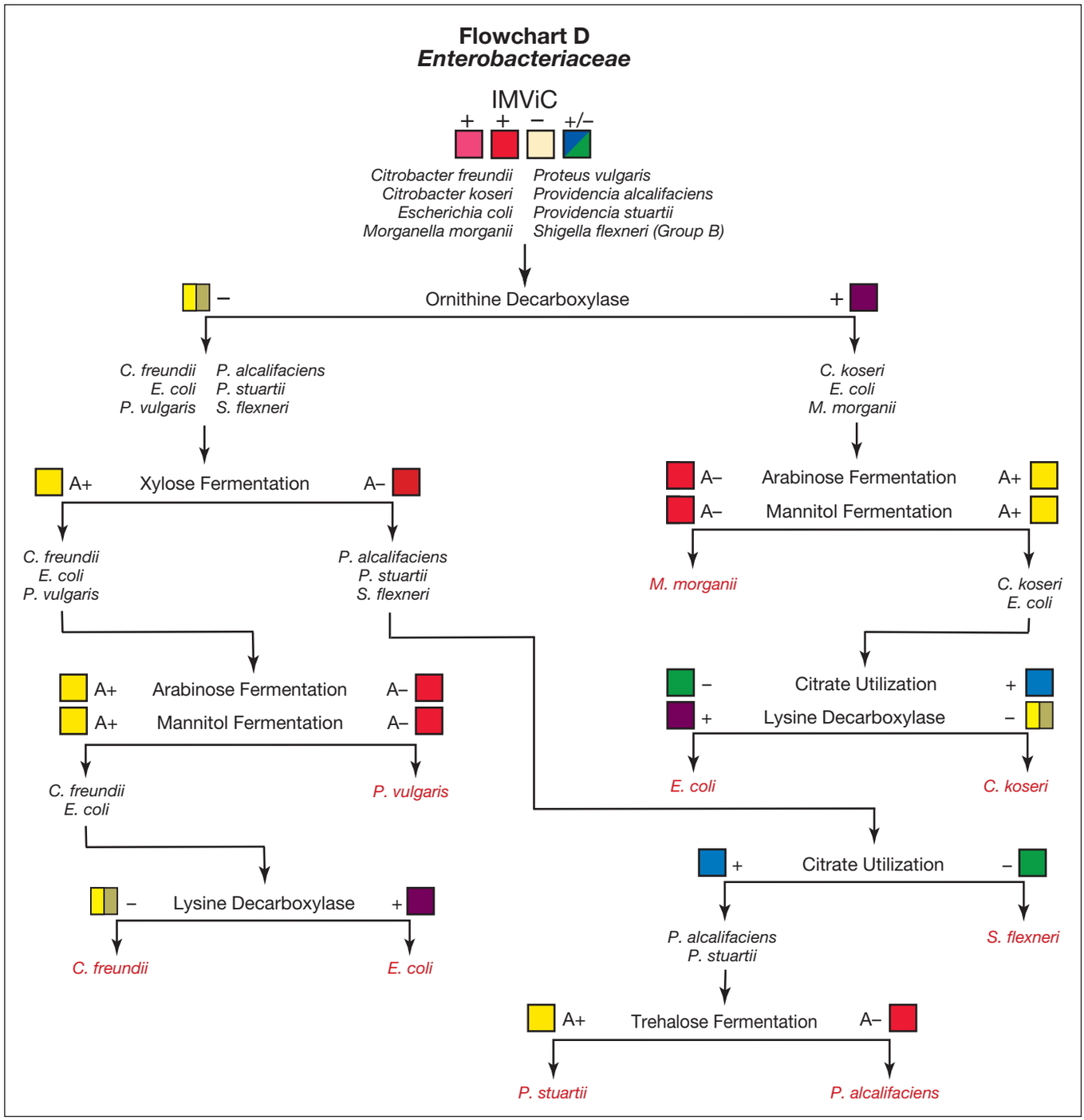


FIGURE **I.6** **Flowchart D for Identifying Indole-Positive, MR-Positive, VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae*** ● See the exercise(s) as listed in Figure 1.2 for instructions on how to run and interpret each test. Positive results are based on 1 to 2 days incubation at 35±1°C. Colors in the icons are not intended to match media exactly. Use controls for this.

APPENDIX J

Medium, Reagent, and Stain Recipes

Media

General Preparation Methods

Unless otherwise specified, prepare all media as follows.

Broths

1. Suspend the dry ingredients in water. Agitate and heat slightly (if necessary) to dissolve completely.
2. Dispense 7.0 mL p
tubes and cap loos
3. Autoclave for 15 m
medium.

Slants and Stabs

1. Suspend the dry ing
and boil until comp
2. Dispense 7.0 mL (o
portions into 16 × 1
loosely.
3. Autoclave for 15 m
medium.
4. Slant the tubes or l
and cool to room t

Plates

1. Suspend the dry ing
and boil until comp
2. Cover loosely and s
pressure (121°C) fo
3. Remove from the autoclave, allow the medium to cool to approximately 50°C, and aseptically pour into sterile 100 mm petri dishes (20.0 mL/plate).
4. Allow the medium to cool to room temperature. Store upside down.

Specific Recipes

Blood Agar (Ex. 25 and Ex. 44)

- Infusion from beef heart (solids) 2.0 g
- Pancreatic digest of casein 13.0 g
- Sodium chloride 5.0 g
- Yeast extract 5.0 g
- Agar 15.0 g
- Defibrinated sheep blood 50.0 mL
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

n water, mix well, and
d. This is blood agar

the autoclave at 15 lb.
utes.

and cool to 45°C.

om-temperature sheep
and mix well.

and allow the medium

HIA) (Plate or Slant)

0 g 7.7 g
g 9.8 g
10.0 g
2.0 g
5.0 g
2.5 g
15.0 g
1.0 L

pH 7.2–7.6 at 25°C

Brain-Heart Infusion Broth (BHIB)

- Calf brains, infusion from 200 g 7.7 g
- Beef heart, infusion from 250 g 9.8 g
- Proteose peptone 10.0 g
- Dextrose 2.0 g
- Sodium chloride 5.0 g
- Disodium phosphate 2.5 g
- Distilled or deionized water 1.0 L

pH 7.2–7.6 at 25°C

APPENDIX J NOT INCLUDED IN PRINT BOOK

Citrate Agar (Simmons) (Ex. 32)

- Ammonium dihydrogen phosphate 1.0 g
- Dipotassium phosphate 1.0 g
- Sodium chloride 5.0 g
- Sodium citrate 2.0 g
- Magnesium sulfate 0.2 g
- Agar 15.0 g
- Bromothymol blue 0.08 g
- Distilled or deionized water 1.0 L

pH 6.7–7.1 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 7.0 mL portions into test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lb. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and position tubes in such a way as to produce a slant nearly to the tube's base.
5. Cool to room temperature.

