

MICROBIOLOGY LAB MANUAL



Sheri Miraglia
City College of San Francisco

Microbiology Lab Manual

Sheri Miraglia

City College of San Francisco

This text is disseminated via the Open Education Resource (OER) LibreTexts Project (<https://LibreTexts.org>) and like the thousands of other texts available within this powerful platform, it is freely available for reading, printing, and "consuming."

The LibreTexts mission is to bring together students, faculty, and scholars in a collaborative effort to provide an accessible, and comprehensive platform that empowers our community to develop, curate, adapt, and adopt openly licensed resources and technologies; through these efforts we can reduce the financial burden born from traditional educational resource costs, ensuring education is more accessible for students and communities worldwide.

Most, but not all, pages in the library have licenses that may allow individuals to make changes, save, and print this book. Carefully consult the applicable license(s) before pursuing such effects. Instructors can adopt existing LibreTexts texts or Remix them to quickly build course-specific resources to meet the needs of their students. Unlike traditional textbooks, LibreTexts' web based origins allow powerful integration of advanced features and new technologies to support learning.



LibreTexts is the adaptable, user-friendly non-profit open education resource platform that educators trust for creating, customizing, and sharing accessible, interactive textbooks, adaptive homework, and ancillary materials. We collaborate with individuals and organizations to champion open education initiatives, support institutional publishing programs, drive curriculum development projects, and more.

The LibreTexts libraries are Powered by [NICE CXone Expert](#) and was supported by the Department of Education Open Textbook Pilot Project, the California Education Learning Lab, the UC Davis Office of the Provost, the UC Davis Library, the California State University Affordable Learning Solutions Program, and Merlot. This material is based upon work supported by the National Science Foundation under Grant No. 1246120, 1525057, and 1413739.

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation nor the US Department of Education.

Have questions or comments? For information about adoptions or adaptations contact info@LibreTexts.org or visit our main website at <https://LibreTexts.org>.

The LibreTexts name, logo, and book covers are trademarked by LibreTexts, Inc. (a 501(c)(3) not-for-profit organization) and protected against unauthorized use.

This text was compiled on 08/22/2026

TABLE OF CONTENTS

Licensing

Experiments

- 1: Introduction to the Microbiology Laboratory
- 2: The Microscope
- 3: Examination of Living Microbes and Environmental Plates
- 4: Colony and Cell Morphology; Simple Stains
- 5: Aseptic Technique and Streak Plates
- 6: Gram Stains
- 7: Acid Fast and Endospore Stains
- 8: Gram Stain Unknown
- 9: Introduction to the Bacterial ID Project
- 10: Carbohydrate Metabolism of Unknowns
- 11: Protein Metabolism of Unknowns
- 12: Completion of the ID Project
- 13: Identification of White Blood Cells
- 14: Antibiotics
- 15: Oxygen Requirements
- 16: Germicides and UV Light
- 17: Food Microbiology

Index

Glossary

Detailed Licensing

Detailed Licensing

Licensing

A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.

This page titled [Licensing](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by [Sheri Miraglia](#).

EXPERIMENTS

- 1: Introduction to the Microbiology Laboratory
- 2: The Microscope
- 3: Examination of Living Microbes and Environmental Plates
- 4: Colony and Cell Morphology; Simple Stains
- 5: Aseptic Technique and Streak Plates
- 6: Gram Stains
- 7: Acid Fast and Endospore Stains
- 8: Gram Stain Unknown
- 9: Introduction to the Bacterial ID Project
- 10: Carbohydrate Metabolism of Unknowns
- 11: Protein Metabolism of Unknowns
- 12: Completion of the ID Project
- 13: Identification of White Blood Cells
- 14: Antibiotics
- 15: Oxygen Requirements
- 16: Germicides and UV Light
- 17: Food Microbiology

1: Introduction to the Microbiology Laboratory

Learning Outcomes

By the end of this lab period, you will be able to

- Articulate appropriate safety procedures in the microbiology lab.
- Wash your hands and disinfect lab surfaces correctly
- Identify the correct ways to dispose of lab waste (compost, biohazard, used slides, culture tubes with media, Petri dishes).
- Identify which supplies you need to purchase and bring to the next laboratory period
- Explain why most professional healthcare programs require an in-person microbiology laboratory experience.

Introduction

Welcome to the microbiology laboratory! We are delighted to have you in our classes and excited to share our passion for microorganisms with you. The microbiology laboratory will give you the opportunity to develop new skills, apply critical thinking to solve problems and work independently on a multi-week project culminating in a written report.

Today we will be reviewing basic microbiology lab safety, and waste disposal methods. We will also determine which supplies you will need to purchase and assign lockers. We often have lab coats that we can loan to students for the duration of the semester.

Be prepared to be quizzed on the content of today's laboratory lecture, particularly the safety rules.

General Lab Safety

1. Read all laboratory material before coming to class. The most important thing you can do to be safe in the lab is to be prepared and know in advance what you will be doing and why!
2. Familiarize yourself with emergency exits, the eyewash station, the fire blanket and extinguisher, the first aid kit, and the broken glass container (Figure 1.1)

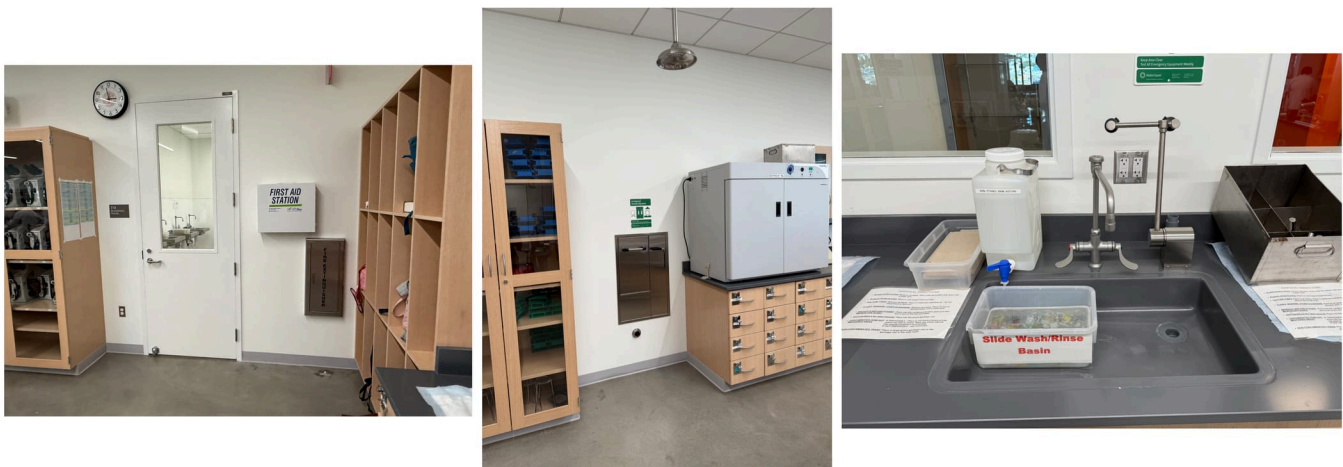


Figure 1.1: Left: Location of the first aid kit and fire extinguisher. Middle: Safety shower and eyewash. Right: ADA-compliant eyewash

3. Wear a lab coat whenever working with microbes. You should bring your lab coat home to wash regularly (it's a good idea to wash the coat in hot water with a little bleach).
4. You must wear closed-toed shoes in the lab at all times - even if lab work is not scheduled on that day. Glassware is routinely broken in labs, and laboratory floors are not a safe place for flip-flops or strappy sandals. For your safety, the instructor will

ask you to go home and return with appropriate footwear if necessary.

5. Wear disposable gloves when working with microbes, and when staining microbes for viewing under the microscope. The biological stains we use will remain on your fingers for days...and they will ruin your nice shoes or clothes.
6. Turn off the Bunsen burners whenever you are not working with them!
7. Notify the instructor if you are pregnant, or become pregnant or have any other medical conditions that might necessitate special precautions.
8. Long hair should be tied back when working in the laboratory.
9. No optional or original experiments are to be conducted without prior approval.
10. Do not taste any chemicals.
11. Do not pour unused chemicals back into the stock bottle.
12. Broken glass should be reported to the instructor who will assist you in the immediate cleanup. Broken glass is to be discarded only in a "broken glass" container.
13. Place plastic Petri dishes, disposable plastic Pasteur pipettes, and other non-sharp disposable items contaminated by bacteria, blood, or body fluids in a disposable autoclave bag for decontamination by autoclaving or place them directly into a 10% bleach solution before reuse or disposal.
14. Wash the laboratory table surface after you have finished the activity when applicable.
15. Sanitize benches BEFORE and AFTER lab. Leave the disinfectant solution on the bench to air dry.
16. Dispose of any trash or chemicals properly.
17. Clean all equipment and return it to the storage area before leaving the laboratory work area.
18. Report any spill, accident, or injury to the instructor immediately, and follow emergency procedures as necessary.
19. Do not sit on lab benches in Microbiology!!! Ewww....
20. When staining bacteria or using any chemicals, wear safety goggles.
21. Place backpacks and purses out of high-traffic areas!
22. If you are not sure of something.....ask for help or clarification before you start!!

Protecting yourself, your lab partners, and your cultures from accidental contamination

- Wipe the desktop with the disinfectant (red squirt bottles) before and after each lab period. Never assume that the class before you disinfected the work area. Allow the disinfectant to evaporate; do not wipe it dry.
- Never lay culture tubes on the table; they always should remain upright in a culture tube rack.
- Report any spills of bacterial cultures immediately to your instructor, and request instructions. In general, cover any large culture spills with paper towels. Soak the towels immediately with disinfectant and allow them to stand for 20 minutes. When you are finished, place the towels in the red biohazard bin.
- If the culture spills on you, let your instructor know and they will help you (this is why you wear a lab coat!).
- If you get a microbial culture in your eyes IMMEDIATELY have a lab partner lead you to the eyewash and rinse your eyes for at least 15 minutes. Speed is important to prevent injury to the eye. Notify your instructor of the spill and your situation, and follow their instructions for handling your care and the spill.
- Take care with computers, phones, and other items that you place on the lab bench. Ensure they remain a safe distance from bacterial cultures and do not become contaminated. We do use and need our computers in the lab on a regular basis.

Safe Disposal of Trash, Contaminated Items, and Glass

In the CCSF Microbiology Lab, we separate waste into various categories.

- Paper towels that are not contaminated with bacteria are disposed of in the green bins - they are compostable. It is okay to throw away paper towels with dye or sanitizing solution on them. (Figure 1.2)
- Gloves and non-recyclable trash can go in the grey or white trash bins. (Figure 1.2)

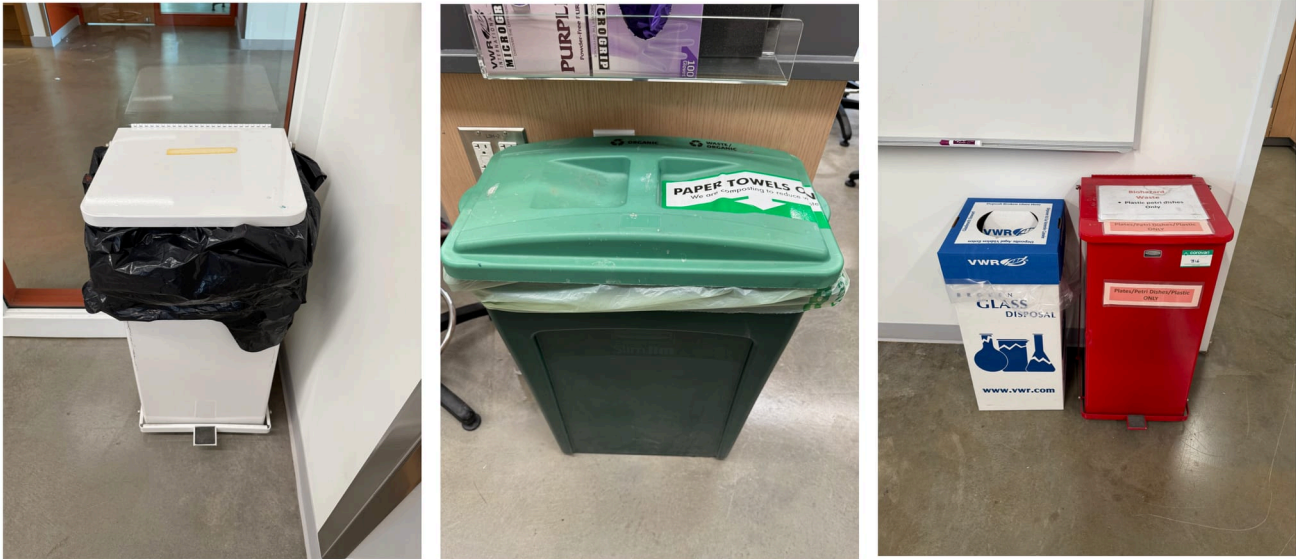


Figure 1.2: White bin (left) are for regular trash. Paper towels are to be placed in the green compost bins (center). In the front of the lab, there is broken glass disposal and non-sharps biohazard disposal

- Contaminated materials (blood, bacteria, body fluids) **that are not sharp** like gloves, paper towels, plastic Petri dishes, and cotton swabs, go in a biohazard bag. (Figure 1.3)
- Sharp objects like needles must be disposed of in a designated sharps container. (Figure 1.3)



Figure 1.3: Non-sharp biohazards (Petri dishes, cotton swabs, contaminated paper towels) can be placed in a biohazard bag in the metal biohazard bin (left). Sharps, such as lancets or microscope slides, must go in a hard-sided sharps container (right).

- Remove all labels from glass culture tubes using alcohol/acetone and a cotton ball. Place them upright in the designated autoclave container. (Figure 1.4)



Figure 1.4: Dispose of culture tubes by removing the labels (use a cotton ball soaked in alcohol/acetone to remove sharpie marks) and placing upright in the metal bins to ensure the contents do not spill.

- Notify the instructor of any broken glass. We use a lot of glass tubes, and glass does occasionally break. Your instructor is the person designated to safely dispose of broken glass. Do not touch or clean up broken glass on your own.

Biosafety Levels

Infectious agents (microorganisms) are divided into four main biosafety levels - BSL-1, BSL-2, BSL-3, and BSL-4. BSL-1 organisms are not known to cause disease in healthy adults. BSL-2 agents are associated with some human diseases, but treatments/vaccines are usually available. BSL-3 and BSL-4 organisms are more exotic and dangerous and require special facilities in order to be safely manipulated.

At City College of San Francisco, we work with BSL-1 and BSL-2 organisms only. Most of our organisms do not typically cause disease in healthy individuals and present a minimal threat to the environment and lab personnel. These include organisms such as *Escherichia coli* (the strains we use), *Bacillus subtilis*, and *Lactococcus lactis* - all of which are BSL-1.

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). We do not work with these organisms in a way that results in splashes or aerosol generation (even though these organisms are not generally known to be transmitted by aerosols). Therefore laboratory work in BIO 120 may be done using standard microbiological practices. BSL-2 organisms that we encounter in BIO 120 include organisms such as *Salmonella enterica* and *Staphylococcus aureus*.

Learn about Biosafety Levels by [completing this very short tutorial](#) which was developed by the Centers for Disease Control and Prevention.

Materials

You must have these by Week 2. You may share some of these with a lab partner to save on costs.

1. A long-sleeved shirt or lab coat that covers you below the waist. Color unimportant.
2. Nitrile or latex gloves (make sure that you are not allergic to latex)
3. Hair ties for long hair
4. Rubber bands
5. Colored pencils or pens (include purple, pink, red, green & blue)
6. A sharpie (thick point) permanent marker or wax pencil
7. Box of microscope slides (one box per student)
8. A blue box for holding microscope slides (CCSF Bookstore)
9. Lens and blotting (bibulous) paper
10. Lab Safety Goggles

This page titled [1: Introduction to the Microbiology Laboratory](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by [Sheri Miraglia](#).

2: The Microscope

Learning Outcomes

By the end of this lab period you will be able to:

- Set up the microscope, and use it to visualize specimens under 4x, 10x, and 40x magnifications
- Calculate the total magnification for each objective
- Use the 4x objective to scan the entire specimen
- Apply the concept of “depth of focus” to determine which silk fiber is on the top, middle, and bottom of a crossed fibers slide.
- Focus on fixed specimens of common free-living protozoa such as paramecium, euglena, and amoeba.

Introduction

Most of life on earth is invisible! Our exploration of tiny organisms began with the first light microscopes developed by [Robert Hooke](#) and [Antonie van Leeuwenhoek](#). Believe it or not, these first simple microscopes work in much the same way as the compound light microscopes that you will be using extensively this semester in our microbiology laboratory.

There are a variety of different types of microscopes in use today, including phase contrast, fluorescence, and dark-field microscopes. We will be using bright-field microscopes in our introductory course, and in fact, these are the most common microscopes used in clinical laboratories as well.

The Compound Microscope - Basics

A microscope works by passing light through a specimen (usually on a glass slide), which travels through lenses that magnify the image (like a magnifying glass) and then to your eye. It's referred to as a “compound microscope” because the light passes through a series of lenses that refract light and magnify the final virtual image that you see when you look through the ocular lenses (Figure 2.2). You can control the amount of light passing through the image by adjusting the light intensity knob or by adjusting the width of the opening of the substage iris diaphragm (Figure 2.1).



Figure 2.1: The major components of a light microscope. Photo credit iStock.com/kwanchaichaiudom

Microscope Lenses and Magnification

The light from the bulb at the base of the microscope first passes through a condenser, which focuses a cone of light right onto the specimen. The light then passes through the specimen into the objective lens which magnifies the image by either 4x, 10x, 40x, or 100x, depending on which objective is in the light path. Finally, the light travels through the 10x ocular lenses and is focused on the back of your retina, where your brain can interpret the virtual, magnified image.

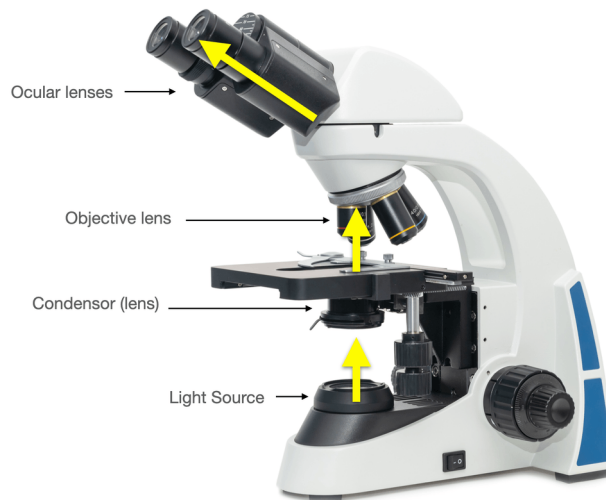


Figure 2.2: The path of light through lenses in a compound light microscope. Photo credit iStock.com/kwanchaichaiudom

The total magnification of the image is determined by multiplying the magnification of the objective lens and the ocular lens.

Table 1 - Microscope objectives, magnifications and uses

Lens	Total magnification with oculars	Alternate Name	Best for:
4x	40x	Scanning objective	Scanning the entire slide to decide the best spots to focus on, low-power observation
10x	100x		Medium power observations
40x	400x	High dry	High power observations
100x	1000x	Oil immersion lens	Highest power observations - needed for very small organisms such as bacteria

Field of View

The field of view is the circular area that is visible when you look through the microscope (Figure 2.3) As you increase magnification, the field of view decreases, that is, you see LESS of your specimen. You will be exploring the field of view with the "Letter E" slide.

Depth of Focus

The depth of focus refers to the thickness of a specimen entirely in focus under a microscope (Figure 2.3). As you increase the magnification the depth of focus decreases. At very high magnifications, only thin slices of your specimen are in focus at any one time. You will be exploring the depth of focus with the crossed silk threads slide.

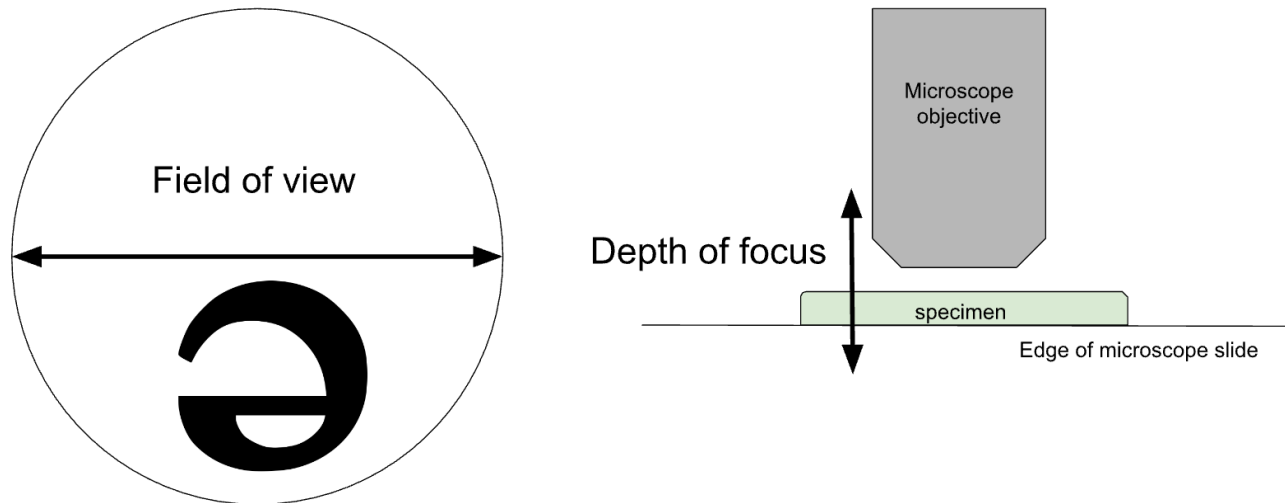


Figure 2.3: Illustrations of the field of view and depth of focus in microscopy.

Resolution vs. Magnification

Just because the image is larger, doesn't mean that you can see much! It's possible to increase magnification, without increasing resolution. In this case, the image would be blurry, and even though it was large, you wouldn't be able to see very much.

The word "resolution" in microscopy refers to the distance that two points in the image must be apart in order to be distinguished from each other. Resolving power is related to the wavelength of light that is used to illuminate the image. The smaller the wavelength of light, the better the resolving power. Resolving power is also impacted by the numerical aperture of the objective lens that is being used to magnify the specimen.

An electron has a much shorter wavelength than even the shortest wavelength visible light (blue light). This is why the resolving power of an electron microscope is so much greater than a light microscope! The best compound light microscopes have a resolving power of about 200 nanometers (nm). However, an electron microscope can resolve between points on an image that are only 0.5 nm apart. Unfortunately, an electron microscope is very expensive and very large! It requires complex sample preparation and a vacuum source. The good news is that we can still see bacteria quite well with a compound light microscope. Bacteria have a wide range of sizes and shapes, but many are around 10 micrometers (10,000 nm). So our compound light microscopes have sufficient resolving power to work with. Unfortunately, smaller microbes such as viruses are too small for our microscopes.

Parfocal Lenses

Our microscopes are parfocal. What does this mean? Once you have focused on any of the objectives (we always start with 4x), the other objectives will also be (mostly) in focus. You do not need to make major focus adjustments after changing objectives - usually the fine focus knob can be used to make small adjustments and obtain a crisp, clear image. In fact, you should never use the coarse focus once you've obtained an in-focus image using the 4x objective.

Materials

- Compound Light Microscope

- Lens paper
- Letter “e” slide
- Crossed threads slide
- Prepared slides of *Amoeba*, *Euglena*, and *Paramecium*
- Your bound lab notebook

Experiment

Letter “e”

Your instructor will walk you through the use of the microscope during this lab. Here are the steps that you will go through, using the “Letter E” slide.

1. Note your station/cabinet number. This will be the number of the microscope that you use all semester. Remove your microscope from the cabinet being sure to grasp it firmly by supporting the microscope base and holding onto the microscope arm.
2. Bring the microscope to your lab bench, and set it carefully down.
3. Make sure the microscope power switch is turned “off” and the light intensity is turned all the way down (counterclockwise).
4. Raise the substage condenser so that it is even with or just a few millimeters below the stage. Open the condenser’s iris diaphragm all the way.
5. Remove the cord from the back of the microscope, and plug the power cord into both the outlet and the back of the microscope.
6. Turn the power on, and increase the light intensity until you can see light coming through the substage condenser from the top of the stage.
7. Your microscope should have the 4x objective lens in place above the stage. If not, rotate the revolving nosepiece to place the 4x objective into position.
8. Place the letter “e” slide onto the microscope stage, securing it with the stage clips.
9. Using the knobs on the side of the stage, move the slide so that the letter “e” is directly over the light.
10. Look through both ocular lenses with both eyes open. Adjust the binocular lenses so that when looking with both eyes open, you see a single image.
11. Bring the letter “e” into focus by adjusting the coarse adjustment knob.
12. Fine-tune the focus of the letter “e” using the fine focus knob. Adjust the brightness by adjusting both the light intensity knob and the condenser’s iris diaphragm until you have a crisp, clear image.
13. Using the knobs on the side of the stage, adjust the position of the letter “e” until it is perfectly centered in the middle of the image.
14. When you have finished drawing/observing the letter “e” under 40x total magnification, move the 10x objective lens into place by rotating the revolving nosepiece.
15. As you get ready to move to a higher magnification, recall that our microscopes are “parfocal” which means that you do not need to significantly readjust the focus each time you change the magnification.
16. Using only the FINE FOCUS KNOB, adjust the focus so that the letter “e” comes back into focus. When you have finished drawing/observing the letter “e” under 100x total magnification, move the 40x objective lens into place by rotating the revolving nosepiece.
17. Using only the FINE FOCUS KNOB, adjust the focus so that the letter “e” comes back into focus. At this higher magnification, only a part of the letter “e” will be visible. When you have finished drawing/observing the letter “e” under 400x total magnification
18. When you are done observing the letter “e” at all three magnification levels, you can remove it from the stage and return it to the slide box in the correct orientation.

Crossed Silk Fibers

The crossed silk fiber slides consist of blue, yellow, and red silk fibers that are crossed in the center. Under low magnification, they appear to be in the same focal plane, but under higher magnification, it’s possible to determine which color fibers are on the top, in the middle, and on the bottom of the stack.

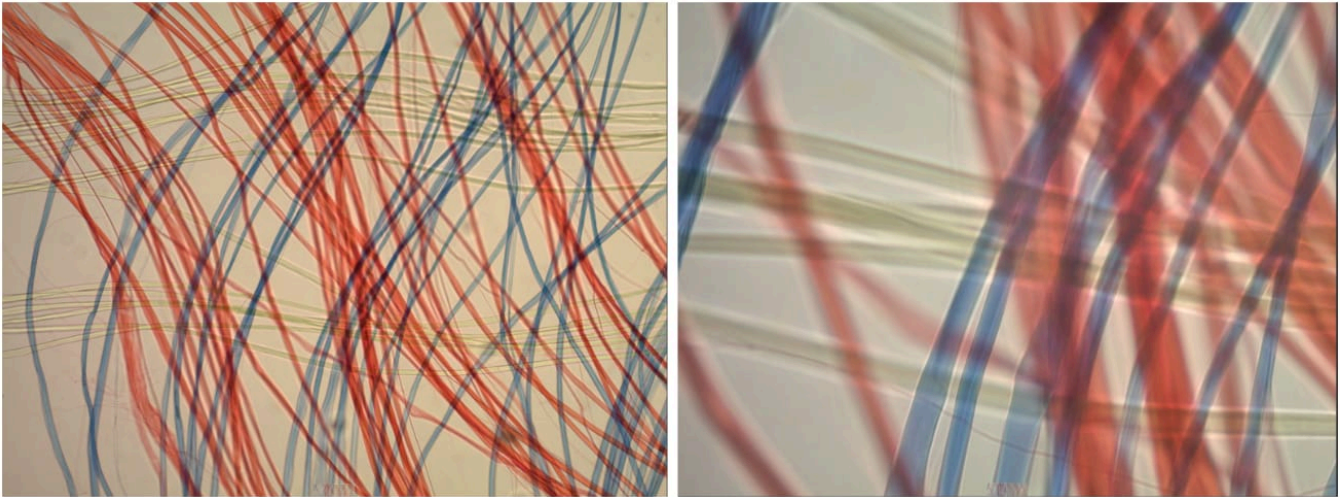


Figure 2.4: Microscope images of crossed silk fibers at 40x (left) and 400x (right) total magnification in a light microscope.

1. Following the same steps as you used for the letter “e”, visualize the crossed fibers using the 4x, 10x, and 40x objectives (Figure 2.4).
2. Be sure to explore how adjusting both the light intensity and aperture of the condenser iris diaphragm impacts the quality of the image at each magnification.
3. Using the fine focus knob, move up and down in the Z-direction through the fibers at each magnification.
4. At which magnification are some fibers in focus while others are not? Use this magnification to determine the order the fibers have been placed on the slide. Note that all these slides are different! So your lab partner’s slides may have silk fibers that are in a different order than yours.

Prepared Slides of Free Living Protozoans

1. As time permits, continue to practice using the microscope by viewing prepared slides of stained protists - *Amoeba*, *Paramecium*, and *Euglena*.
2. Explore how the quality of the image changes as you increase the magnification
3. Explore the effect of opening and closing the iris diaphragm while also adjusting the light intensity.
4. Draw one picture of each organism in your lab notebook using the magnification where you are happiest with the image quality.

Data

Complete the Data Table below:

Table 2 - Student data table for recording information about microscope objectives

Objective	Alternate name	Magnification of Objective	Magnification of Oculars	Total Magnification	When would you use this objective?
4x					
10x					

Objective	Alternate name	Magnification of Objective	Magnification of Oculars	Total Magnification	When would you use this objective?
40x					
100x					

Sketch/record your observations in your lab notebook

- Letter E slide - Draw at 40, 100 and 400x
- Protozoa slides - sketch the protozoa that you view under the microscope. In each case, indicate which objective you chose that gave you the best image.

Silk Fibers Slide - Put the crossed fibers in order:

Table 3: Student data table for recording silk fiber results

Silk Fiber	Your result
Top (closest to the objective)	
Middle	
Bottom (closest to the stage)	

3: Examination of Living Microbes and Environmental Plates

Learning Outcomes

By the end of the lab period you will be able to:

- Prepare wet mounts of live organisms including pond organisms and termites
- Identify *Euglena*, *Paramecium*, *Amoeba*, and *Trichonympha* by their morphology and how they move.
- Use our microscopes to follow a living organism as it moves through the field of view in the microscope.
- Inoculate agar plates with environmental samples and samples from the human body.

Introduction

Part I - Free Living Protozoa

For the most part, we will be using our time in the microbiology lab to explore prokaryotic cells (bacteria). They are easy to work with, visible under a light microscope, and many species are pathogenic organisms relevant to human health. In fact, many non-pathogenic bacteria are also relevant to human health, but in beneficial ways!

However, there are other groups of organisms that we won't be able to work with this semester. These include viruses, which are too small to see using a light microscope. There are also much larger organisms including pathogenic worms such as tapeworms, which are visible to the naked eye.

The protozoans are a group of single-celled organisms that include both parasitic forms that cause disease in humans, and "free-living" forms that live in pond water. There are also protozoa that live in symbiosis with their hosts in a mutualistic manner (both the protozoan and the host benefit from the relationship). Free-living protozoa are readily available and really fun to look at using our microscopes because they can be viewed while they are alive which allows us to practice our microscopy skills as we use the scanning objective to both locate them and chase them around the field of view as they move!

Today we will be looking at 4 different types of protozoans. All of these organisms are eukaryotes and belong to the domain "Eukaryota". Their cells are organized in a way that is very similar to your cells (as you are also in the domain Eukaryota). They have membrane-bound nuclei and organelles, and they are much larger than bacteria. A typical bacterial cell is only about 1-10 microns, while a large amoeba can be up to 750 microns!

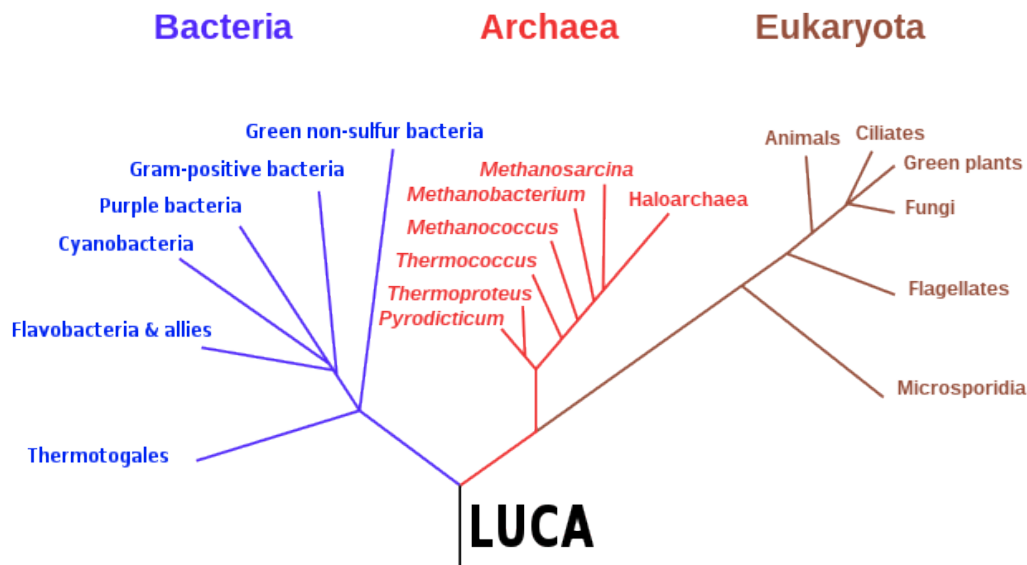


Figure 3.1: The three domains of life. Single-celled eukaryotic organisms such as protozoa and fungi are in the domain Eukaryota. [Chiswick Chap, CC BY-SA 4.0](#), via Wikimedia Commons

Amoeba

Have you ever heard of a brain-eating amoeba? *Naegleria fowleri* is the terrifying parasite that is being referred to! However, today in the lab, we can very safely look at the harmless *Amoeba proteus*. This organism moves around using extensions of the plasma membrane called “pseudopods” or “false feet” which stretch out to surround and engulf their food in the environment. The way amoebas move to trap and ingest their food is very similar to the way white blood cells trap and destroy invaders that have made their way into the human body.

Paramecium

The *Paramecium* has been so widely explored by scientists that it is often called the “white rats” of ciliates to highlight its role as a lab animal. Ciliates are organisms that are covered by cilia that cover their outer surface. The organism can move when the cilia move back and forth. You will be surprised to see how fast these organisms can get around when you try to chase them around the microscope slide.

Euglena

Euglena is the smallest of the protozoa that we will look at today and are unique in that they contain chloroplasts and can photosynthesize! This means they can make their own food in addition to eating and digesting food in the environment. *Euglena* is also different from *Amoeba* and *Paramecium* because they move by rotating their flagella located at either end of the organism.

Trichonympha

Trichonympha is not a free-living organism but instead is a symbiont with termites. Termites eat wood, but they don’t have the enzymes they need to digest it. They rely on microbes such as *Trichonympha* (a protist) and bacteria to break down the cellulose in wood into glucose that the termite can use for energy. The relationship is mutually beneficial - the termite chews on the wood and breaks it down into tiny particles. Then those particles are broken down in the termite gut by the microbes into a form of food (glucose) that everyone can use. How do baby termites get “inoculated” with these microbes they need to digest food? They eat fluid that comes out the posterior of the parent termite (proctodeal trophallaxis) which is rich in the microbes they need.

Part II - Environmental Plates

Microbes are all around us - on the surface of your cell phone, the bottom of your shoes, door handles, the soil, etc. We are also covered in microbes both inside and outside of our bodies. In fact, all of life is so dependent on the microbes in our environment

that it would be an apocalypse if they all suddenly disappeared!

Today we take a quick and imperfect sampling of some microbes on and around us. Why imperfect? We will be using nutrient agar to culture microbes that we capture on a sterile cotton swab. However most organisms won't grow on nutrient agar, so we will only see the ones that do. It's still lots of fun, and we'll look at the organisms we do grow under the microscope in Lab #4.

Materials

Per Class

- Prepared slides of *Amoeba*, *Euglena*, and *Paramecium*
- Living cultures of *Amoeba*, *Euglena*, and *Paramecium*
- Termites
- Pond samples
- Transfer pipets
- Forceps

Per Student

- Clean glass slides and coverslips
- Compound microscope
- Lens paper
- 2 agar plates
- 4 cotton or calcium alginate swabs

Experiment

Observe Living Protozoa

1. Obtain a microscope and place it on the bench. Check to be sure the stage is all the way down and the scanning objective is in place.
2. Your instructor will show you how to prepare wet mounts of the protozoan specimens. Sketch what you see in the data sheet or lab notebook.
3. Use forceps to transfer a termite into a drop of water on a clean slide. Add a coverslip, and use the blunt end of the forceps or your finger to squash the termite. Mash the intestine to release *Trichonympha* and other protozoa and bacteria.
4. Examine drops of pond water samples. Sketch and describe the organisms you find.
5. When you are finished observing specimens, clean the objective lenses with lens paper. Return all lenses and adjustments to their storage positions before putting the microscope away.

Prepare Environmental/Body Parts Plates

1. Choose 2 different environments to investigate. Use a sharpie to draw a line at the bottom of each agar plate to divide it into two halves, and label each half with the environment you plan to sample (Figure 3.2).
2. If you are collecting from a dry surface, dip the cotton swab in sterile water (if available), and then swab the surface.
3. Open the lid of the agar plate, and light streak back and forth across the agar in the labeled region. Discard the swab.
4. Choose 2 body parts to investigate. Any body parts are acceptable except vaginas and anuses, with a goal not to inadvertently grow up any of the more significant pathogens.
5. Label the bottom of the plates with your first initial and last name, and the date. Incubate at 37°C for 48 hours or at 30°C for 5 days.