Coagulase Medium (Rabbit) (Ex. 38)

- Commercially available as rabbit plasma plus an anticoagulant, such as citrate or EDTA. Reconstitute per the vendor's instructions.

Decarboxylase Medium (Møller) (Ex. 33)

- Peptone 5.0 g
- Beef extract 5.0 g
- Glucose (dextrose) 0.5 g
- Bromocresol purple 0.01 g
- Cresol red 0.005 g
- Pyridoxal 0.005 g
- L-lysine, L-ornithine, or L-arginine 10.0 g
- Distilled or deionized water 1.0 L

pH 5.8–6.2 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved. (Use only one of the listed L-amino acids.)
2. Adjust pH by adding NaOH if necessary.
3. Dispense 7.0 mL volumes into test tubes and cap.
4. Sterilize in the autoclave at 15 lb. pressure (121°C) for 10 minutes.
5. Remove from the autoclave and cool to room temperature.

Enriched Tryptic Soy Agar (See Tryptic Soy Agar)

Eosin Methylene Blue Agar (EMB) (Levine) (Ex. 24)

- Pancreatic digest of gelatin 10.0 g
- Lactose 10.0 g[1]
- Dipotassium phosphate 2.0 g
- Agar 15.0 g
- Eosin Y 0.4 g
- Methylene blue 0.065 g
- Distilled or deionized water 1.0 L

pH 6.9–7.3 at 25°C

[1] An alternative recipe replaces the 10.0 g of lactose with 5.0 g of lactose and 5.0 g of sucrose.

Glucose Salts Broth (GSB) (Ex. 17)

- Glucose 5.0 g
- Sodium chloride 5.0 g
- Magnesium sulfate 0.2 g
- Ammonium dihydrogen phosphate 1.0 g
- Dipotassium phosphate 1.0 g
- Distilled or deionized water 1.0 L

Kligler Iron Agar (KIA) (Ex. 37)

- Pancreatic digest of casein 10.0 g
- Peptic digest of animal tissue 10.0 g
- Lactose 10.0 g
- Dextrose (glucose) 1.0 g
- Ferric ammonium citrate 0.5 g
- Sodium chloride 5.0 g
- Sodium thiosulfate 0.5 g
- Agar 15.0 g
- Phenol red 0.025 g
- Distilled or deionized water 1.0 L

pH 7.2–7.6 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lb. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and slant in such a way as to form a deep butt.
5. Allow the medium to cool to room temperature.

MacConkey Agar (MAC) (Ex. 23)

- Pancreatic digest of gelatin 17.0 g
- Pancreatic digest of casein 1.5 g
- Peptic digest of animal tissue 1.5 g
- Lactose 10.0 g
- Bile salts 1.5 g
- Sodium chloride 5.0 g
- Neutral red 0.03 g
- Crystal violet 0.001 g
- Agar 13.5 g
- Distilled or deionized water 1.0 L

pH 6.9–7.3 at 25°C

Mannitol Salt Agar (MSA) (Ex. 22)

- Beef extract 1.0 g
- Peptone 10.0 g
- Sodium chloride 75.0 g
- D-Mannitol 10.0 g
- Phenol red 0.025 g
- Agar 15.0 g
- Distilled or deionized water 1.0 L

pH 7.2–7.6 at 25°C

Methyl Red-Voges Proskauer (MR-VP) Broth (Ex. 28)

- Buffered peptone 7.0 g
- Dipotassium phosphate 5.0 g
- Dextrose (glucose) 5.0 g
- Distilled or deionized water 1.0 L

pH 6.7–7.1 at 25°C

Motility Test Agar (Ex. 36)

- Beef extract 3.0 g
- Pancreatic digest of gelatin 10.0 g
- Sodium chloride 5.0 g
- Agar 4.0 g
- Triphenyltetrazolium chloride (TTC) 0.05 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

Mueller-Hinton II Agar (Ex. 43)

- Beef extract 2.0 g
- Acid hydrolysate of casein 17.5 g
- Starch 1.5 g
- Agar 17.0 g
- Distilled or deionized water 1.0 L

pH 7.2–7.4 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Cover loosely and sterilize in the autoclave at 121°C (15 lb. pressure) for 15 minutes.
3. Remove from the autoclave, cool to approximately 50°C.
4. Aseptically pour into sterile petri dishes to a depth of 4 mm.
5. Allow the medium to cool to room temperature.

Nitrate Broth (Ex. 31)

- Beef extract 3.0 g
- Peptone 5.0 g
- Potassium nitrate 1.0 g
- Distilled or deionized water 1.0 L

pH 6.8–7.2 at 25°C

1. Suspend the ingredients in water, mix well, and warm until completely dissolved.
2. Transfer 8.0 mL portions to test tubes, and cap loosely. (Add inverted Durham tubes before capping, if desired.)
3. Sterilize in the autoclave at 15 lb. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and allow the medium to cool to room temperature.

Nutrient Agar (NA) (Plate or Tube)

- Beef extract 3.0 g
- Peptone 5.0 g
- Agar 15.0 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

NA Tubes

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 7 mL or 10 mL portions into test tubes, and cap loosely.
3. Autoclave for 15 minutes at 121°C to sterilize the medium.
4. Cool to room temperature with the tubes in an upright position (10 mL) for agar deep tubes. Cool with the tubes on an angle (7 mL) for agar slants.

A

Nutrient Broth (NB)

- Beef extract 3.0 g
- Peptone 5.0 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

Nutrient Gelatin (Ex. 39)

- Beef extract 3.0 g
- Peptone 5.0 g
- Gelatin 120.0 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

1. *Slowly* add the dry ingredients to the water while stirring.
2. Warm to >50°C and maintain temperature until completely dissolved.
3. Dispense 7.0 mL volumes into test tubes and cap loosely.
4. Sterilize in the autoclave at 15 lb. pressure (121°C) for 15 minutes.
5. Remove from the autoclave immediately and allow the medium to cool to room temperature in the upright position.

O–F Medium (Hugh and Leifson) (Ex. 26)

Basal Medium

- Pancreatic digest of casein 2.0 g
- Sodium chloride 5.0 g
- Dipotassium phosphate 0.3 g
- Agar 2.5 g
- Bromothymol blue 0.03 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

O–F Carbohydrate Solution

- Carbohydrate (glucose, lactose, sucrose) 1.1 g
- Distilled or deionized water to total 10.0 mL

1. Suspend the dry basal medium ingredients, *without the carbohydrate*, in water, mix well, and boil to dissolve completely. This is basal medium.
2. Divide the medium into 10 aliquots of 100.0 mL each.
3. Cover loosely and sterilize in the autoclave at 121°C for 15 minutes.
4. Prepare carbohydrate solution, cover loosely, and autoclave at 118°C for 10 minutes.
5. Allow both solutions to cool to 50°C.
6. Aseptically add 10.0 mL sterile carbohydrate solution to a basal medium aliquot, and mix well.
7. Aseptically transfer 7.0 mL volumes to sterile test tubes, and allow the medium to cool to room temperature.

Phenol Red (Carbohydrate) (PR) Broth (Ex. 27)

- Pancreatic digest of casein 10.0 g
- Sodium chloride 5.0 g
- Carbohydrate (use any; e.g., glucose, lactose, sucrose, etc.) 5.0 g
- Phenol red 0.018 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in water, mix well, and warm slightly to dissolve completely.
2. Dispense 7.0 mL volumes into test tubes.
3. Insert inverted Durham tubes into the test tubes and cap loosely.
4. Sterilize in the autoclave at 116–118°C for 15 minutes.
5. Remove from the autoclave and allow the medium to cool to room temperature.

Purple Broth (Optional, Ex. 27)

- Peptone 10.0 g
- Beef extract 1.0 g
- Sodium chloride 5.0 g
- Bromocresol purple 0.02 g
- Carbohydrate (use any; e.g., glucose, lactose, sucrose, etc.) 10.0 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

1. Suspend the dry ingredients in water, mix well, and warm slightly to dissolve completely.
2. Dispense 9.0 mL volumes into test tubes.
3. Insert inverted Durham tubes into the test tubes and cap loosely.
4. Sterilize in the autoclave at 118°C for 15 minutes.
5. Remove from the autoclave and allow the medium to cool to room temperature.

Skim Milk Medium (Ex. 15)

- Skim Milk Powder 100.0 g
- Distilled or deionized water 1.0 L

1. Suspend and mix the milk powder in water and heat to approximately 50°C to dissolve completely.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 113°–115°C for 20 minutes.
4. Remove from the autoclave and allow the medium to cool to room temperature.

Sporulating Agar (Ex. 16)

- Pancreatic digest of gelatin 6.0 g
- Pancreatic digest of casein 4.0 g
- Yeast extract 3.0 g
- Beef extract 1.5 g
- Dextrose 1.0 g
- Agar 15.0 g
- Manganous sulfate 0.3 g
- Distilled or deionized water 1.0 L

Final pH 6.650.2 at 25°C

Starch Agar (SA) (Ex. 34)

- Beef extract 3.0 g
- Soluble starch 10.0 g
- Agar 12.0 g
- Distilled or deionized water 1.0 L

pH 7.3–7.7 at 25°C

Sulfur-Indole-Motility (SIM) Medium (Ex. 36)

- Pancreatic digest of casein 20.0 g
- Peptic digest of animal tissue 6.1 g
- Ferrous ammonium sulfate 0.2 g
- Sodium thiosulfate 0.2 g
- Agar 3.5 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

Thioglycollate Broth (Fluid) (FTB) (Ex. 18)

- Yeast extract 5.0 g
- Casitone 15.0 g
- Dextrose (glucose) 5.5 g
- Sodium chloride 2.5 g
- Sodium thioglycolate 0.5 g
- L-cystine 0.5 g
- Agar 0.75 g
- Resazurin 0.001 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 10.0 mL into sterile screw-cap test tubes.
3. Autoclave for 15 minutes at 15 lb. pressure (121°C) to sterilize. Allow the medium to cool to room temperature before inoculating.

Triple Sugar Iron Agar (TSIA) (Ex. 37)

- Pancreatic digest of casein 10.0 g
- Peptic digest of animal tissue 10.0 g
- Dextrose (glucose) 1.0 g
- Lactose 10.0 g
- Sucrose 10.0 g
- Ferrous ammonium sulfate 0.2 g
- Sodium chloride 5.0 g
- Sodium thiosulfate 0.2 g
- Agar 13.0 g
- Phenol red 0.025 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lb. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and slant in such a way as to form a deep butt.
5. Allow the medium to cool to room temperature.

Tryptic Soy Agar (TSA) (Plate or Tube)

- Tryptone 15.0 g
- Soytone 5.0 g
- Sodium chloride 5.0 g
- Agar 15.0 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

Tryptic Soy Broth (TSB)

- Tryptone 17.0 g
- Soytone 3.0 g
- Sodium chloride 5.0 g
- Dipotassium phosphate 2.5 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

Tryptic Soy Broth Plus 1% Glucose (Ex. 45)

- Tryptic Soy Broth (see Tryptic Soy Broth recipe)
- Glucose 10 g

Urea Broth (Rustigian and Stuart) (Ex. 35)

- Yeast extract 0.1 g
- Potassium phosphate, monobasic 9.1 g
- Potassium phosphate, dibasic 9.5 g
- Urea 20.0 g
- Phenol red 0.01 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

1. Suspend the dry ingredients in the water and mix well.
2. Filter sterilize the solution. *Do not autoclave*.
3. Aseptically transfer 1.0 mL volumes to small sterile test tubes and cap loosely.