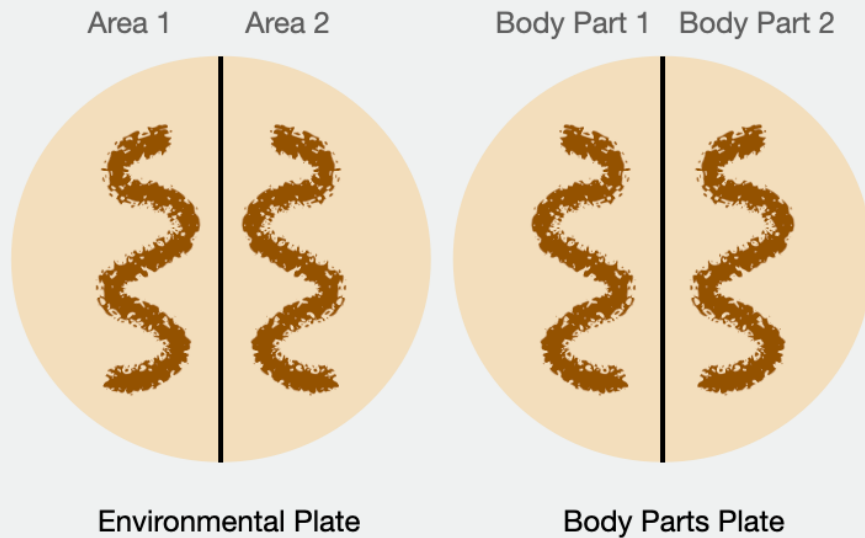


Figure 3.2: Diagram of how to prepare your environmental and body part plates

Data

Sketch the protozoa that you view under the microscope. Indicate the total magnification for each sketch, and label any structural characteristics you can identify (nucleus, cell membrane, flagella, pseudopods, etc.).

Questions

1. What features did the cells you observed have in common? How were they different?
2. If you observed the same organism on a prepared slide and a wet mount, how did the images compare?

4: Colony and Cell Morphology; Simple Stains

Learning Outcomes

By the end of this laboratory period you will be able to:

- Differentiate between colony morphology and cell morphology, and describe the colony morphology of bacteria grown on a solid surface
- Prepare a bacterial smear for staining
- Perform a simple stain, and explain why they are useful
- Visualize individual bacterial cells using oil immersion microscopy and describe their cell morphology

Introduction

Colony morphology

On a solid surface, a single bacterium can grow into millions very quickly as they divide. Many of the common organisms we use in the microbiology lab can be seen on a nutrient agar plate within 24 hours of inoculation. When a single bacterium is placed on the surface of solid media, it will divide and divide and divide until there are millions of that specific bacteria - enough that you can see them. We call these “colonies” of bacteria. Depending on the species, the colonies can look really different from each other - this is referred to as “**colony morphology**”.

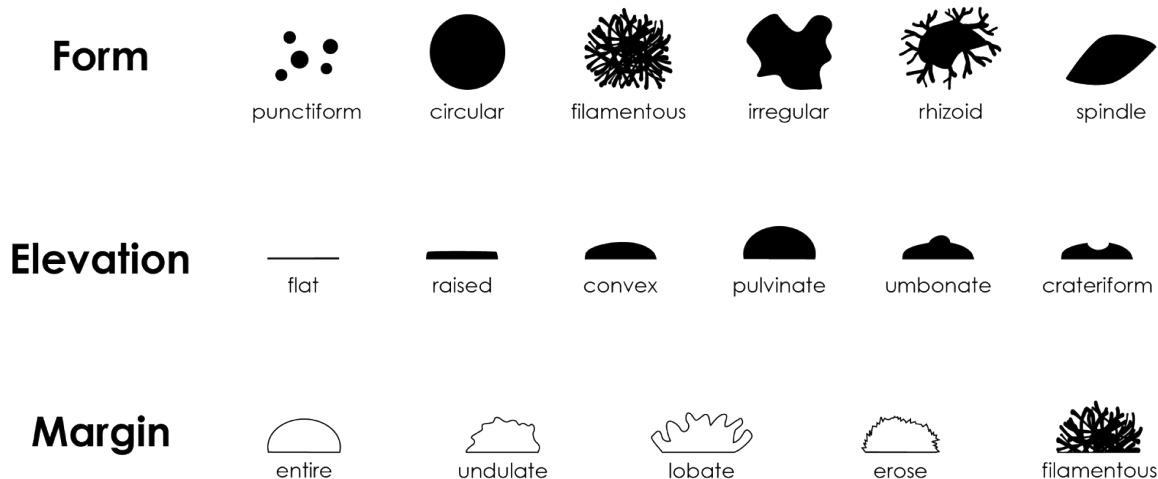


Figure 4.1: Illustration of the various ways that you can describe colony morphology of organisms grown on solid media. [Macedo](#), CC BY-SA 4.0, via Wikimedia Commons

There is some subjectivity to assigning descriptions to colonies - and bacteria can look very different depending on factors such as the type of media used and the temperature organisms are grown at! The best way to view colonies is to remove the lid and hold the plate at an angle to allow the light to catch the colony surface. A magnifying glass can also help visualize colony characteristics. Here are some examples of common organisms that you will see in the microbiology lab (Figure 4.2)



Figure 4.2: Morphology examples of (clockwise starting from top left): *E. coli*, *B. subtilis*, *S. epidermidis*, *Proteus vulgaris* (showing swarming), *K. pneumoniae*, and *S. aureus*. *S. aureus* and *S. epidermidis* are grown on sheep's blood agar, all other organisms are grown on nutrient agar.

Cell Morphology

To view individual bacterial cells, you will need to use a microscope. Bacteria are so small that they test the limit of resolution in our light microscope. The best way to view most bacteria is using oil immersion microscopy, which you will learn to do in today's lab.

Despite the tremendous genetic diversity seen in prokaryotes, cell morphology falls into just a few categories. Bacterial cells form cocci (round), bacillus (rod), and curved structures. Detailed diagrams and descriptions of bacterial cell morphology can be found in [OpenStax Microbiology, Chapter 3.3](#), so we will not go over that in detail here. However, it's good to be familiar with some common shapes and arrangements seen in our laboratory (and ones you are likely to see in today's lab!).

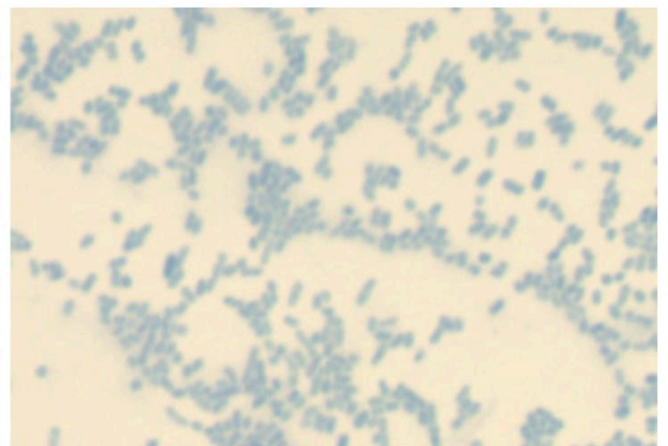
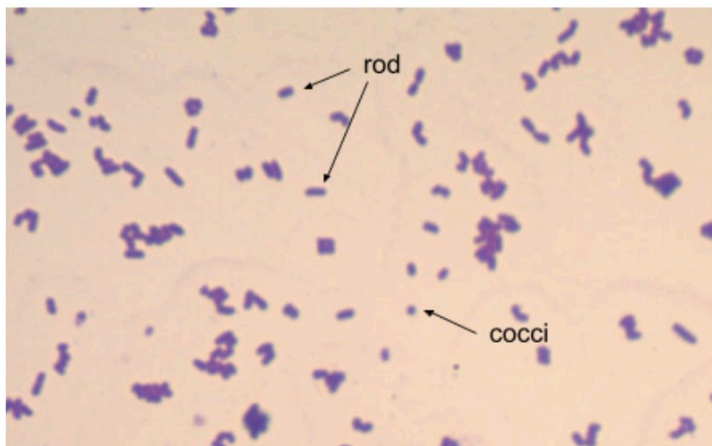
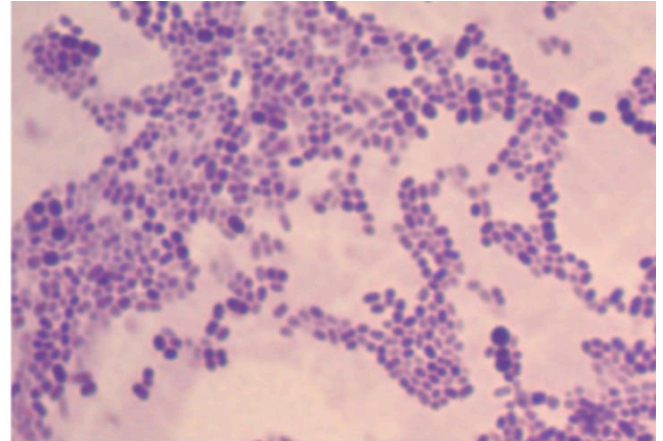
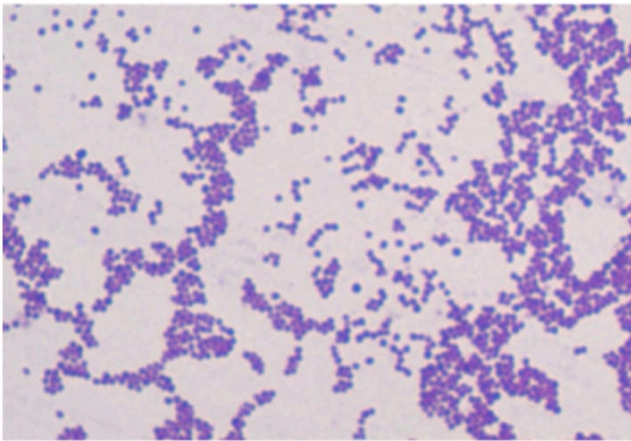
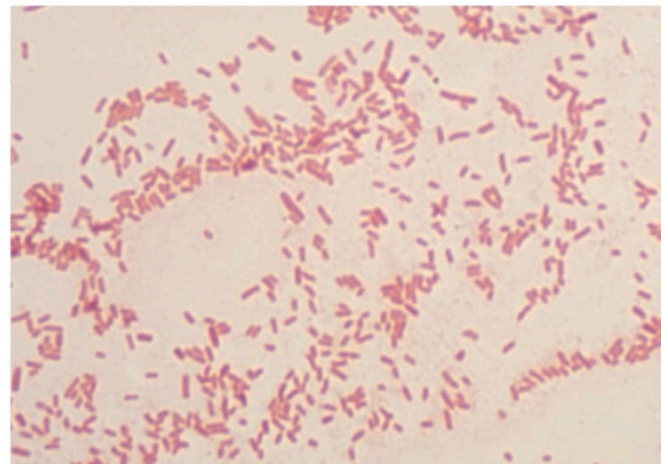
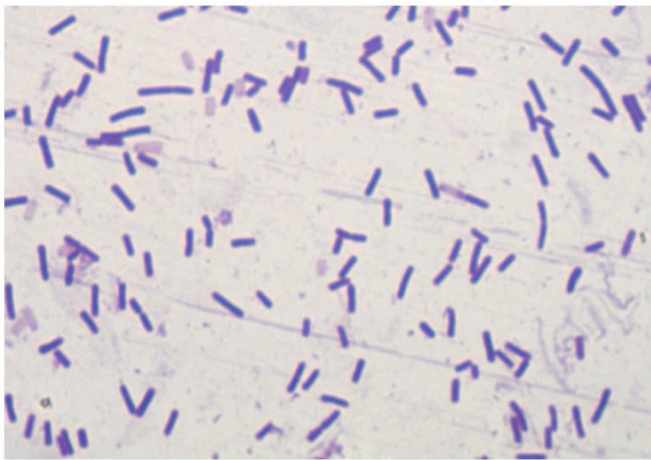


Figure 4.3: Clockwise from top left: Cell morphology of *B. cereus* stained with crystal violet, *E. coli* stained with safranin, *L. lactis* stained with crystal violet and showing coccobacillus morphology, *P. vulgaris* stained with methylene blue, *C. xerosis* stained with crystal violet showing pleomorphic cocci and rod morphology and *S. aureus* stained with crystal violet.

Preparation of a Bacterial Smear

In order to view bacteria under a microscope, you first need to get them onto a microscope slide and heat-fix them to kill them and help them adhere to the slide.

Procedure

1. Make a circle using a sharpie around the colony from which you will be taking bacteria for your smear (Figure 4.4)



Figure 4.4

1. Label the edge of a clean microscope slide with the name of the specimen.
2. Add a drop of water to the slide. Be sure to keep track of which side of the slide you are making your smear! This will be important when you go to view the slide under the microscope. Note: many staining procedures use decolorizers with alcohol which will remove the writing on the slide made with a sharpie (Figure 4.5).



Figure 4.5

3. Using an inoculation loop or needle, gather a very small amount of bacteria from the colony. Be sure to use aseptic technique when collecting the specimen.
4. Use the needle or loop to deposit the bacteria onto the slide in the water droplet. Agitate the loop to get an even distribution of bacteria within the water drop (Figure 4.6).

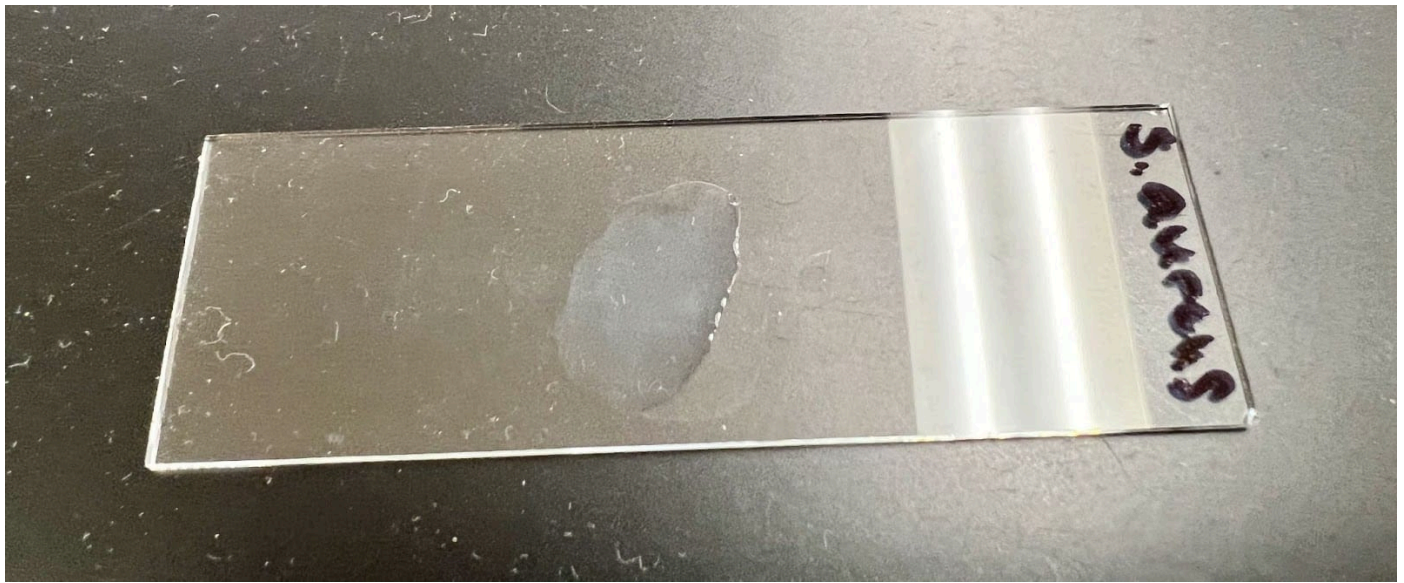


Figure 4.6

5. Place the slide on a hot plate. This will allow the water to evaporate and simultaneously heat-fix the smear. You can also do this by passing the smear over a Bunsen burner flame (carefully!).
6. When the slide is dry you should see a slightly opaque film on the surface of the slide. This slide is ready to be stained (Figure 4.7).



Figure 4.7

Simple Stains

It is nearly impossible to see the bacteria in your smear without some kind of stain. Bacteria are so tiny and unstained that they are nearly transparent when viewed with a light microscope. The simplest kind of stain uses one type of colored dye that will bind to the surface of the bacteria cells and allow them to be visualized under the microscope. Some of the most common dyes used for simple stains include crystal violet, safranin, and methylene blue. All are “basic” dyes that are positively charged and will adhere to the negatively charged surface of the bacterial cell.

Procedure

1. Immerse the smear into the staining reservoir for approximately 1 minute.
2. Rinse the surface of the stained smear with water to remove any excess stain
3. Blot dry
4. The principles of oil immersion microscopy are shown in Figure 4.4 below.

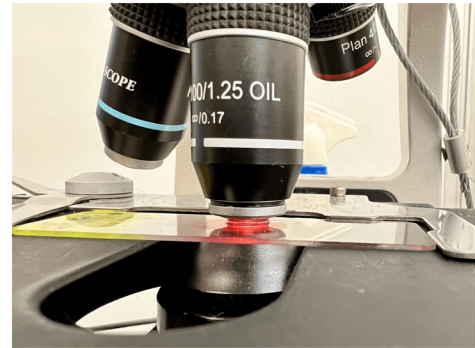
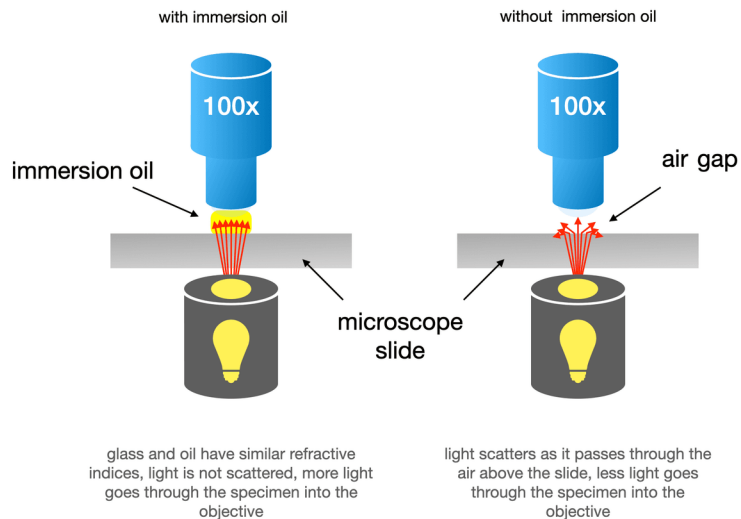


Figure 4.8: Left - Principles of oil immersion microscopy. Right - A microscope slide with immersion oil visible between the slide and the 100x objective.

Oil Immersion Microscopy

When you use the 4x, 10x, and 40x microscope objectives, light passes through the underside of the slide and reaches an air gap before it goes into the objective lens. This means that less light is getting into the objective lens, and this reduces the resolution of the image. When we reach the 100x objective, this loss of resolution significantly impacts our ability to view the specimen. This is resolved by placing a thin layer of oil between the microscope slide and the objective lens. The refractive index of the oil is similar to that of the glass slide and objective lenses, and results in more light making it through the objective and ultimately to the eyepiece.