Voges-Proskauer Broth
(See Methyl Red Voges-Proskauer Broth)

Reagents

Kovac's Reagent (Ex. 36)

- Amyl alcohol 75.0 mL
- Hydrochloric acid, concentrated 25.0 mL
- *p*-dimethylaminobenzaldehyde 5.0 g

Methyl Red Test Reagent (Ex. 28)

Methyl Red

- Methyl red dye 0.1 g
- Ethanol 300.0 mL
- Distilled water to bring volume to 500.0 mL

1. Dissolve the dye in the ethanol.
2. Add water to bring the total volume up to 500 mL.

Nitrate Test Reagents (Ex. 31)

Reagent A

- Sulfanilic acid 0.8 g
- 5N Acetic acid (30%) 100.0 mL

Reagent B

- N,N-Dimethyl-α-naphthylamine 0.6 g
- 5N Acetic acid (30%) 100.0 mL

Oxidase Test Reagent (Ex. 30)

- Tetramethyl-*p*-phenylenediamine dihydrochloride 1.0 g
- Distilled or deionized water 100.0 mL

Voges-Proskauer Reagents A and B (Ex. 28)

VP Reagent A (Barritt's)

- α-naphthol 5.0 g
- Absolute ethanol to bring volume to 100.0 mL

1. Dissolve the α-naphthol in approximately 95 mL of ethanol.
2. Add ethanol to bring the total volume up to 100 mL.

VP Reagent B (Barritt's)

- Potassium hydroxide 40.0 g
- Distilled water to bring volume to 100.0 mL

1. Dissolve the potassium hydroxide in approximately 60 mL of water. (***Caution!*** This solution is highly concentrated and will become hot as the KOH dissolves. It should be prepared in appropriate glassware on a stirring hot plate.) Allow it to cool to room temperature.
2. Add water to bring the volume up to 100 mL.

Solutions

McFarland Turbidity Standard (0.5) (Ex. 43)

- Barium chloride ($BaCl_2 \cdot 2H_2O$) 1.175 g
- Sulfuric acid, concentrated (H_2SO_4) 1.0 mL
- Distilled or deionized water ≅200.0 mL

1. Pour approximately 90 mL of water into a small Erlenmeyer flask.
2. Add the $BaCl_2$ and mix well.
3. Remeasure and add water to bring the total volume up to 100 mL.
4. Add the H_2SO_4 to approximately 90 mL of water.

5. Remeasure and add water to bring the total volume up to 100 mL.
6. Add 0.5 mL of the $BaCl_2$ solution to 99.5 mL of H_2SO_4 and mix well.
7. While keeping the solution well mixed (the barium sulfate will precipitate and settle out) distribute 7 mL to 10 mL volumes into very clean screw-cap test tubes.

10× Phosphate Buffered Saline (PBS) Stock Solution (Ex. 45)

- Na_2HPO_4, anhydrous, reagent grade — 12.36 g
- $NaH_2PO_4 = H_2O$, reagent grade — 1.80 g
- NaCl, reagent grade — 85.00 g
- Dissolve ingredients in distilled water to a final volume of 1.0 L.

1× PBS Working solution (0.01 M phosphate, pH 7.6) (Ex. 45)

- 10× PBS Stock solution — 100 mL
- dH_2O — 900 mL

Stains

Acid-Fast Stain, Modified Kinyoun Procedure (Ex. 14)

Carbolfuchsin (Primary Stain)

- Basic fuchsin — 1.5 g
- Phenol — 4.5 g
- Ethanol (95%) — 5.0 mL
- Isopropanol — 20.0 mL
- Distilled or deionized water — 75.0 mL

1. Dissolve the basic fuchsin in the ethanol and add the isopropanol.
2. Mix the phenol in the water.
3. Mix the solutions together and let stand for several days.
4. Filter before use.

Decolorizer

- H_2SO_4 — 1.0 mL
- Ethanol (95%) — 70.0 mL
- Distilled or deionized water — 29.0 mL

Brilliant Green (Counterstain) (See also Methylene Blue)

- Brilliant green dye — 1.0 g
- Sodium azide — 0.01 g
- Distilled or deionized water — 100.0 mL

Acid-Fast Stain, Ziehl-Neelson Procedure (Ex. 14)

Carbolfuchsin (Primary Stain)

- Basic fuchsin — 0.3 g
- Ethanol — 10.0 mL
- Distilled or deionized water — 95.0 mL
- Phenol — 5.0 mL

1. Dissolve the basic fuchsin in the ethanol.
2. Dissolve the phenol in the water.
3. Combine the solutions and let stand for a few days.
4. Filter before use.

Decolorizer

- Ethanol — 97.0 mL
- HCl (concentrated) — 3.0 mL

Methylene Blue (Counterstain)

- Methylene blue chloride — 0.3 g
- Distilled or deionized water — 100.0 mL

Capsule Stain Procedure (Ex. 15)

Congo Red Stain (Primary Stain)

- Congo red dye — 5.0 g
- Distilled or deionized water — 100.0 mL

Maneval's Stain (Counterstain)

- Phenol (5% aqueous solution) — 30.0 mL
- Acetic acid, glacial (20% aqueous solution) — 10.0 mL
- Ferric chloride (30% aqueous solution) — 4.0 mL
- Basic fuchsin (1% aqueous solution) — 2.0 mL

Carbolfuchsin (See Acid Fast Stain Procedure)

Congo Red Stain (See Capsule Stain Procedure)

Crystal Violet Stain (1%) (Ex. 45)

- Gram Crystal Violet (Modified Hucker's) — 50.0 mL
- Distilled or deionized water — 50.0 mL

1. Mix thoroughly.
2. Filter as needed.

Gram Stain Procedure (Ex. 13)

Gram Crystal Violet (Modified Hucker's) (Primary Stain)

A

Solution A

- Crystal violet dye (90%) 2.0 g
- Ethanol (95%) 20.0 mL

Solution B

- Ammonium oxalate 0.8 g
- Distilled or deionized water 80.0 mL

1. Combine solutions A and B. Store for 24 hours.
2. Filter before use.

Gram Decolorizer

- Ethanol (95%)
- Can use 75% ethanol and 25% acetone, but decolorization time must be reduced

Gram Iodine (Mordant)

- Potassium iodide 2.0 g
- Iodine crystals 1.0 g
- Distilled or deionized water 300.0 mL

1. Dissolve the potassium iodide in the water *first*.
2. Dissolve the iodine crystals in the solution.
3. Store in an amber bottle.