Procedure

1. Set up the microscope as you normally would, bringing the smear into focus at 4x using the coarse focus knob. Bacterial smears do not have coverslips! Using the 4x objective, scan your smear looking for a place where the smear is thin, but there is also light coming through patches of cells. You are looking for a place on the slide where the cells are in a single layer and there is space in between them.
2. Rotate the revolving nosepiece bringing the 10x objective into place. Use the fine focus to re-focus the image as needed and re-center the slide or adjust position further to find a nice thin part of the smear where the cells are in a monolayer.
3. Rotate the revolving nosepiece bringing the 40 x objective into place. Use the fine focus to re-focus the image as needed and re-center the slide or adjust position further to find a nice thin part of the smear where the cells are in a monolayer.
4. Rotate the revolving nosepiece so that the specimen is between the 40x and 100x objectives. Place a drop of immersion oil right on top of the slide above where the light is passing through.

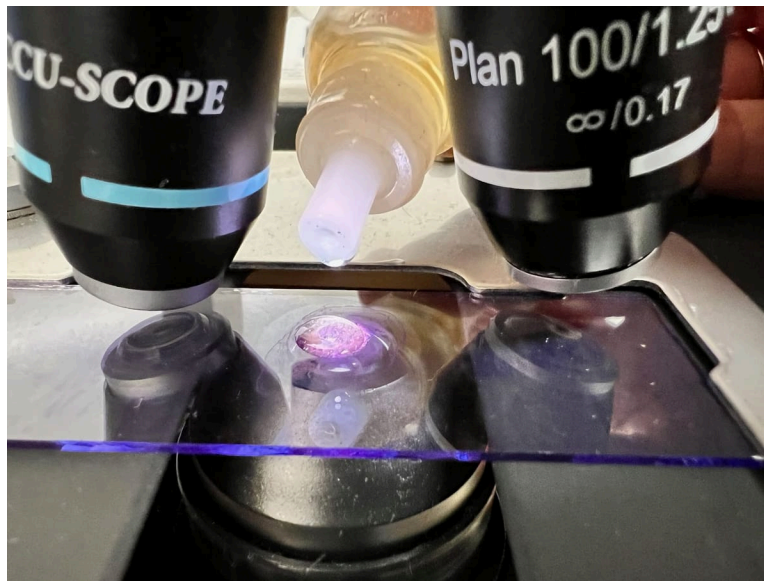


Figure 4.9: A drop of immersion oil is placed right on top of the specimen before advancing to the 100x objective.

5. Complete the rotation so that the 100x objective is in position. There should be no air between the specimen and the objective lens. DO NOT rotate the 40x objective into the oil by accident!
6. When you are done photographing or drawing what you see, use lens paper to remove all oil from the 100x objective. Discard that lens paper and when you are ready to put your microscope back, use a fresh piece of lens paper to clean the objectives starting at 4x, then 10x, then 40x, and finally clean the 100x one more time. Have your instructor check your microscope before putting it away.

Materials

Per Student Group

- Magnifying glass and colony counter
- Metric ruler
- Environmental plates from Lab 3
- Clean glass microscope slides
- Methylene blue stain
- Safranin stain
- Crystal violet stain
- Disposable gloves
- Squirt bottle with water
- Clothespins as slide holders
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper

Experiment

1. Use the terms in Figure 4.1 and in the text, to describe some representative colonies on your environmental plates. Measure colony diameters (in mm) with a ruler and include them with your descriptions in the table on the datasheet or lab notebook.
2. Follow the procedural diagram in Figure 4.34 to prepare smears of two different organisms on a slide. Be sure not to mix them. Make a second slide with the remaining two organisms at the same time so they can be air-drying simultaneously. Emulsions should be about the size of a dime.
3. Heat-fix each slide as described in Figure 4.34. Grasp the slide with a slide holder and pass the smears through the upper part of a flame at least 2-3 times. Avoid overheating the slide.
4. Place the slide on the metal stain rack over the sink. Cover the smears with the stain using the following times:

5. Crystal violet: stain for 30 to 60 seconds
6. Safranin: stain for up to 1 minute
7. Methylene blue: stain for 30 to 60 seconds
8. Grasp the slide with a slide holder and hold it at an angle. Gently rinse the slide with
9. distilled water. Aim the water bottle above the smears, let the water run down the smears, and rinse. Squirting water directly on the smears will risk losing the bacteria on the slide.
10. Gently blot dry in a tablet of bibulous paper. Do not rub.
11. Observe the slide using the oil-immersion lens. Record your observations of cell morphology and arrangement in the datasheet.
12. Draw your environmental plates and your lab partner's plates. Choose at least 3 species of microbes that you find most interesting, and describe the colony morphology of these species.

Data

Colony morphology table

Table 1 - Student Data, Colony Morphology

Organism	1	2	3
Form			
Elevation			
Margin			
Pigmentation			
Texture			
Diameter (mm)			
Bacterium or fungus?			
Sketch or photograph			

Cell morphology table:

Table 2 - Student Data, Cell Morphology

Organism	1	2	3
Cell shape			
Arrangement			
Basic dye used			
Bacterium or fungus?			
Sketch or photograph			

Questions

1. From your environmental plate, which environment sample contains the most diverse microorganisms? Why do you think this is the case?
2. From the body part plate, do you see the same bacteria from the two parts of your body that you sampled? How can you tell? And why do you think this is the case?
3. Why are basic dyes more effective for bacterial staining than acidic dyes?
4. Are your bacterial smears thin enough for you to see individual bacterial or fungal cells and their arrangements? If not, what can you do to improve your smear preparation technique?

5: Aseptic Technique and Streak Plates

Learning Outcomes

By the end of this lab period, you will be able to

- You will be able to define “mixed culture” and “pure culture”.
- You will be able to articulate the principles of aseptic transfer.
- You will know how to aseptically transfer bacteria from one pure culture to another using broths, slants, and deeps.
- You will know the proper way to prepare streak plates to isolate pure cultures from a mixed culture.

Introduction

As we saw in Lab #4, when we take environmental or body part samples, and put them into growth media, more than one type of organism will usually grow. If you put them in liquid media, they will grow together and you'll have a mixed culture.

When a single organism (species) is present in growth medium, we use the term “pure culture”. Because there are microbes everywhere, special care must be taken to maintain a pure culture and prevent it from becoming contaminated with other organisms. Since bacteria are living organisms, over time they will use up the nutrients that are in culture media. Many bacteria also produce acid as a metabolic byproduct. To maintain a pure culture for longer periods, organisms must be “sub-cultured” by moving them to fresh media.

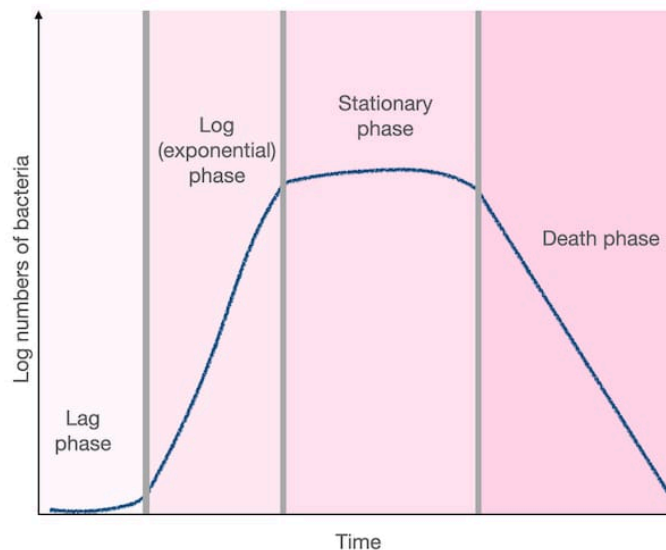


Figure 5.1: Bacterial growth curve showing the stages of growth over time. Eventually, all of the bacteria in a culture will die without subculturing into fresh media.

Types of Media

Microbiologists use a variety of different media to grow microorganisms, and we will be using a wide variety of these media over the course of the semester, including some media that selects for or differentiates between the growth of specific types of bacteria.

However, there are some kinds of non-selective media that allow you to grow a wide variety of microbes. In our microbiology laboratory, we grow most of our organisms in [Nutrient Agar](#) (NA/NB) or [Trypticase Soy Agar \(TSA\)](#). Nutrient agar (NA) and Nutrient Broth (NB) use the same formulation, except that agar has been added to form a solid medium to make NA. NA/NB is a simple

medium and does not contain a lot of extra nutrients. Because of this, only organisms that are easy to grow will typically thrive. Fortunately, many of the organisms that we use grow well in NA/NB. Trypticase Soy Agar (TSA) contains somewhat more nutrients than NA, and can be used to grow organisms that are more fastidious - that is, require a richer medium with additional nutrients to grow well.

When liquid or solid media is made, it can be prepared in a variety of formats. When a large culture volume is needed, bacteria can be grown in large flasks. However, when only small amounts of bacteria are needed, we typically use culture tubes. Culture tubes can be used to prepare both liquid (broth) media and solid media. Solid media is frequently prepared in flat plates known as Petri plates or [Petri dishes](#) when a larger surface is needed. In today's lab, we will be using Nutrient Broths (NB), Nutrient Agar (NA) Deeps, NA Slants, and NA plates. We will also be using a Trypticase Soy Agar (TSA) plate.

Agar slants, in particular, are very useful for growing and maintaining stock cultures for several weeks. Agar deeps can be used to look at bacterial motility as well as the oxygen dependence of organisms as there is always more oxygen at the surface of the deep than at the middle and bottom. Petri plates are used for the isolation of bacteria, as well as for differentiation and quantification (counting) of bacteria.

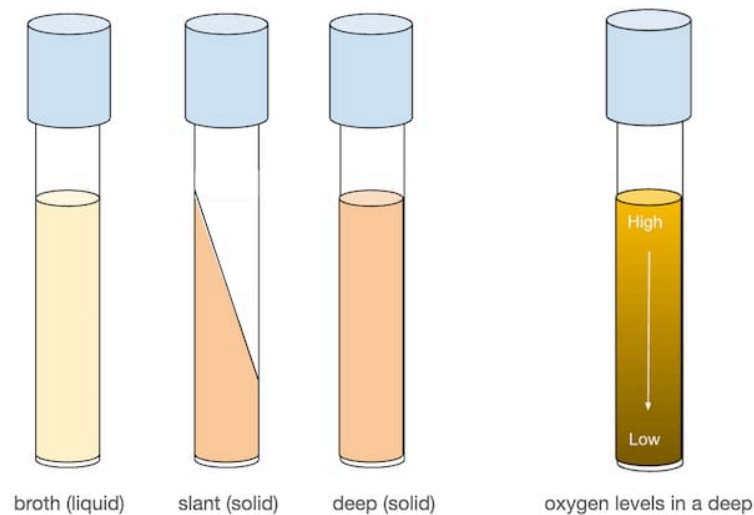


Figure 5.2: Examples of different types of media in culture tubes.

Growth in liquid culture

Individual bacteria are far too tiny to see without a microscope! However, in the lab when grown under ideal conditions, we can see bacteria when there are a lot of them. In a liquid culture, bacteria can be seen by the cloudy appearance of the broth after inoculation. Sometimes the entire broth is quite cloudy or **turbid**. In other situations, bacteria can form a **pellicle**, which is a floating biofilm of bacteria at the liquid-air interface of the culture. You may also find that bacteria grown in broth need a vigorous shake to redistribute the organisms throughout the tube as most have fallen to the bottom of the tube. This is referred to as "**sediment**".

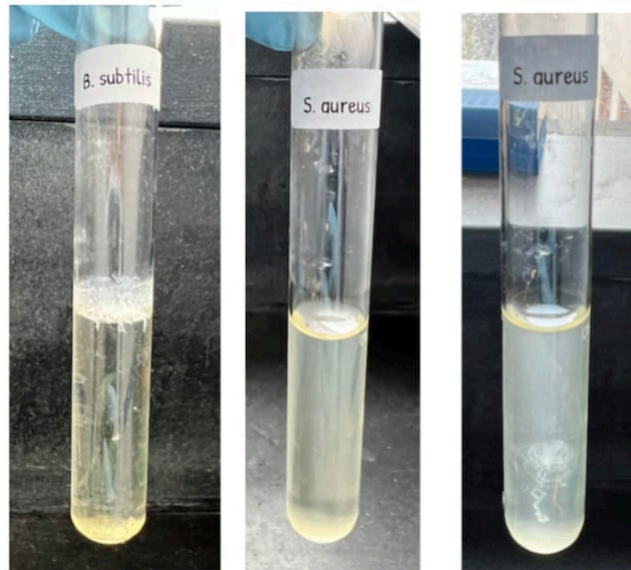


Figure 5.3: Left - a visible pellicle forms at the surface of a *B. subtilis* broth culture. Middle - characteristic turbidity seen in *S. aureus* broths. Right - sediment from the bottom of the *S. aureus* broth can be seen by gently re-suspending the cells at the bottom of the tube.

Aseptic Transfers

Today, you will be given some cultures prepared by your instructor and asked to aseptically transfer them to new media - a process called “subculturing”. Your instructor will demonstrate the process for you in class. It is critical that the cultures are transferred into media that has been sterilized and that you use proper aseptic technique in order to avoid contamination.

The steps for making an aseptic transfer are as follows:

Part I

1. Label the media that you are inoculating with the date, organism name, and your name.
2. Light your Bunsen burner or ethanol lamp to produce a steady flame.
3. Sterilize your inoculating loop. Hold it in your dominant hand as you would a pencil (Figure 5.4).

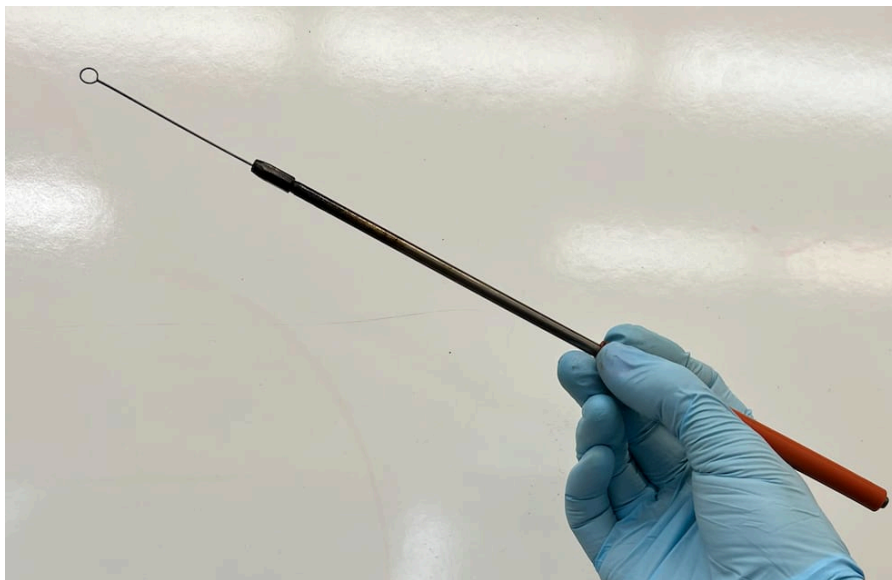


Figure 5.4

4. Grasp the culture tube containing the bacterial culture in your non-dominant hand.

5. Using your dominant hand, use your ring finger and pinky to remove the metal culture tube cap (Figure 5.5).



Figure 5.5

5. Pass the top of the glass culture tube through the flame 2 or 3 times. This warms up the air inside the tube, forcing it to expand and move upward, and reduces the likelihood of an environmental organism contaminating the culture (Figure 5.6).



Figure 5.6

6. Using your sterile inoculating loop, collect bacteria from the broth or deep. You need only touch the loop to the bacteria - plenty of organisms will remain on the loop even if you cannot see them (Figure 5.7).



Figure 5.7

7. Pass the top of the glass culture tube through the flame 2 or 3 times again.
8. Replace the culture tube cap (it should still be in your dominant hand).

Part II

1. Grasp the culture tube containing the fresh media in your non-dominant hand.
2. Using your dominant hand, use your ring finger and pinky to remove the metal culture tube cap.
3. Pass the top of the glass culture tube through the flame 2 or 3 times.
4. Inoculate the new media by touching it with the loop. If this is a slant, place the loop at the bottom of the slant and drag it up over the slant surface.
5. Pass the top of the glass culture tube through the flame 2 or 3 times again.
6. Replace the culture tube cap (it should still be in your dominant hand).
7. Sterilize the inoculating loop.

Preparation of a Streak Plate

What happens when you have two or more organisms mixed together and you want to isolate one or more to grow in a pure culture? In this case, we use a “streak plate” to grow colonies originating from individual bacteria.

Streaking for isolation can be done in several ways, but we will focus on one of the most common methods, the three-sector streak method. When creating a streak plate, the goal is to dilute the bacteria in each step so that each “streak” contains fewer individual organisms than the prior one. Eventually, there are so few bacteria that as you streak, individual organisms are spread out far apart from one another. As they grow in the incubator, these individual organisms will grow into discrete colonies originating from a single organism (species).

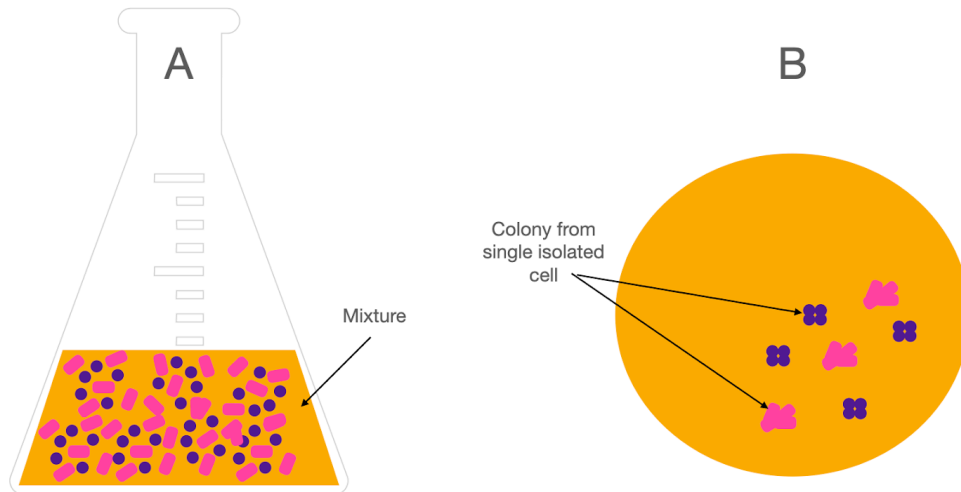


Figure 5.8: Bacteria grown in a mixed culture (A) can be isolated by streaking them out on a solid surface (B).