Gram Safranin (Counterstain)

- Safranin O 0.25 g
- Ethanol (95%) 10.0 mL
- Distilled or deionized water 100.0 mL

1. Dissolve the safranin O in the ethanol.
2. Add the water.

Methylene Blue Stain (See Acid Fast, Z-N Procedure)

Nigrosin Stain (Ex. 12)

- Nigrosin 10.0 g
- Distilled or deionized water 100.0 mL

Safranin Stain (See Gram Stain Procedure) (Ex. 11)

Spore Stain Procedure (Ex. 16)

Malachite Green (Primary Stain)

- Malachite green dye 5.0 g
- Distilled or deionized water 100.0 mL

Safranin (see Gram Stain Procedure)



GLOSSARY

A

absorbance A measurement, using a spectrophotometer, of how much light entering a substance is not transmitted.

acetoin A four-carbon intermediate in the conversion of pyruvic acid to 2,3-Butanediol; the compound detected in the Voges-Proskauer test.

acetyl-CoA Compound that enters the Krebs cycle by combining with oxaloacetate to form citrate; may be produced from pyruvate in carbohydrate metabolism or from oxidation of fatty acids.

acidic stain Staining solution with a negatively charged chromophore.

acidophile Microorganism adapted to a habitat below pH 5.5.

adenosine triphosphate (ATP) In hydrolysis of ATP to adenosine diphosphate (ADP), supplies the energy necessary to perform most work in the cell.

aerobe Microorganism that requires oxygen for growth.

aerobic respiration A type of energy releasing metabolism in which oxygen is the final electron acceptor in the pathway. It also has the highest potential ATP yield of all catabolic and exergonic reactions.

aerosol Droplet nuclei that remain suspended in the air for a long time.

aerotolerance Designation of an organism based on its ability to grow in the presence of oxygen.

aerotolerant anaerobe Anaerobe that grows in the presence of oxygen but does not use it metabolically.

alkaliphile Microorganism adapted to a habitat above pH 8.5.

alpha (α) hemolysis The greening around a colony on blood agar as a result of partial destruction of red blood cells; typical of certain members of *Streptococcus*.

amphitrichous Describes a flagellar arrangement with flagella at both ends of an elongated cell.

amylase Family of enzymes that hydrolyze starch.

anaerobe Microorganism that cannot tolerate oxygen.

anaerobic respiration A type of energy releasing metabolism in which an inorganic compound other than oxygen is the final electron acceptor. These include NO_3, NO_2, SO_4, and CO_2. The ATP yield is higher than fermentation, but not as high as aerobic respiration.

antibiotic Refers to an antimicrobial substance produced by a microorganism such as a bacterium or fungus.

antimicrobial/antimicrobic Describes any substance that kills microorganisms, natural or synthetic.

aseptic Describes the condition of being without contamination.

autotroph An organism that is able to make all of its organic molecules using CO_2 as the source of carbon. *See also* heterotroph.

B

bacillus Rod-shaped cell.

basic stain Staining solution with a positively charged chromophore.

β-hemolysis The clearing around a colony on blood agar resulting from complete destruction of red blood cells; typical of certain members of *Streptococcus*.

β-lactamase Enzyme found in some bacteria that breaks a bond in the β-lactam ring of β-lactam antibiotics, rendering the antibiotic ineffective; the source of resistance against these antibiotics.

biofilm A layer of bacterial cells adhering to and reproducing on a surface.

biosafety level (BSL) One of four sets of minimum standards for laboratory practices, facilities, and equipment to be used when handling organisms at each level; BSL-1 requires the least care, BSL-4 the most.

C

capnophile Microaerophile that requires elevated CO_2 levels.

capsule Insoluble, mucoid, extracellular material surrounding some bacteria.

carbohydrate One of four families of biochemicals; characterized by containing carbon, hydrogen, and oxygen in the ratio 1:2:1.

cardinal temperatures Minimum, optimum, and maximum temperatures for an organism.

casein Milk protein that gives milk its white color.

catalase Enzyme produced by some bacteria that catalyzes the breakdown of metabolic H_2O_2.

chemoheterotroph Any organism that uses chemicals for energy and organic compounds as a carbon source. Usually the same organic compounds serve both purposes.

chemolithotroph Any organism that gets its energy from chemicals and its electrons from an inorganic source. No eukaryotes are chemolithotrophic, only some prokaryotes (e.g., sulfur and ammonia-oxidizing bacteria) are chemolithotrophs.

chromogenic reducing agent A substance that produces color when it gives up electrons (becomes oxidized).

chromophore The charged region of a dye molecule that gives it its color.

coagulase *See* bound coagulase; free coagulase.

coagulase-reacting factor Plasma component that reacts with free coagulase to trigger the clotting mechanism.

coccus Spherical cell.

coenzyme Nonprotein portion of an enzyme that aids the catalytic reaction (usually by accepting or donating electrons).

coliform Member of *Enterobacteriaceae* that ferments lactose with production of gas within 48 hours at 37°C.

colony Visible mass of cells produced on culture media from a single cell or single CFU (cell forming unit); a pure culture.

colony forming unit Term used to define the cell or group of cells (e.g., staphylococci, streptococci) that produces a colony when transferred to plated media.

commensal Describes a synergistic relationship between two organisms in which one benefits from the relationship and the other is affected neither negatively nor positively.

common source epidemic An epidemic in which the disease is transmitted from a single source (such as water supply) and is not transmitted person-to-person.

complex medium A medium in which at least one nutritional ingredient is of unknown composition or amount. Media with any plant or animal extract are always complex.

confirmatory test One last test after identification of an unknown, to further support the identification.

conjugate acid A compound (one of a conjugate pair) that donates a proton in solution.

conjugate base A compound (one of a conjugate pair) that accepts a proton in solution.

conjugate pair A compound that alternates between acid and base forms by losing or gaining one proton. Example: HCl/Cl^-.

counterstain Stain applied after decolorization to provide contrast between cells that were decolorized and those that weren't.

culture A liquid or solid medium with microorganisms growing in or on it.

cyst "Resting stage" in life cycle of certain protozoans. *See also* trophozoite.

cytochrome A class of iron-containing enzymes in electron transport chains; characterized by a porphyrin ring structure.

cytochrome oxidase An electron carrier in the electron transport chain of aerobes, facultative anaerobes, and microaerophiles that makes the final transfer of electrons to oxygen.

cytoplasm The semifluid component of cells inside the cellular membrane in which many chemical reactions take place.