Steps to make a streak plate:

1. Sterilize the inoculating loop.
2. Touch the loop to the agar surface to cool it down. You may hear a “sizzle” as the hot loop is rapidly cooled by contact with the agar.
3. Inoculate the loop by dipping it into broth, or touching it to the surface of a bacterial colony. You don’t need to be able to see any globs of bacteria on the loop! There are plenty there by simply touching the surface once.
4. Create the first streaks following the pattern below (Figure 5.9)

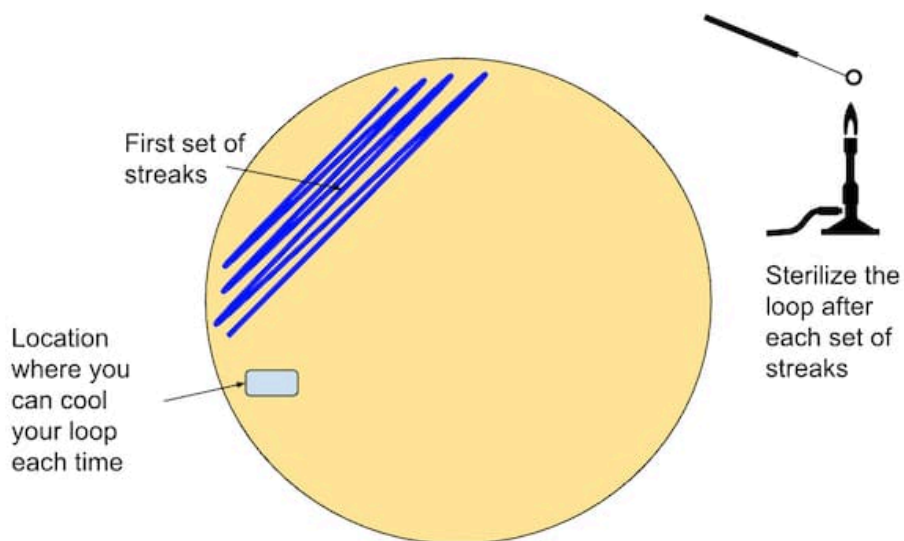


Figure 5.9

1. Sterilize the inoculating loop again.
2. Touch the loop to the agar surface (again) to cool it down.
3. Create the second streaks following the pattern below. Be sure to pass the loop through the original (1st) streaks 1-5 times in order to spread the bacteria out further (Figure 5.10)

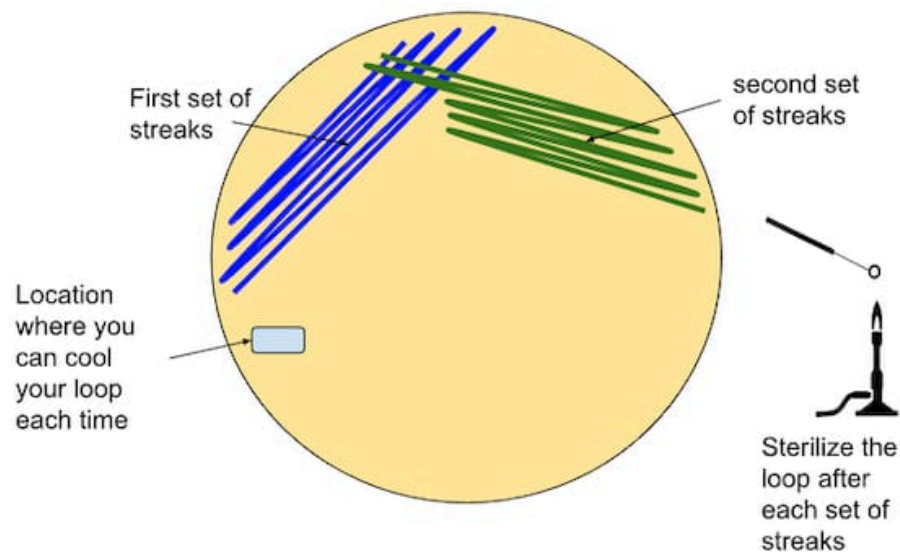


Figure 5.10

1. Sterilize the inoculating loop again.
2. Touch the loop to the agar surface (again) to cool it down.
3. Create the third streaks following the pattern below. Be sure to pass the loop through the original (2nd) streaks 1-5 times in order to spread the bacteria out further. (Figure 5.11)

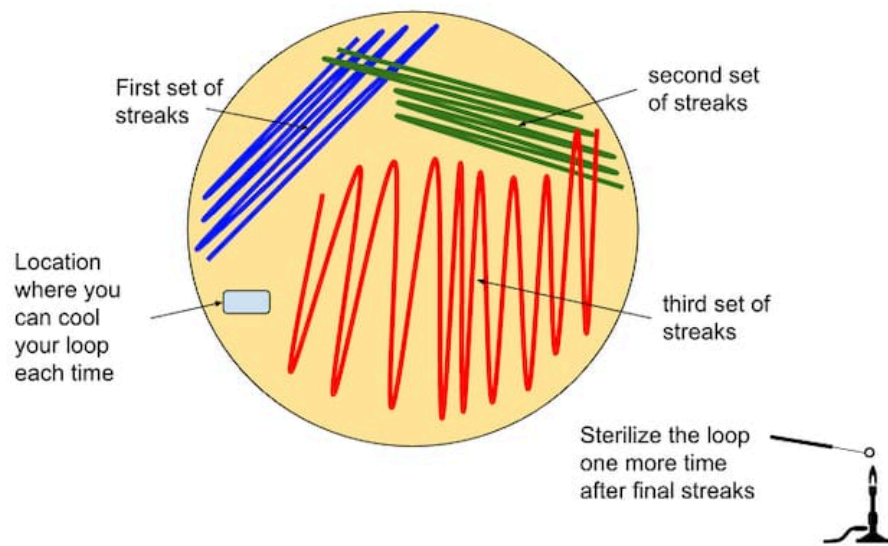


Figure 5.11

Materials

Per student:

- Two uninoculated Nutrient agar "deeps"
- Two uninoculated Nutrient agar "slants"
- Two uninoculated Nutrient "broths"
- Two Nutrient agar plates
- One TSA plate
- An inoculating loop

- An inoculating needle

Per student pair:

Cultures of:

- *Serratia marcescens*
- *Chromobacterium violaceum*
- *Bacillus subtilis*
- *Staphylococcus aureus*
- *Proteus mirabilis*

Experiment

1. Label each uninoculated tube/plate with your first and last name, date, and lab section.
2. Inoculate two nutrient agar (NA) deeps, two NA slants, and two nutrient broths (NB).
 1. Subculture *S. aureus* to a **slant** and a **deep**.
 2. Subculture *S. marcescens* to a **broth** and a **deep**.
 3. Subculture *B. subtilis* to a **slant** and a **broth**.
3. Label each new culture with the name of the organism that you used!
4. Prepare two streak plates, using *S. aureus* for one, and *S. marcescens* for the other.
5. Prepare a line inoculation on a **TSA plate** of the organism *P. mirabilis*.
6. Place your new tubes in racks, and loosely place a rubber band around each set of tubes so that they are less likely to get separated. Incubate at 35-37 degrees until the next lab period.
7. Incubate your three plates upside down (lids on the bottom) at 35-37 degrees until the next lab period.
8. Discard the old cultures. Be sure to clean the labels off of the tubes before placing them upright in the metal bins. Ask for help if you don't remember how to properly discard your tubes.

Data

No data will be collected this lab period, but we will be using these subcultures next lab period to prepare gram stains. You might find it helpful to consider a few questions.

Questions

1. Why do we incubate the new plates upside down (lid on the bottom)?
2. Why do we flame the lip of each culture tube before and after inserting an inoculating loop or needle?
3. For which type(s) of media do we use an inoculation loop vs an inoculation needle?
4. When are the appropriate times to sterilize your loop or needle?
5. What is the purpose of a streak plate?
6. Why do we cool off the inoculation loop before using it to create streaks on a streak plate?
7. Why do we flame the inoculation loop between each set of streaks on a streak plate?
8. When you prepare a streak plate, which set of streaks (1, 2, or 3) has the highest bacteria count? Which has the lowest? Why is this important in preparing a streak plate?

6: Gram Stains

Learning Outcomes

By the end of this lab period, you will be able to

1. Define “differential” stains and explain how this general concept is different from a “simple” stain.
2. Explain the purpose of both a “primary” stain and a “counterstain”.
3. Troubleshoot Gram-stains that don’t work properly.

Introduction

We use many different types of staining techniques in microbiology that allow us to visualize these tiny organisms, and also to differentiate between some of the major groups. One of the most common ways to differentiate between two major groups of bacteria is using the Gram stain. The Gram staining technique allows you to quickly categorize bacteria as having either a Gram-positive or a Gram-negative cell wall. You can review the features of both Gram-positive and Gram-negative cell walls in our OpenStax Microbiology textbook in [Chapter 3.3, Unique Characteristics of Prokaryotic Cells](#).

Differential Stains

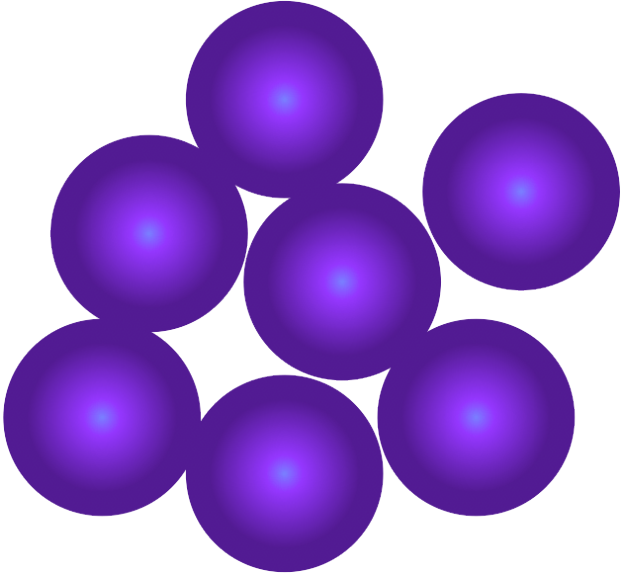
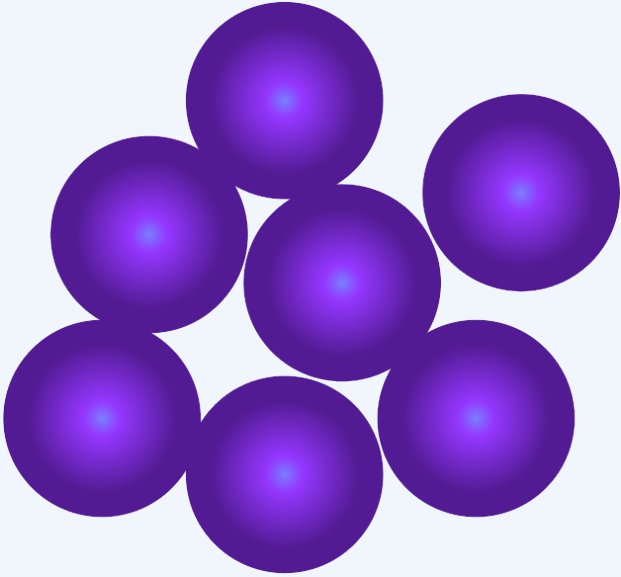
As we learned about in an earlier lab, a simple stain involves adding one dye to a bacterial smear in order to visualize them. The Gram-stain, however, requires additional steps and results in Gram-positive cells staining a deep purple, while Gram-negative cells stain a lighter red or pink color. Therefore the Gram stain is a “differential stain” because it “differentiates” between Gram-positive and Gram-negative bacteria.

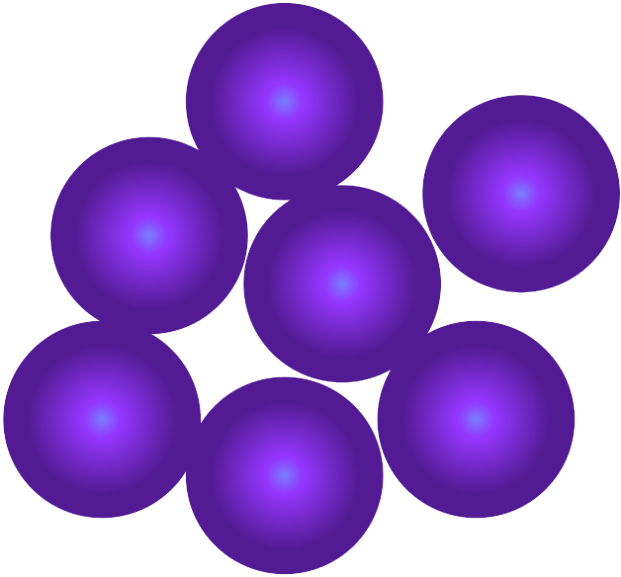
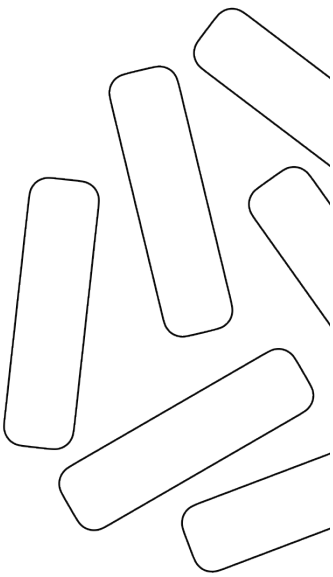
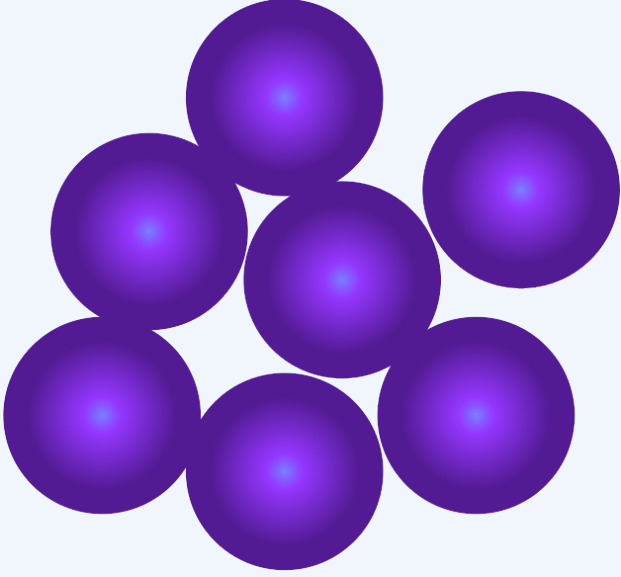
In the gram stain, two dyes are used, crystal violet and safranin. Crystal violet is first applied to the bacterial smear, staining all cells. This first step, if no other steps were completed, would be a simple stain! However, in the Gram stain, the stained smear is then treated with Gram’s iodine. The iodine crosslinks the crystal violet molecules, effectively trapping them within the thick peptidoglycan layer of a Gram-positive cell wall. However, the thinner peptidoglycan layer in a Gram-negative cell is not as effective at trapping the dye, and a “decolorizing” step with ethyl alcohol strips the outer Gram-negative cell wall membrane, effectively releasing the crystal violet from those cells. At this stage, Gram-positive cells are deep purple, but Gram-negative cells will be transparent. In order to visualize them, a “counterstain”, safranin is added. Safranin is a much less saturated dye, and it won’t change the deep purple color of a Gram-positive cell, but it will stain the Gram-negative cells so that we can see them. At the end of a Gram-stain, all the Gram-positive cells will be deep purple and the Gram-negative cells will be a light red or pink.

Gram Stain Process

Let’s review the 4 main steps in performing a Gram stain (Table 1). Note that after each step, the bacterial smear is rinsed with distilled water in order to remove excess stain or reagent.

Table 1: Gram Staining Process

Number	Process	Effect	Result for G+ cell	Result for G- cell
1.	Stain the smear with Crystal violet	All cells will be stained a deep purple.		
2	Immerse stained smear in Gram's Iodine (mordant)	Iodine crosslinks the crystal violet trapping it inside G+ cells		

Number	Process	Effect	Result for G+ cell	Result for G- cell
3	Decolorize with 95% ethyl alcohol	Alcohol strips the outer membrane from G-cells, and the crystal violet is released		
4	Counterstain with safranin	Safranin is needed to stain the now-transparent G- cells.		

Typical Gram Stain Results (Figure 6.1)

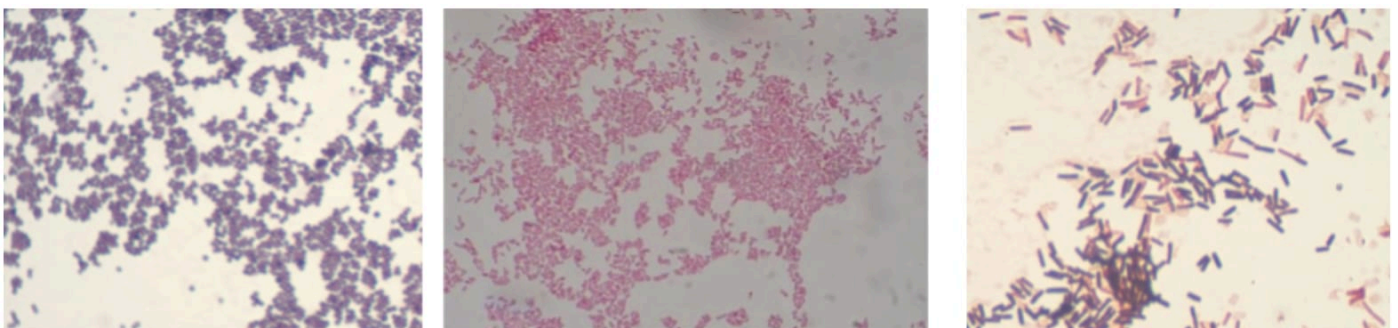


Figure 6.1: Gram Staining. Left - *S. aureus*, a Gram-positive coccus. Middle - *E. coli*, a Gram-negative rod (Dr Graham Beards, CC BY-SA 4.0, via Wikimedia Commons). Right - *B. subtilis*, a Gram-positive rod exhibiting Gram stain variability

Materials

Per Student

- Compound microscope with oil-immersion lens
- Clean glass microscope slides
- Gram-stain solutions
- Gram crystal violet
- Gram iodine
- 95% ethanol
- Gram safranin
- Squirt bottle with water
- Bibulous paper
- Disposable gloves
- Clothespins as slide holder

Per Student Group

Bacterial culture (these will be your sub-cultures from the Aseptic Technique Lab)

- *Staphylococcus aureus*
- *E. coli*
- *Bacillus subtilis*
- *Serratia marcescens*
- *Chromobacterium violaceum*
- *Proteus mirabilis*

Experiment

1. Using your subcultures from the Aseptic Technique Lab, prepare and heat-fix smears of Gram-positive and Gram-negative bacteria onto a single microscope slide.