D

deaminase Enzyme that catalyzes the removal of the amine group (NH_2) from an amino acid.

decarboxylase Enzyme that catalyzes the removal of the carboxyl group (COOH) from an amino acid.

defined medium A growth medium in which the exact amount and chemical formula (and thus identity) of each ingredient are known.

deoxyribonuclease A family of hydrolytic enzymes that depolymerize DNA into polynucleotides. The precise location of hydrolysis within the sugar-phosphate backbone depends on which DNase is acting. These are usually secreted enzymes.

differential medium Growth medium that contains an indicator (usually color) to detect the presence or absence of a specific metabolic activity.

differential stain Staining procedure that allows distinction between cell types or parts of cells; often involves more than one stain, but not necessarily.

dilution blank A test tube containing a measured volume of sterile diluent (water, saline, or buffer) used to serially dilute a concentrated solution or broth.

dilution factor Proportion of original sample present in a new mixture after it has been diluted; calculated by dividing the volume of the original sample by the total volume of the new mixture. (Subsequent dilutions in a serial dilution must be multiplied by the dilution factor of the previous dilution.)

diploid Defines a cell that has two complete sets of genetic information; one stage in the life cycle of all eukaryotic microorganisms (which also have a haploid [one set] stage).

disaccharide A sugar made of two monosaccharide subunits; e.g., the monosaccharides glucose and galactose can combine to make the disaccharide lactose.

disk diffusion test (Kirby-Bauer Test) This is one way to check susceptibility and resistance of a microbe to an antibiotic. A paper disk containing an antibiotic is placed on a plate inoculated to produce confluent growth of a bacterium. Susceptibility is determined by the size of the zone of inhibition of growth around the disk.

Durham tube A small, inverted test tube used in some liquid media to trap gas bubbles and indicate gas production.

E

electromagnetic energy The energy that exists in the form of waves (including x-rays, ultraviolet, visible light, and radio waves).

electron donor A compound that can be oxidized to transfer electrons to another compound, which in turn becomes reduced.

electron transport chain A series of membrane-bound electron carriers that participate in oxidation-reduction reactions whereby electrons are transferred from one carrier to another until given to the final electron acceptor (oxygen in aerobes, other inorganic substances in anaerobes) in respiration.

endergonic Any metabolic reaction in which the products have more potential energy than the reactants. These are usually associated with anabolic (synthesis) reactions.

endospore Dormant, highly resistant form of bacterium; produced only by species of *Bacillus*, *Clostridium*, and a few others.

enteric bacteria Informal name given to bacteria that occupy the intestinal tract.

Enterobacteriaceae A group of Gram-negative rods that also are oxidase-negative, ferment glucose to acid, have polar flagella if motile, and usually are catalase-positive and reduce nitrate to nitrite.

enzyme A protein that catalyzes a metabolic reaction by interacting with the reactant(s) specifically; each metabolic reaction has its own enzyme.

eukaryote Type of cell with membranous organelles, including a nucleus, and having 80S ribosomes and many linear molecules of DNA.

exergonic Any metabolic reaction in which the reactants have more potential energy than the products. These are usually associated with catabolic (degradative) reactions.

exoenzyme Enzyme that operates in the external environment after being secreted from the cells.

extracellular Pertains to the region outside a cell.

extreme thermophile Organism that grows best at temperatures above 80°C.

F

facultative anaerobe Microorganism capable of both fermentation and respiration; grows in the presence or absence of oxygen.

facultative thermophile Microorganism that prefers temperatures above 40°C but will grow in lower temperatures.

false negative Test result that is negative when the sample is actually positive; usually a result of lack of sensitivity in the test system.

false positive Test result that is positive when the sample is actually negative; usually a result of lack of specificity of the test system.

fastidious (microorganism) Describes a microorganism with strict physiological requirements; difficult or impossible to grow unless specific conditions are provided.

fatty acid A long chain, organic molecule with a carboxylic acid at one end with the rest of the carbons being bonded to hydrogens.

fermentation Metabolic process in which an organic molecule acts as an electron donor and one or more of its organic products act as the final electron acceptor, marking the end of the metabolic sequence (differs from respiration in that an inorganic substance is not needed to act as final electron acceptor).

final electron acceptor (FEA) The molecule receiving electrons (it becomes reduced) at the end of a metabolic sequence of oxidation/reduction reactions.

flavin adenine dinucleotide (FAD) Coenzyme used in oxidation/reduction reactions that acts as an electron acceptor or donor, respectively.

flavoprotein A flavin-containing protein in the electron transport chain, capable of receiving and transferring electrons as well as entire hydrogen atoms; sometimes bypasses normal route of transfer and reduces oxygen directly, forming hydrogen peroxide and superoxide radicals.

free coagulase An enzyme produced and secreted by some microorganisms that initiates the clotting mechanism in plasma; seen as a solid mass in the coagulase tube test or clumps in the slide test.

free energy (of formation) The energy released by a molecule in a chemical reaction that is able to do work (e.g., produce ATP). It is equivalent to the energy required to form the molecule from its individual elements.

free living A term used to describe nonparasitic organisms.

G

gametangium A structure that produces gametes; used in describing fungi.

gamete Reproductive cell that must undergo fusion with another gamete to continue the life cycle; usually are haploid. *See also* spore.

germ theory Theory holding that diseases and infections are caused by microorganisms; first hypothesized in the 16th century by Girolamo Fracastoro of Verona.

glycolysis Metabolic process by which a glucose molecule is split into two 3-carbon pyruvic acid molecules, producing two ATP (net) and two $NADH_2$ molecules.

group A streptococci α-hemolytic members of the genus *Streptococcus*, characterized by possessing the Lancefield group A antigen; belong to the species *S. pyogenes*, an important human pathogen.

group B streptococci β-hemolytic members of the genus *Streptococcus*, characterized by possessing the Lancefield group B antigen; belong to the species *S. agalactiae*.

group D streptococci Streptococci possessing the Lancefield group D antigen; include *S. bovis* and species of the genus *Enterococcus*.

H

haploid Describes a cell that has one complete set of genetic information; all eukaryotic microorganisms have a haploid stage and a diploid (two sets) stage in their life cycle; all prokaryotes are haploid.

hemoglobin Iron-containing protein of red blood cells that is responsible for binding oxygen.

hemolysin A class of chemicals produced by some bacteria that break down hemoglobin and produce hemolysis.

heterotroph An organism that requires carbon in the form of organic molecules. *See also* autotroph.

host An organism that serves as a habitat for another organism such as a parasite or a commensal.

hydrolysis The metabolic process of splitting a molecule into two parts, adding a hydrogen ion to one part and a hydroxyl ion to the other. (One water molecule is used in the process.)

hypha (*pl.* hyphae) A filament of fungal cells.

I

IMViC Acronym representing the four tests used in identification of *Enterobacteriaceae*: Indole, Methyl Red, Voges-Proskauer, and Citrate.

incubation The process of growing a culture by supplying it with the necessary environmental conditions.

index case First occurrence of an infection or disease that results in an epidemic.

infectious disease A transmissible illness or infection.

inoculum The organisms used to start a new culture or transferred to a new place.

inorganic molecule A molecule that does *not* contain carbon and hydrogen.

intracellular Within the cell; as an intracellular enzyme catalyzing reactions inside the cell.

iron-sulfur protein Iron-containing electron carrier in electron transport chains. Differentiated from cytochromes by the lack of porphyrin ring structure.

isolate (*v.*) The process of separating individual cell types from a mixed culture; (*n.*) the group of cells resulting from isolation.

K

karyogamy The process of nuclear fusion that occurs after fertilization (plasmogamy) in sexual life cycles.

Krebs cycle A cyclic metabolic pathway found in organisms that respire aerobically or anaerobically.

L

ligate To join together.

limit of resolution The closest two points can be together for the microscope lens to make them appear separate; two points closer than the limit of resolution will blur together.

lipid A fat.

lophotrichous Describes a flagellar arrangement with a group of flagella at one end of an elongated cell.

M

medium A substance used for growing microbes; may be liquid (usually a broth) or solid (usually agar).

meiosis The process in which the nucleus of a diploid eukaryotic cell divides to make four haploid nuclei.

mesophile A microorganism that grows best at temperatures between 15°C and 45°C.

microaerophile A microorganism that requires oxygen, but can't tolerate atmospheric levels of it.

minimum inhibitory concentration (MIC) The lowest concentration of an antimicrobial substance required to inhibit growth of all microbial cells it contacts; on an agar plate, typically the outer edge of the zone of inhibition where the substance has diffused to the degree that it no longer inhibits growth.

mitosis The process in which a nucleus divides to produce two identical nuclei.

mixed acid fermentation Vigorous fermentation producing many acid products including lactic acid, acetic acid, succinic acid, and formic acid, and subsequently lowering the pH of the medium to pH 4.4 or below.

mold Informal grouping of filamentous fungi. *See also* yeast.

monotrichous Describes a flagellar arrangement consisting of a single flagellum.

morbidity Epidemiological measurement of incidence of a disease; typically accompanied by "incidence rate," referring to the incidence of a disease over time.

morphology The shape of an organism.

mortality Epidemiological measurement of death caused by a disease; typically accompanied by "incidence rate," referring to the incidence of death from a disease over time.

mutagen A substance that causes mutation in DNA; most mutagens are carcinogens.

mutation Alteration in a cell's DNA.

mutualistic Describes a synergistic relationship between two organisms in which both benefit from the interaction.

mycelium A mass of fungal filaments (hyphae).

N

neutrophile Microorganism adapted to a habitat between pH 5.5 and 8.5.

nicotinamide adenine dinucleotide (NAD) A coenzyme used in oxidation/reduction reactions that acts as an electron acceptor or donor, respectively.

nitrate A highly oxidized form of nitrogen; NO_3.

nitrate reductase An enzyme produced by all members of *Enterobacteriaceae* (and others) that catalyzes the reduction of nitrate (NO_3) to nitrite (NO_2).

nitrification The chemical process in which nitrogen compounds, such as ammonia and nitrite are oxidized to form nitrate. Typically, different bacteria perform these two steps in which ammonia is first oxidized to nitrite, which is subsequently oxidized to nitrate.

nitrite NO_2, an oxidized form of nitrogen.

noninfectious diseases Conditions not caused by microorganisms; examples are stroke, heart disease, and emphysema.

O

objective lens The microscope lens that first produces magnification of the specimen in a compound microscope.

obligate (strict) aerobe Microorganism that requires oxygen to survive and grow.

obligate (strict) anaerobe Microorganism for which oxygen is lethal; requires the complete absence of oxygen.

obligate thermophile Microorganism that grows only at temperatures above 40°C.

ocular lens The lens the microscopist looks through; produces the virtual image by magnifying the real image.

ocular micrometer A uniformly graduated linear scale placed in the microscope ocular used for measuring microscopic specimens.

opportunistic pathogen A microorganism not ordinarily thought of as pathogenic (i.e., most enterics) that will cause infection when out of its normal habitat.

organic molecule A molecule made of reduced carbon—that is, containing at least carbon and hydrogen.

osmosis Diffusion of water across a semipermeable membrane.

oxidation/reduction Chemical reaction in which electrons are transferred. (The molecule losing the electrons becomes oxidized; the molecule gaining the electrons becomes reduced.)

oxidative deamination The metabolic process in which an amino acid has its amine removed, producing ammonia and an organic acid.

oxidative phosphorylation The process by which an electron transport chain is used to add a phosphate to ADP to make ATP.

oxidizing agent A substance that removes electrons from (oxidizes) another. *See also* reducing agent.

P

parasite An organism that lives symbiotically with another, but to the detriment of the other organism (called a host).

peptidoglycan The insoluble, porous, cross-linked polymer comprising bacterial cell walls; generally thick in Gram-positive organisms and thin in Gram-negative organisms.

peptone A digest of protein used in formulating some bacteriological media.

peritrichous Describes a flagellar arrangement in which flagella arise from the entire surface of the cell.

pH The measure of a solution's alkalinity or acidity; the negative logarithm of the hydrogen ion concentration.

plasma The noncellular (fluid) portion of blood; consists of serum (including serum proteins) and clotting proteins.

plasmid Small, circular, extrachromosomal piece of DNA found in prokaryotic cells; often carries genes for antibiotic resistance.

plasmogamy The cytoplasmic fusion of gametes at the time of fertilization. *See also* karyogamy.

polar flagellum A single flagellum at one end of an elongated cell.

precipitate An insoluble material that comes out of a solution during a precipitation reaction.

presumptive identification Tentative identification of an isolate based on one or more key test results.

primary stain The first stain applied in many differential staining techniques; usually subjected to a decolorization step that forms the basis for the differential stain.

prokaryote Type of cell lacking internal compartmentalization (membranous organelles, including a nucleus) and having 70S ribosomes and a circular molecule of DNA.

propagated transmission Conveying a disease person-to-person.

proteolytic Refers to catabolism of protein; e.g., a proteolytic enzyme.

pseudohypha A chain of fungal cells produced by budding (rather than by cytokinesis that produces two equally sized cells) and which is characterized by constrictions at cell junctions.

psychrophile A microorganism that grows only at temperatures below 20°C, with an optimum around 15°C.

psychrotroph A microorganism that grows optimally at temperatures between 20 and 30°C but will grow at temperatures as low as 0°C and as high as 35°C.

pure culture Microbial culture containing only a single species.

putrefaction The process of digesting dead organic material; decay.

pyruvic acid (pyruvate) A three-carbon compound produced at the end of glycolysis that may enter a respiration or a fermentation pathway; also serves as a starting point for synthesis of certain amino acids and an entry point for their digestion.