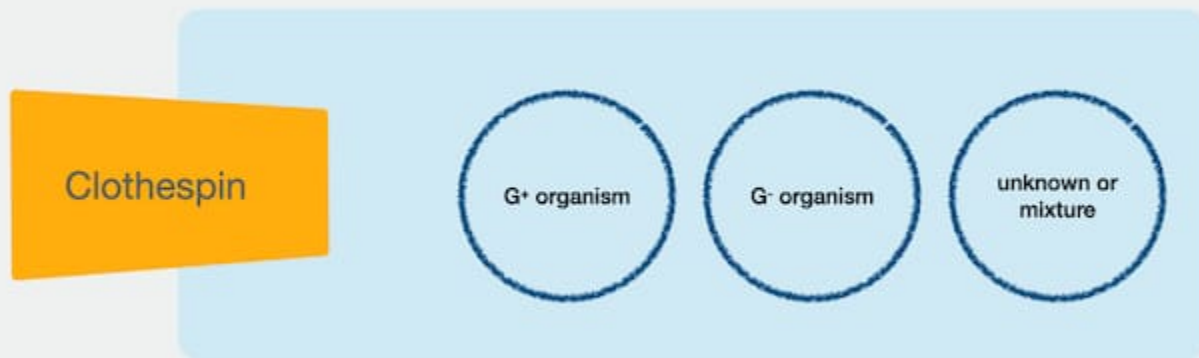


Figure 6.2: Example of how to organize specimens on the microscope slide prior to staining

2. Because Gram stains require practice, prepare several sets of slides and let them air-dry simultaneously. Then they will be ready when you need them.
3. Using clothespins as slide holders, immerse the slide in the crystal violet staining solution. Stain for one minute.
4. Grasp the slide with the clothespin and hold it at an angle. Gently rinse the slide with distilled water. Aim the water bottle above the smears, let water run down the smears, and rinse. Squirting water directly on the smears will risk losing the bacteria on the slide.
5. Immerse the slide in Gram iodine and stain for one minute.
6. Grasp the slide with the clothespin and hold it at an angle. Gently rinse the slide with distilled water.

7. Immerse the slide in 95% alcohol, and decolorize for 20 seconds. It can help to gently agitate the slide during this step. It is critical that you do not leave the slide in the alcohol for longer than 20 seconds. This can lead to over-decolorization, and the Gram-positive cells appearing to be Gram-negative.
8. Grasp the slide with the clothespin and hold it at an angle. Gently rinse the slide with distilled water.
9. Counterstain the smears by immersing in safranin for one minute.
10. Grasp the slide with the clothespin and hold it at an angle. Gently rinse the slide with distilled water.
11. Gently blot dry with bibulous paper. Do not rub. When dry, observe under oil immersion.
12. Evaluate the quality of the stain result.

Troubleshooting

It's not trivial to produce a gorgeous Gram stain, but it's very satisfying when you do! Here are a few tips to help you improve your Gram stains this lab period. Remember, if your gram-positive organism appears purple and your gram-negative organism appears pink, your technique is fine.

Table 2: Troubleshooting the gram stain process

Problem	Solution
The gram-positive organism appears uniformly pink or red	You have over-decolorized your smear. Try again, reducing the decolorization time.
The gram-negative organism appears uniformly purple	You have under-decolorized your smear. Try again, increasing the decolorization time.
A pure culture appears to have both purple and pink/red cells in the same smear	Sections of your smear was too thick. This can trap the crystal violet preventing decolorization from working properly. Prepare another smear before staining, and be sure to make it very, very thin. You want a single layer of cells across much of the smear.
Bacillus subtilis or Bacillus cereus, which are gram-positive, appears pink instead of purple	These species are highly gram-variable. This means that they only reliably retain the crystal violet when the culture is fresh, typically 24 hours old or less. Since most of our lab periods are 48 or more hours apart, we will find that in our hands, these organisms often appear mixed, and sometimes appear mostly gram-negative.

Data

Record your staining results. Sketch the cellular morphology and arrangement of each bacteria. If you prefer, you may capture images using your cell phone. If drawing, use colored pencils to illustrate your staining results, as well as the overall morphology of each organism. Include the total magnification for each sketch. Note: This should be 1000x...it's not possible to really see these organisms well without using the oil immersion lens. But it's good practice to record total magnification regardless.

7: Acid Fast and Endospore Stains

Learning Outcomes

By the end of this lab period you will be able to:

- Distinguish between acid-fast and non-acid-fast bacteria in a stained sample.
- Identify vegetative cells and endospores in a stained sample.
- Employ the use of a steam bath to assist penetration of the primary stain into resistant cells and structures.
- Describe the clinical relevance of both acid-fast and spore-forming microorganisms.

Introduction

Today we are going to learn how to perform two new differential stains. The stains themselves are unrelated to each other, but both stains require heat to drive the primary stain into the bacteria, so it makes sense for us to learn about them on the same day.

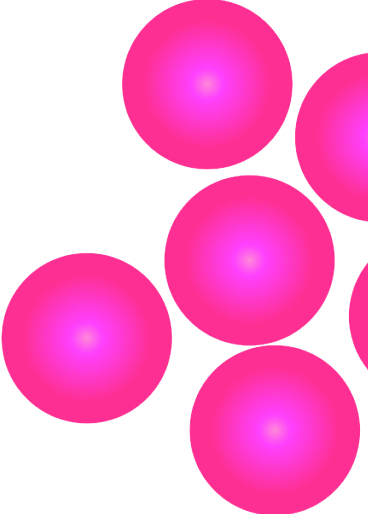
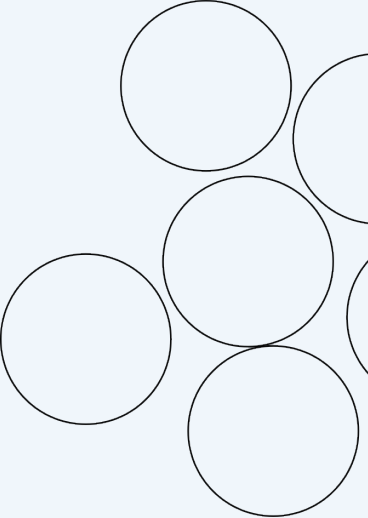
Acid-Fast Stains

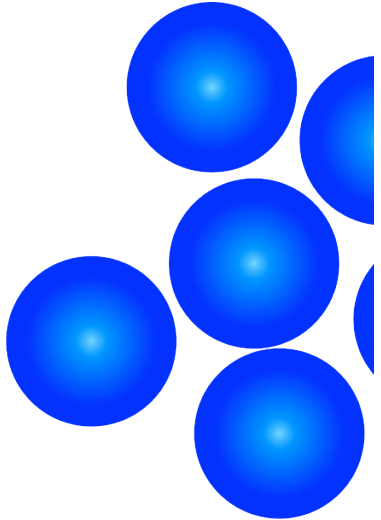
The Acid-Fast staining technique allows us to differentiate between “acid-fast” and “non-acid-fast” bacteria. There is an important clinical reason for us to learn about this technique, and that’s because it’s used to identify *Mycobacterium tuberculosis* in sputum smears. *Mycobacterium tuberculosis* is an incredibly serious and prevalent pathogen, and is the [13th leading cause of death worldwide, according to the WHO](#). A cousin of *Mycobacterium tuberculosis*, *Mycobacterium leprae*, causes leprosy. Prior to the availability of effective treatments, individuals with leprosy were banished to [leper colonies](#), a practice with a long and tragic history. Today, we will work with the non-pathogenic *Mycobacterium smegmatis*.

Mycobacteria can be difficult to stain because their outer cell wall is rich in mycolic acids which are very hydrophobic (waxy), making it difficult for stain to penetrate. Common aqueous (water-based) stains such as methylene blue or crystal violet are repelled by the hydrophobic nature of the mycolic acids. In order to stain these cells, a primary stain called carbolfuchsin is used. This dye is hydrophobic just like the mycolic acids, and it is able to penetrate the cell walls of acid-fast organisms. We also help the process along by staining acid-fast organisms in a steam bath. The higher temperature further facilitates the entry of the dye into the cells.

Non-acid fast organisms can be decolorized using an acid-alcohol decolorizer and counterstained with methylene blue.

Table 1: Acid-Fast Staining Process

Number	Process	Effect	Result for acid-fast cell	Result for non-acid-fast cell
1.	Stain the smear with carbolfuchsin for 10 minutes over a steam bath	All cells will be stained bright fuchsia color.		
2.	Decolorize with acid alcohol (3% HCl in 95% ethyl alcohol)	Acid-alcohol strips the color from non-acid-fast cells		

Number	Process	Effect	Result for acid-fast cell	Result for non-acid-fast cell
3.	Counterstain with methylene blue	Methylene blue is needed to stain the now-transparent non-acid-fast cells.		

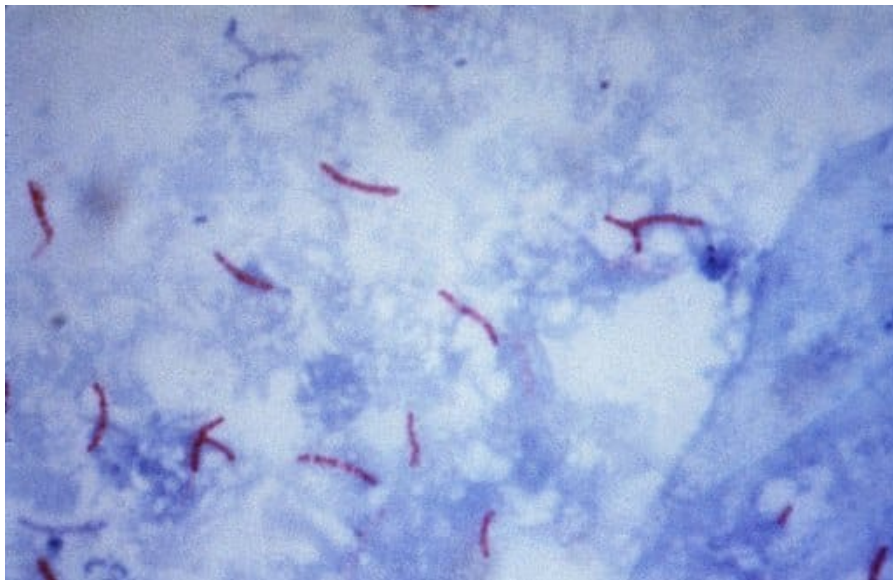


Figure 7.1: Acid Fast Stain of *Mycobacterium tuberculosis*. CDC/Dr. George P. Kubica, Public domain, via Wikimedia Commons

Endospore Stains

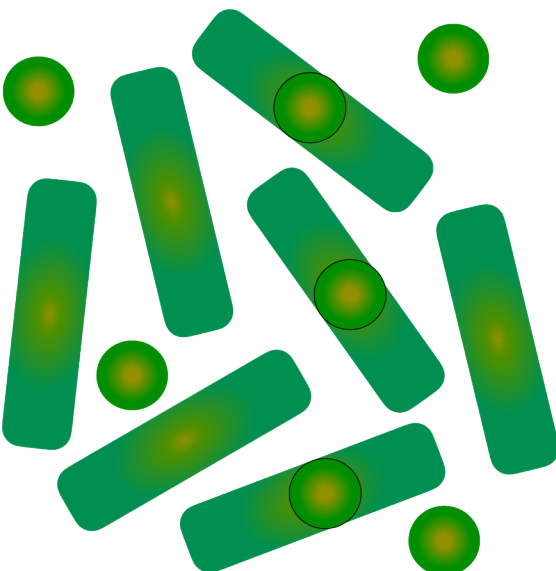
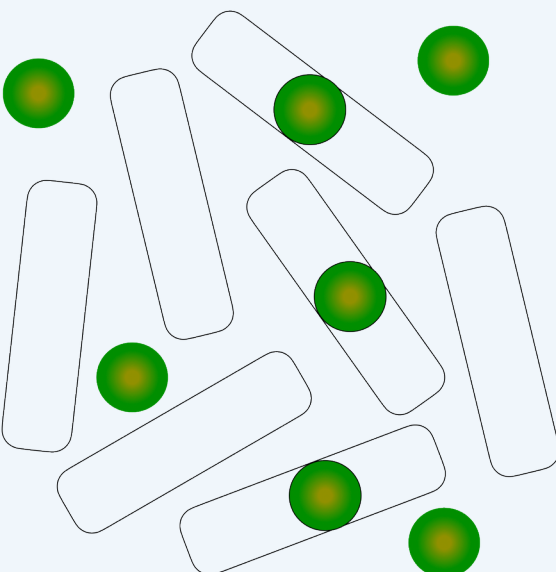
Another differential staining technique, which also requires heat to assist penetration of the primary stain, is the endospore stain. Endospores are structures made by some (not all!) Gram-positive bacteria to withstand long periods of harsh conditions. Endospores consist of bacterial DNA in a tough protective coat of protein (keratin) and allow the organism to remain dormant until conditions become favorable for bacterial metabolism and growth. You can review endospores in our OpenStax Microbiology textbook in [Chapter 3.3, Unique Characteristics of Prokaryotic Cells](#).

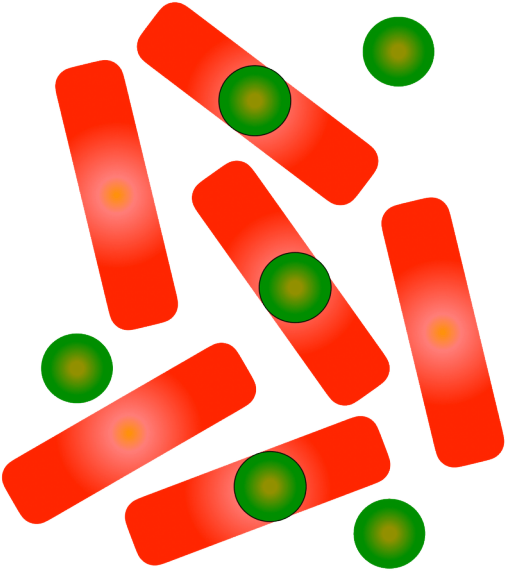
Endospores that form in the center of a cell are called central endospores and are characteristic of the *Bacillus* genus. Members of the *Clostridium* genus produce terminal endospores.

Spore-forming organisms are responsible for some very significant pathogens. *Bacillus cereus* and *Clostridium botulinum* are associated with foodborne illness. *Clostridium tetani* causes tetanus and *Clostridium perfringens* is associated with both foodborne illness as well as wound infections that can lead to gas gangrene.

Like the acid-fast cell wall, the keratin in the spore coat is very resistant to staining, so it is difficult for biological dyes to penetrate spores. Heat (in the form of a steam bath) is used to force the primary stain, malachite green, into the spore. Vegetative cells can be decolorized with distilled water and counterstained with safranin.

Table 2: Endospore Staining Process

Number	Process	Effect	Result for spores and vegetative cells
1.	Stain the smear with malachite green for 10 minutes over a steam bath	All cells and spores will be stained green	
2.	Decolorize with distilled water	Water strips the color from vegetative cells	

Number	Process	Effect	Result for spores and vegetative cells
3.	Counterstain with safranin	Safranin is needed to stain the now-transparent vegetative cells	

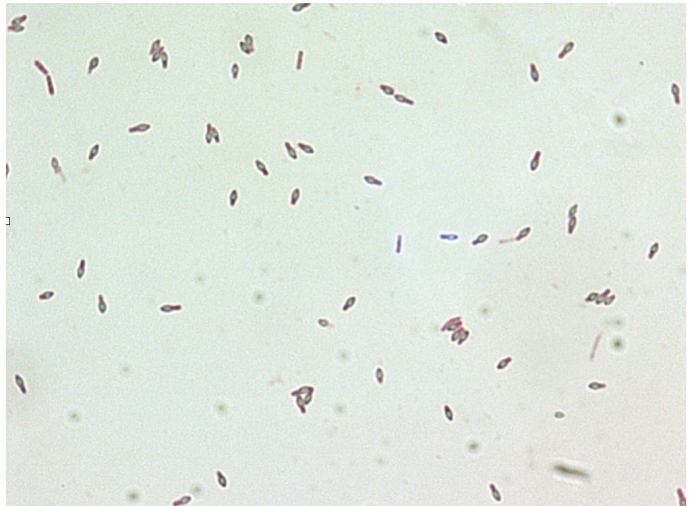
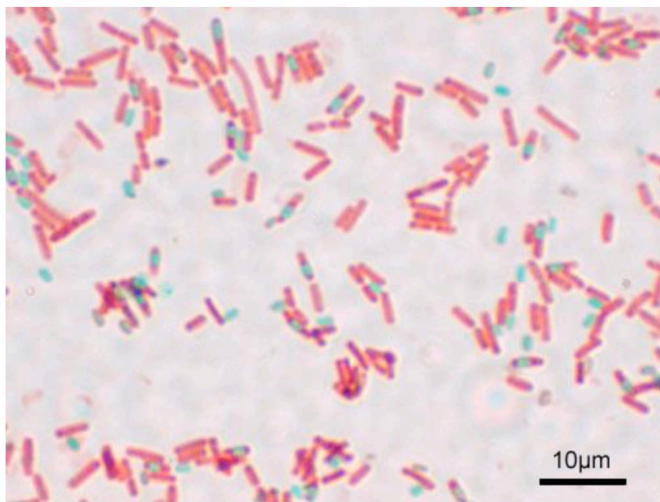


Figure 7.2: Endospore stains. Left - *B. subtilis* showing central endospores. Y tambe (original uploader), CC BY-SA 3.0, via Wikimedia Commons Right - *Clostridium botulinum* showing terminal endospores. CDC/ Courtesy of Larry Stauffer, Oregon State Public Health Laboratory, public domain.

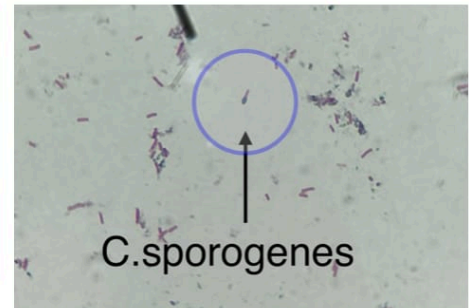
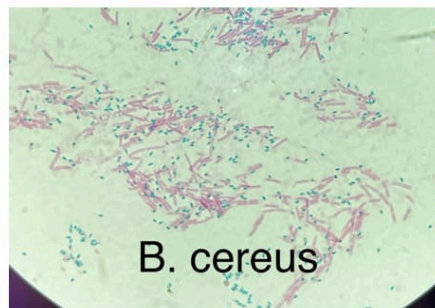
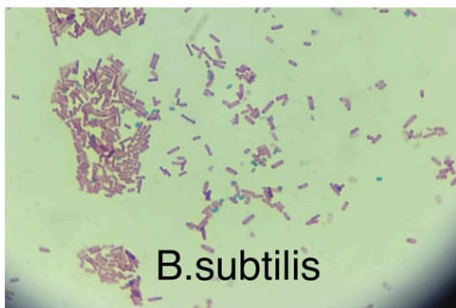


Figure 7.3: Endospore stains from the CCSF Microbiology Lab. Left - *B. subtilis* showing vegetative cells and free spores. Middle - *B. cereus* showing vegetative cells and free spores. Right- *C. sporogenes* showing terminal endospores. Photo credits: Doris Inson, used with permission

Materials

Per Student Group

- Carbol-fuchsin stain
- Methylene blue stain
- Acid alcohol (95% ethanol + 3% HCl)
- Malachite green stain
- Safranin stain
- Squirt bottle with water
- 1 L beaker to boil water
- Bibulous paper
- Clothespins as slide holders
- Disposable gloves
- Compound microscope with oil-immersion lens
- Immersion oil
- Lens paper
- Bacterial cultures

- *Mycobacterium smegmatis*
- *Staphylococcus aureus*
- *Bacillus subtilis* (> 72 hours)
- *Clostridium sporogenes* (> 72 hours)

Experiment

1. Both the acid-fast and endospore stains require the use of steam to drive the dyes into bacterial cells (Figure below TBD). Due to time constraints, each student will perform one of the two staining methods. Decide with your lab partner who is to perform the acid-fast stain, and who is to perform the endospore stain.
2. The student performing the acid-fast stain should collect *Mycobacterium smegmatis* and *Staphylococcus aureus*, and the student performing the endospore stain should collect *Bacillus subtilis* and *Clostridium sporogenes*.
3. To prepare the smear for the acid-fast stain, add a drop of water on the slide, and prepare a smear of *S. aureus*, dry and heat-fix the slide on the hot plate. Take a small amount of *M. smegmatis* and put it directly on top of the *S. aureus* smear. Because *Mycobacterium* cells tend to stick together in clumps, use the inoculation loop to chop up the *M. smegmatis* clumps, as if you were mincing garlic. The finer the mincing, the better your staining result will be.
4. To prepare the smears for the endospore stain, add two drops of water on the same slide, and make smears of *B. subtilis* and *C. sporogenes* separately. Dry and heat-fix on the hot plate.
5. Set up the heating device with the Bunsen burner, metal stand, mesh, and 1-liter beaker with ~ 500 ml water in it. Heat up the water until boiling, and reduce the heat to maintain a low rolling boil.