Q

quinoidal (compound) A color-producing compound containing quinone as its central structure.

R

real image Magnified image of a specimen produced by the objective lens of a microscope; the real image is magnified again by the ocular lens to produce the virtual image.

reducing agent A substance that donates electrons to (reduces) another. *See also* oxidizing agent.

reductase An enzyme that catalyzes the transfer of electrons from donor molecule to acceptor molecule, thereby reducing the acceptor.

reduction *See* oxidation/reduction.

refraction The bending of light as it passes from a medium with one refractive index into another medium with a different refractive index.

reservoir A nonhuman host or other site in nature serving as a perpetual source of pathogenic organisms.

resolution The clarity of an image produced by a lens; the ability of a lens to distinguish between two points in a specimen; high resolution in a microscope is desirable.

resolving power *See* limit of resolution.

respiration Metabolic process by which an organic molecule acts as an electron donor and an inorganic substance—such as oxygen, sulfur, or nitrate—acts as the final electron acceptor in an electron transport chain, marking the end of the metabolic sequence (differs from fermentation, which uses one of its own organic products as the final electron acceptor).

reversion In carbohydrate fermentation tests, the phenomenon of a microorganism fermentively depleting the carbohydrate and reverting to amino acid metabolism, thereby neutralizing acid products with alkaline products; produces a false negative.

rhizoid A root-like structure used for attachment of some fungi to the substrate.

S

saprophyte A heterotroph that digests dead organic matter; a decomposer.

selective medium Growth medium that favors growth of one group of microorganisms and inhibits or prevents growth of others.

sensitivity This is a measure of a test's ability to detect small amounts of the item being tested for. The better the sensitivity, the fewer the false negatives (due to smaller amounts triggering a positive reaction), and the more useful the test is.

serial dilution Series of dilutions used to reduce the concentration of a culture and thereby produce between 30 and 300 colonies when plated, providing a means of calculating the original concentration.

serum Fluid portion of blood minus the clotting factors.

solute The dissolved substance in a solution.

solution The mixture of dissolved substance (solute) and solvent (liquid).

solvent The liquid portion of a solution in which solute is dissolved.

specificity This is a measure of a test's ability to produce a positive response only when reacting with the particular item being tested for. The better the test's specificity, the fewer the false positives (due to better discrimination), and the more useful it is.

spirillum Spiral-shaped cell.

sporangium Structure that produces spores.

spore In bacteria, a dormant form of a microbe protected by specialized coatings produced under conditions of, and resistant to, adverse conditions; also known as an endospore; in fungi and plants, spores are specialized reproductive cells; frequently a means of dissemination.

spread plate technique Method of plating bacteria in which the inoculum is transferred to an agar plate and spread with a sterile bent-glass rod or other spreading device.

stolons Surface hyphae of some molds (e.g., *Rhizopus*) that attach to the substrate with rhizoids.

streptolysin Hemolysin (blood hemolyzing exotoxin) produced and secreted by members of *Streptococcus*.

superoxide dismutase Enzyme produced by some bacteria that catalyzes the conversion of superoxide radicals to hydrogen peroxide.

T

thermophile A microorganism that grows best at temperatures above 40°C.

titer A measurement of concentration of a substance or particle in a solution; used in measurements of phage concentration.

trend line A line drawn on a graph to show the general relationship between X and Y variables; also known as a regression line if the trend line is linear (not curved).

triacylglycerol *See* triglyceride.

tricarboxylic acid cycle *See* Krebs cycle.

trichome A filament of cyanobacterial cells. It does not include the sheath, if present.

triglyceride A molecule composed of glycerol and three long chain fatty acids.

trophozoite "Feeding" stage in the life cycle of certain protozoans. *See also* cyst.

tryptophan An amino acid.

tryptophanase Enzyme that catalyzes the hydrolysis of tryptophan into indole and pyruvic acid; detected in the indole test.

turbid A solution that is cloudy.

turgor pressure The pressure inside a cell that is required to maintain its shape, tonicity, and necessary biochemical functions.

2,3-butanediol fermentation The end product of a metabolic pathway leading from pyruvate through acetoin with the associated oxidation of NADH; detected in the Voges-Proskauer test.

U

undefined medium *See* complex medium.

urease Enzyme that catalyzes the hydrolysis of urea into two ammonias and one carbon dioxide.

utilization medium A differential medium that detects the ability or inability of an organism to metabolize a specific ingredient.

V

variable A factor in a scientific experiment that is changed; the control and experimental groups in a good experiment differ in only one variable, the one being tested.

vegetative cell An actively metabolizing cell.

virtual image The image produced when the ocular lens of a microscope magnifies the real image; appears within or below the microscope.

Voges-Proskauer test A differential test used to identify organisms that are capable of performing a 2,3-butanediol fermentation.

W

wavelength Measurement of a wave from crest to crest, usually in nanometers—as in electromagnetic energy.

X

X-Y scatter plot Graph presenting the relationship between two variables.

Y

yeast An informal grouping of unicellular fungi. *See also* mold.

Z

zone of inhibition On an agar plate, the area of non-growth surrounding a paper disk containing an antimicrobial substance. (The zone typically ends at the point where the diffusing antimicrobial substance has reached its minimum inhibitory concentration, beyond which it is ineffective.)

zygospore The product of fertilization and the site of meiosis in some molds.

zygote The product of gamete fusion (plasmogamy) and nuclear fusion (karyogamy); a fertilized egg.

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