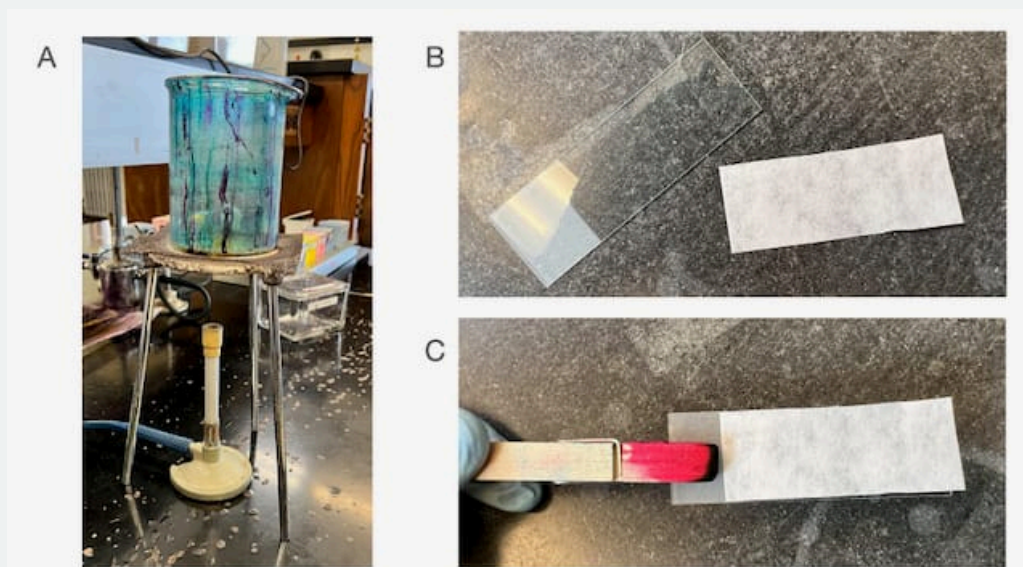


Figure 7.3: Setting up the acid-fast and endospore stains. A. Steam-bath apparatus. B. Cut bibulous paper or paper towel to fit the microscope slide. C. Place the bibulous paper over the dried/fixed smear to help keep the smear moist with stain during primary staining.

For the acid-fast stain:

6. Cover the smear with a strip of bibulous paper cut slightly smaller than the slide. Place the slide over the steaming device (beaker containing boiling water).
7. Saturate the bibulous paper with carbolfuchsin stain. Heat steam for 10 minutes. Be sure to keep the paper moist with stain. If the bibulous paper looks dry, add a few drops of the stain.
8. Remove the bibulous paper. Hold the slide on an angle over the sink and gently rinse both sides with distilled water until the runoff is clear.
9. Continue holding the slide with a slide holder. Decolorize with acid-alcohol until the run-off is clear. Use caution when handling the acid-alcohol.
10. Still holding the slide on an angle, gently rinse with distilled water into the sink.
11. Place the slide on the staining rack and counterstain with methylene blue for one minute.
12. Gently rinse the slide with distilled water into the sink.
13. Carefully blot the slide dry with fresh bibulous paper. Do not rub.

For the endospore stain:

1. Cover the smears with a strip of bibulous paper cut slightly smaller than the slide. Place the slide over the steaming device (beaker containing boiling water).
2. Saturate the bibulous paper with malachite green stain. Heat steam for 10 minutes. Be sure to keep the paper moist with stain. If the bibulous paper looks dry, add a few drops of the stain.
3. Remove the bibulous paper. Hold the slide on an angle over the sink and gently rinse both sides with distilled water until the runoff is clear.
4. Place the slide on the staining rack and counterstain with safranin for one minute.
5. Gently rinse the slide with distilled water into the sink.
6. Carefully blot the slide dry with fresh bibulous paper. Do not rub.
7. Observe using the oil-immersion lens. Record your observations of cell morphology and arrangement, acid-fast reaction, endospore presence, position, and shape.

Data

Record your staining results. Sketch the cellular morphology and arrangement of each bacteria. Use colored pencils to indicate staining results or take photographs to record what you saw. Include the total magnification for each sketch.

Questions

1. What is the role of steaming the smear during primary staining for both the endospore and acid-fast staining techniques? What might the results look like if the primary staining was done at room temperature?
2. Why does the endospore stain exercise call for an older culture of *Bacillus*? What would you expect the difference in the results to look like between a very fresh culture and an older culture?
3. Describe the clinical relevance of organisms that are acid-fast. What common pathogens are acid-fast organisms?
4. Describe the clinical relevance of some spore-forming organisms.

8: Gram Stain Unknown

Learning Outcomes

By the end of this lab period you will be able to:

- Compile data on a single microorganism from multiple sources (visual analysis of colony morphology, cell morphology, and Gram stain results).
- Use a dichotomous key to identify an unknown organism based on multiple features or characteristics.

Introduction

At this point in the semester, you have learned how to isolate microorganisms on streak plates, produce subcultures aseptically, and perform several different kinds of useful differential stains. We are almost ready to embark on our capstone Bacterial ID project!

Today, we will complete a “mini” unknown project using gram staining and morphological features of the bacterial colonies as well as their cell shape and arrangement. We will also learn how to read a dichotomous key.

Dichotomous keys allow us to identify an organism by asking questions about specific features. Does the organism have wings? Does it have feathers? Typically, a dichotomous key is a series of questions that allow you to narrow down the possible options until a single species of organism has been identified.

We are going to use the following dichotomous key to differentiate between six different species of bacteria.

1. *Proteus mirabilis*
2. *Escherichia coli*
3. *Micrococcus luteus*
4. *Pseudomonas aeruginosa*
5. *Staphylococcus aureus*
6. *Bacillus subtilis*

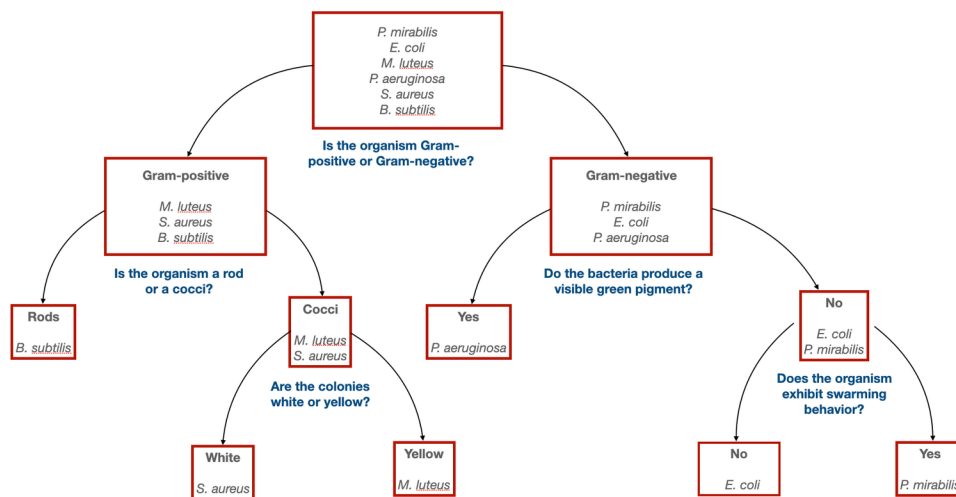


Figure 8.1: Dichotomous key for use in identifying the unknowns in today's lab.

Materials

- Inoculating Loop
- Bunsen Burner
- Slide Warmer
- Gram Stain Solutions (Crystal Violet, Gram's Iodine, Decolorizer, Safranin)
- Slides
- Microscope
- Blinded (labeled with numbers but not names!) cultures of each of the following organisms both on NA slants and plates.
 - *Proteus mirabilis* (TSA plate is used)
 - *Micrococcus luteus*
 - *Bacillus subtilis* or *Bacillus megaterium*
 - *Pseudomonas aeruginosa* (Mueller Hinton plate is used)
 - *Escherichia coli*
 - *Staphylococcus aureus*

Experiment

1. Your instructor will provide you with two unknowns on slants.
2. Prepare smears and Gram-stain each one. Your lab partner will gram-stain the same two organisms independently so that you can compare your results.
3. After looking at your stained bacteria under the 100x objective on your microscope, complete the chart below for your organisms.
4. Look at the growth on the plates on the instructor's desk to see more colony morphology and growth characteristics.
5. You will fill out the rest of the chart in class as we discuss everyone's results.

Data

Table 1: Student Data Table

Unknown Number	Gram Stain Results	Cell Shape	Color/Growth on Media	Swarming Growth?	Organism Identification
1					
2					
3					
4					
5					
6					

9: Introduction to the Bacterial ID Project

Learning Outcomes

By the end of this laboratory (3 lab periods) you will be able to

- Explain the difference between selective and differential media
- Describe the differences between TSA, PEA, and MAC media
- Verify the existence of a pure culture by Gram staining.

Introduction

Today we will begin the capstone project of the microbiology laboratory - the Bacterial ID project. Today you will be given a single tube containing a mixture of one Gram-positive and one Gram-negative organism. Over the next six weeks, you will first isolate the two organisms, generating two pure cultures, and then perform extensive biochemical tests to determine which two organisms you have been given.

The identification of microorganisms in clinical samples used to be based solely on the kinds of testing we will be performing. However, with the advent of rapid genomic sequencing, clinical laboratories can often both identify organisms and determine their sensitivity to common antibiotics in a single step. Regardless, there is a lot to be learned about the biology of microorganisms in these biochemical tests and it remains a worthwhile exercise for us. Most microbiology students find this project to be a fun challenge!

Table 1: Possible Unknown Organisms

Possible Unknown Organisms	
Gram-positives	Gram-negatives
<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
<i>Staphylococcus epidermidis</i>	<i>Proteus mirabilis</i>
<i>Bacillus cereus</i>	<i>Providencia stuartii</i>
<i>Corynebacterium xerosis</i>	<i>Salmonella enterica</i>
<i>Bacillus circulans</i>	<i>Proteus vulgaris</i>
<i>Lactococcus lactis</i>	<i>Shigella sonnei</i>

Possible Unknown Organisms

<i>Micrococcus luteus</i>	<i>Klebsiella pneumoniae</i>
<i>Enterococcus faecalis</i>	<i>Klebsiella aerogenes</i> (formerly <i>Enterobacter aerogenes</i>)
<i>Staphylococcus aureus</i>	<i>Citrobacter freundii</i>
	<i>Morganella morganii</i>
	<i>Alcaligenes faecalis</i>

Overview of the isolation process

Your goal for the first 3 lab periods is to successfully isolate your gram-negative organism from your gram-positive organism. Hopefully, you've gained confidence over the past few weeks in performing high-quality streak plates! This skill will be extremely valuable during isolation. We will also employ the use of two types of "selective media" to stack the odds in favor of successful isolations. In today's lab, you will be streaking out your unknown mixture on three types of media:

1. TSA (Trypticase Soy Agar)
2. PEA (Phenylethyl alcohol agar)
3. MAC (MacConkey agar)

You will also prepare a gram stain from your mixed-culture broth to get a first peek at your two organisms!

In the second lab period, you will be evaluating the growth on TSA, PEA, and MAC, and Gram-staining individual colonies on those plates. If you have successfully isolated one or both of your organisms, you will make a slant(s) of the pure culture(s) (Figure 9.1).

There will be a third and final lab period to complete the isolation of both unknowns and confirm that you have one Gram-positive and one Gram-negative by Gram-staining in order to move on to biochemical testing.

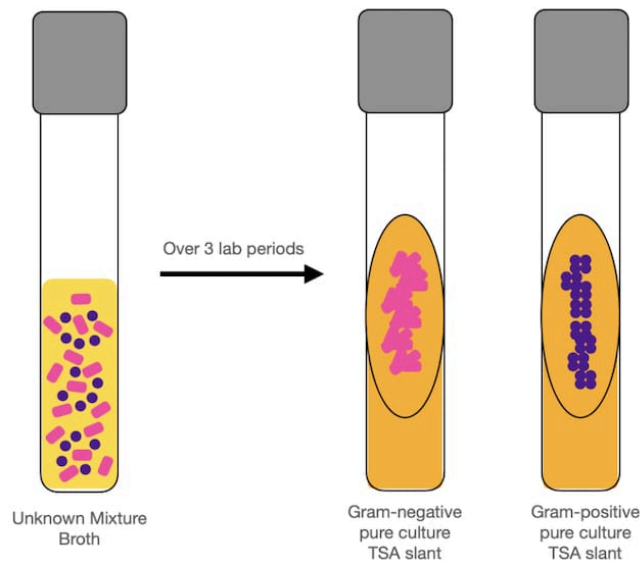


Figure 9.1: In the course of this lab we will isolate your Gram-negative and Gram-positive organisms and subculture them on TSA slants.

TSA (Trypticase Soy Agar)

TSA is a general-purpose media that is non-selective. Both of your organisms (Gram-positive and Gram-negative) will grow well on TSA. We use TSA instead of Nutrient Agar (NA) because TSA is a slightly richer medium and our more fastidious unknown organisms grow more reliably on TSA than NA.

PEA (Phenylethyl alcohol agar)

Phenylethyl alcohol agar is a type of “selective” media. When media is selective, it allows some organisms to grow, while others are prevented. In the case of PEA, the alcohol is disruptive to the outer cell membrane of Gram-negative cells and they have a very difficult time growing under the conditions that are present in PEA. Thus, PEA selects *for* Gram-positive organisms and *against* Gram-negative bacteria.

PEA’s selection is not perfect. Some of our Gram-negative bacteria do grow a little bit on PEA, so it’s extremely important to perform your streak plate correctly in order to grow individual colonies that have originated from a single organism. You will want to confirm that these colonies are truly Gram-positive! Ideally, you will use only part of the colony to create your smear, and once you’ve confirmed that it’s correct, you can use the remainder to make your slant.

MAC (MacConkey agar)

MacConkey agar (MAC) is also selective, but in this case, it selects *for* Gram-negative bacteria and *against* Gram-positive organisms. In the case of MAC, bile salts and crystal violet dye prevent the growth of Gram-positives. MAC reliably selects against Gram-positive bacteria, but you will still want to confirm that these colonies are truly Gram-negative! Again, you will use only part of the colony to create your smear, and once you’ve confirmed that it’s correct, you can use the remainder to make your slant.

MAC is also “differential”. It differentiates between organisms that ferment lactose and those that do not ferment lactose. As we learned in the lecture, acids are commonly produced during fermentation, and these acids will lower the pH of the surrounding medium. MAC also contains a dye called “neutral red” which is a pH indicator. As the pH drops below pH 6.8, the dye turns a pink or red color resulting in distinctive pink or purple colored media. Organisms that do not ferment lactose leave the media looking brown (Figure 9.2).



Figure 9.2: MacConkey media inoculated with a lactose fermentor (left) and a non-fermentor (right). [Medimicro](#), Public domain, via Wikimedia Commons

Materials

- Mixed bacteria culture containing one Gram-positive and one Gram-negative bacteria
- Inoculating loop
- Bunsen burner
- Gloves
- 1 x TSA Plate
- 1 x PEA Plate
- 1 x MAC Plate
- Gram Staining Materials
- Microscope with oil immersion lens
- TSA slants (at least 2 per student)

Experiment

Day One:

1. Obtain and label plates (TSA, PEA, and MAC). TSA and PEA look alike, so be sure to label them with a Sharpie as you collect them (Figure 9.3).
2. Obtain your unknown mixed culture (containing one Gram-positive and one Gram-negative bacteria) from your instructor.
3. Gently resuspend the mixture to evenly distribute the organisms.
4. Gram stain the mixed culture to see the initial morphology of bacteria when together. Be sure to carefully mix the broth to resuspend any bacteria that have settled to the bottom of the tube. Using a sterile Pasteur pipette, put three drops onto your microscope slide and dry on the hot plate to prepare your smear from the broth. Draw or photograph your initial Gram-stain to include in your final report.
5. Using aseptic streak plate technique, streak the mixed culture onto each of the three plates.
6. Incubate the plates at 37 degrees C for 48 hours, or at 30 degrees C for 5 days.



Figure 9.3: Uninoculated MAC, PEA, and TSA media. Note that you cannot tell TSA and PEA apart by looking at it! Be sure to bring your sharpie with you and label each plate as you collect it.

Day Two:

1. Remove plates from the incubator. Observe and record the colony morphology. What color are the colonies? How big are they? Does either of your organisms exhibit swarming characteristics? Can you tell your two organisms apart by looking at their growth (often you can)?
2. Photograph EACH plate carefully - these pictures are a required element of your final ID project!
3. Using a sharpie, make a circle around colonies that are likely to be your Gram-positive and your Gram-negative organism. If the colonies are large enough, use only half (or less) of the colony to make bacterial smears for staining.
4. Gram-stain your smears, and confirm that you have colonies that contain only your Gram-positive bacteria and only your Gram-negative bacteria with no contaminating organisms. In addition to a uniform Gram-stain, observe the cell size and shape. Are you confident that the colony you selected contains only one organism?
5. Draw or photograph your Gram-stains. These pictures are a required element of your final ID project!
6. Once you have confirmed that you have isolated colonies of your Gram-positive bacteria and your Gram-negative bacteria, use the SAME colonies that you Gram-stained to create two slants - one that contains your Gram-negative bacteria and one with your Gram-positive bacteria.
7. Incubate the slants at 37 degrees C for 48 hours, or at 30 degrees C for 5 days.
8. If cultures are not pure, re-streak a colony onto the appropriate isolation plate - you have one additional lab period to successfully isolate any organisms that have eluded you so far. Incubate any new plates at 37 degrees C for 48 hours, or at 30 degrees C for 5 days.
9. Save all of your plates - your instructor will provide the class with a bin to store them in for the duration of the project. You may need them again if at some point your slants become contaminated.

Day Three

You are not finished with your isolations until you have confirmed that one of your slants contains your purified Gram-negative organism and the other contains your purified Gram-positive organism. Be sure to Gram-stain both slants before proceeding to the next lab. Note: It is very easy to accidentally contaminate your slants by using poor aseptic technique when making Gram stains or subculturing. Be fastidious with your technique EVERY time you stick a loop into the slant for any reason!

Data

By the end of this lab, you should have

1. Your Gram-positive and Gram-negative organisms on separate slants.
2. Photographs or drawings of your initial Gram-stain of the mixture
3. Photographs of all three of your streak plates (TSA, PEA, and MAC)
4. Photographs or drawings of your Gram-stains of your isolated colonies

5. Photographs or drawings of the Gram-stains of your two slants.

All of this data will be part of the final report you submit for the Bacterial ID project.

Creating a Test Table

This is a great time to begin working on your final report! You do this by creating a “test table” where you can begin collecting and interpreting all of your data. You will be turning in this table twice before submitting your final project. Your instructor may have a [template](#) they have shared with you on Canvas that you can use to build your table and report.

For now, you can record all of your results so far. This table includes some examples to get you started, but you will want to put everything in your own words and add both pictures and written descriptions to the table.

Your instructor may ask you to also include upcoming tests in your test table. Be sure to refer to the specific assignment instructions and rubric found in the course Canvas shell.

Bacterial ID Project Test Table - Example

Table 2: Example Data Table for Unknown Project Report

Name of TEST	Name of media, description - how it works	Gram - Test Result, description, and pictures	Gram + Test Result, description, and pictures
	- What does a + result look like? - What does a - result look like?		
Growth on TSA	An all-purpose medium that is non-selective. Both Gram-positive and Gram-negative organisms should grow.	<i>Put the picture of your TSA plate here!</i> <i>Include a written description!</i>	<i>Put the picture of your TSA plate here!</i> <i>Include a written description - morphology, color, etc!</i>
Growth on PEA	<i>fill in with your description!</i>	No growth.	<i>Put the picture of your PEA plate here!</i> <i>Include a written description - morphology, color, etc!</i>

Name of TEST	Name of media, description - how it works	Gram - Test Result, description, and pictures	Gram + Test Result, description, and pictures
	• What does a + result look like? • What does a - result look like?		
Growth on MacConkey Agar	Bile salts and crystal violet inhibit the growth of gram-positive organisms, organisms that ferment lactose reduce the pH and turn the neutral red dye pink. An organism that is both gram - and ferments lactose grows and turns pink. An organism that is both Gram- and does not ferment lactose grows and does not change color. An organism that is gram-positive does not grow.	<i>Put the picture of your MAC plate here! Include a written description - morphology, color, etc!</i>	No growth
Gram Stains	<i>describe how these work in your own words and what you expect to see from a G+ and G- organism</i>	<i>Gram stain picture here! Describe the cell morphology too!</i>	<i>Gram stain picture here! Describe the cell morphology too!</i>

10: Carbohydrate Metabolism of Unknowns

Learning Outcomes

By the end of this lab (2 lab periods) you will be able to:

- Describe the pattern of carbohydrate metabolism for each of your organisms for the tests that we completed.
- Articulate how pH indicators such as phenol red, Bromothymol blue, methyl red, etc. are used to track carbohydrate utilization.
- Explain how differences in the expression of specific enzymes (such as amylase, citrate permease, catalase, etc.) can be used to differentiate between bacteria species.

Introduction

Now that you have isolated your two unknowns, the next step is to work on figuring out which two organisms you were assigned! The first step will be to perform a series of tests that are based on the utilization of carbohydrates. While all bacteria can use glucose as a source of energy, not all bacteria can ferment glucose or other simple carbohydrates. Those that can ferment carbohydrates often produce different fermentation products that can be used to distinguish between organisms.

The tests that we will be setting up today are:

1. Phenol red broths containing
 1. Glucose
 2. Lactose
 3. Sucrose
 4. Mannitol
2. Methyl Red Test and Voges-Proskauer (MR-VP) tests
3. Citrate utilization
4. Starch hydrolysis
5. Catalase

Phenol Red Broths

As we've learned in the lecture, many bacteria can undergo fermentation. In fermentation, glucose is first broken down into pyruvate. To regenerate NAD⁺ for repeated rounds of glycolysis, these bacteria undergo fermentation producing products such as lactic acid and/or ethanol and CO₂.

When bacteria are “fed” sugars other than glucose, the bacteria need to be able to change them into either glucose or into other intermediates that are part of the glycolytic pathway. For a molecule like sucrose, which is a disaccharide composed of glucose and fructose, the enzyme invertase, found in some bacteria, can break the bond between glucose and fructose. The glucose enters glycolysis directly, and the fructose is phosphorylated and enters a few steps down the pathway. This means that the bacteria only need a few extra enzymes in order to use sucrose for food - they don't need entirely new pathways. However, not all bacteria have all of these “extra” enzymes, so determining which of these carbohydrates can be fermented by your unknown bacteria can be very helpful in determining which one you've got.

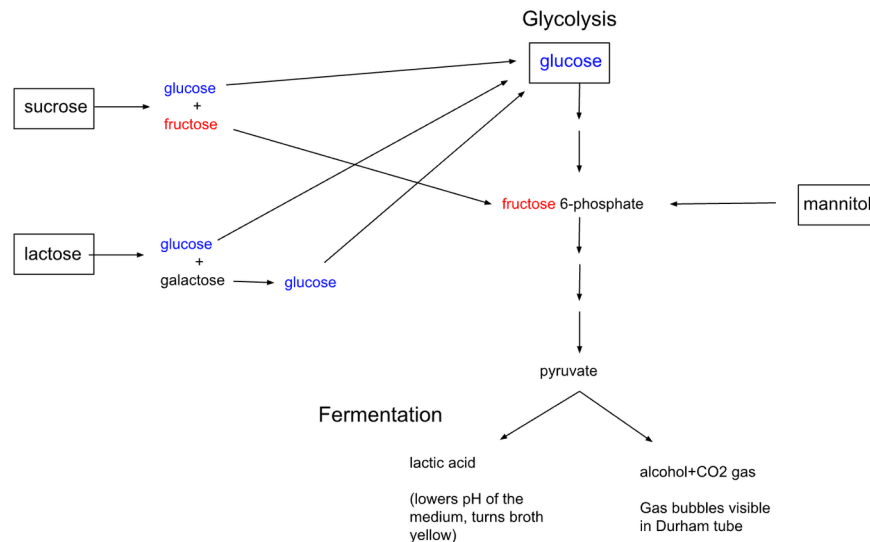


Figure 10.1: Glucose, mannitol, sucrose and lactose can be fermented using glycolysis and fermentation pathways with just one or two additional steps (enzymes).

Each phenol red broth contains ONE of the four sugars (glucose, lactose, sucrose or mannitol) soluble protein and phenol red indicator. Phenol red is a dye that is red between pH 6.8 and 7.4 and yellow below pH 6.8. The indicator will turn pink as the pH increases above 7.4. If acid is produced by fermentation of the sugar, the broths will turn yellow after 24-48 hours of incubation.

Phenol red broths also contain a small upside down test-tube called a Durham tube. The purpose of the Durham tube is to capture any gas that might be produced by fermentation. Some of our organisms that can ferment sugars produce only acid, but some can produce acid AND gas. You will want to look at the Durham tube carefully after incubation to determine if gas has been produced.

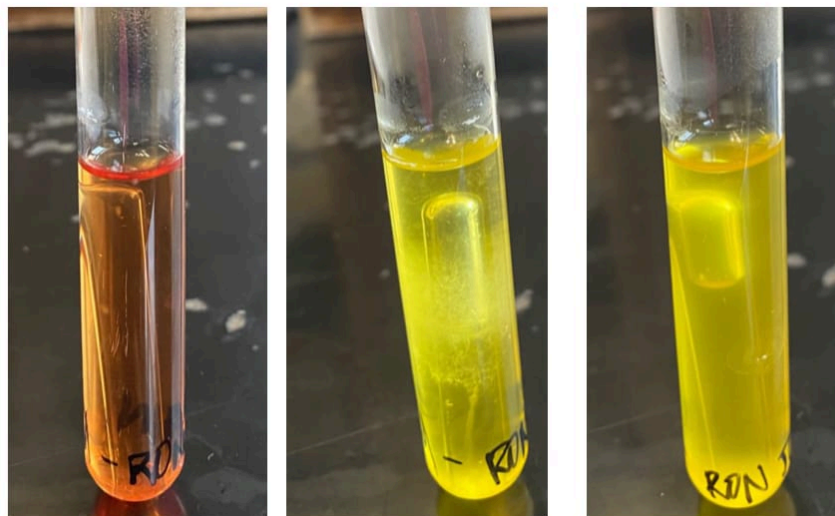


Figure 10.2: Phenol Red Glucose Fermentation Broth Results. Left - no acid produced. Middle - fermentation occurred producing acid. Right - fermentation occurred producing acid and gas. Photo credits - Ron John Vargas, used with permission.

MR-VP

The Methyl red (MR) and Voges Proskauer (VP) tests are done by first inoculating one tube of MR-VP media and incubating for 24-48 hours. The resulting culture is then split into 2 tubes and one is tested with methyl red, the other with VP reagents. So we tend to refer to them as “MR-VP”, but in fact, there are two tests here!

Methyl Red (MR)

Not all bacteria produce lactic acid and ethanol/CO₂ as byproducts of fermentation. Some bacteria are also heterofermentative, meaning they can produce more than one fermentation product! The methyl-red test allows us to identify organisms that produce a variety of acids also called “mixed-acid” fermentation. In this case, pyruvate is also converted to acetic acid, formic acid, and succinic acid. These acids are capable of lowering the pH below 4.4, where the methyl red indicator will turn red. If the acids are not produced, the pH will remain above 4.4 and there will not be a color change.

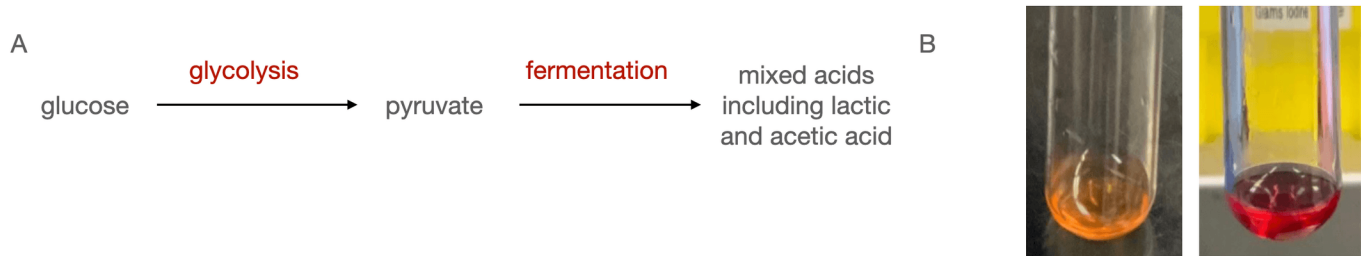


Figure 10.3:A. The pathway that produces mixed acids. B. A methyl-red positive result is shown on the right. A MR negative result is shown on the left. Photo credits - I.H., used with permission

Voges-Proskauer (VP)

Some organisms produce a different molecule called acetoin as an intermediate during 2,3 butanediol fermentation. The VP reagents (VP Reagent 1 and VP Reagent 2) react with the acetoin and other media components to produce a red color.

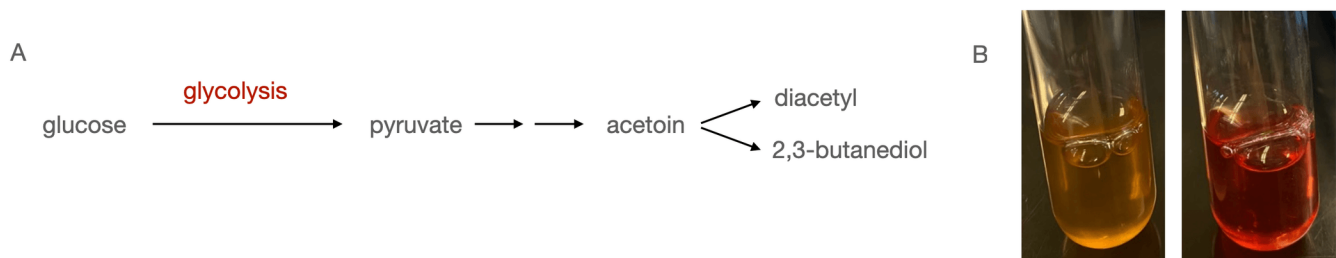


Figure 10.4:A. Acetoin is an intermediate in the breakdown of glucose to 2,3-butanediol. B. A VP positive result is shown on the right. A VP negative result is shown on the left. Photo credits - Maithy Nguyen, used with permission

Citrate Utilization

You probably recall that citrate is an intermediate in the Krebs Cycle. It is formed when oxaloacetate reacts with Acetyl CoA. It turns out that bacteria can and will use citrate as a source of food if glucose is not available, but only IF the citrate can be brought into the cell. This is accomplished when the organism can make an enzyme called citrate permease.

Simmons citrate agar contains citrate as the only source of carbon (food) and ammonium phosphate as the only source of nitrogen. For an organism to survive on this medium, it must possess the citrate permease enzyme. When citrate is used, basic byproducts including sodium carbonate are produced, increasing the pH. The organisms also convert the ammonium phosphate in the medium to ammonia and ammonium hydroxide, both of which also increase the pH of the agar. To detect this increase in pH, a dye called Bromothymol blue is also added to the medium. At a pH of 7.6, the medium changes color from green to a brilliant, sapphire blue.

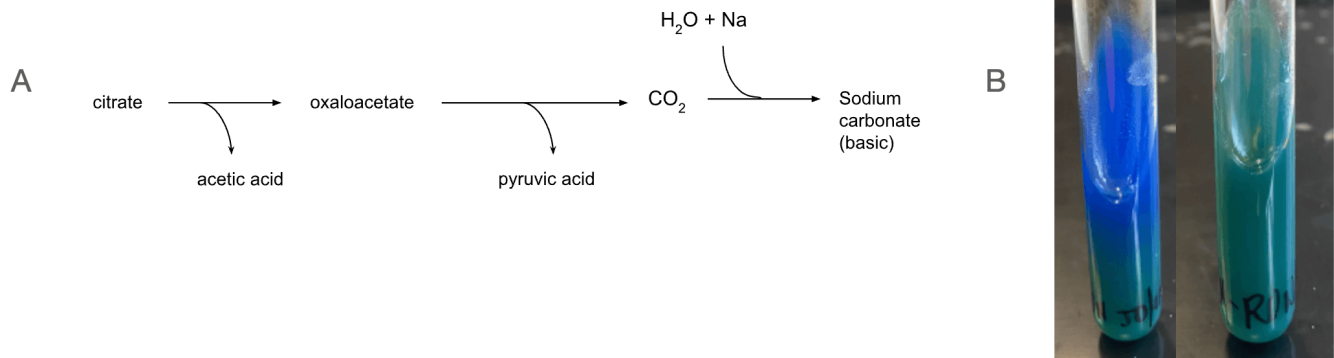


Figure 10.5: A. When citrate is used as a source of carbon, basic products such as sodium carbonate are produced, raising the pH. B. A citrate-positive result is shown on the left. A citrate-negative result is shown on the right. Photo credits - Ron John Vargas, used with permission.

Starch Hydrolysis Test

Think back to early in the semester when we discussed [monomers and polymers](#). We learned that starch is a very large polymeric molecule composed of thousands of tiny glucose molecules linked together by alpha 1,4- and alpha 1,6-glycosidic bonds. Recall that starch is enormous and far too big to cross into a bacterial cell. However, some bacteria secrete enzymes into the environment which can break starch down into glucose molecules. These molecules can easily pass into the cell.

To determine if an organism produces these enzymes, we grow organisms on a starch plate. Starch molecules can be detected by adding Lugol's iodine to the plate surface. The iodine will react with starch and turn it a dark blue/black. If bacteria secrete the enzymes that digest starch, then all of the starch around the bacterial growth will be digested and there will be a clear "zone" around the growth. This zone is easy to see by holding the plate up to the light or observing the plate over a lightbox.

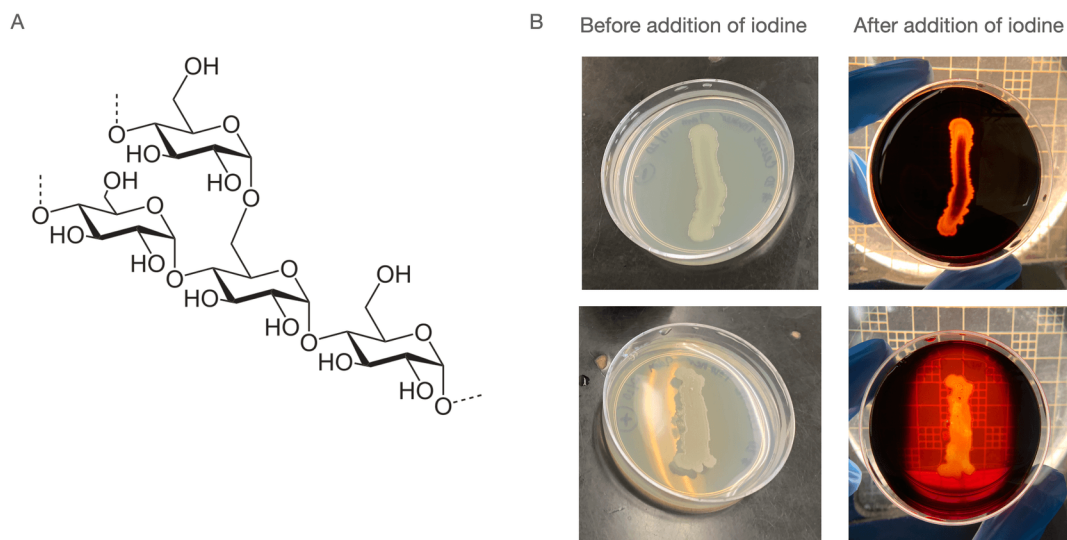


Figure 10.6: A - A small section of a starch molecule showing alpha 1,4 and alpha 1,6 glycosidic linkages. Citation: Starch. (2023, June 4). In *Wikipedia*. [Starch molecule](#) [en.Wikipedia.org] B. Starch hydrolysis detection on starch plates. The organism on the top does not hydrolyze starch. The organism on the bottom does hydrolyze starch as evidenced by the large zone of clearing around the organism. Photo credits - Celeste Towner, used with permission.

Catalase Test

You will recall that the enzyme catalase is found in obligate aerobes and facultative anaerobes. It converts hydrogen peroxide, which is an oxidizing agent, into water and oxygen gas. Hydrogen peroxide is often formed as a byproduct of aerobic respiration, and it is quite toxic to cells if it is not broken down.

Because oxygen gas is produced when catalase breaks down hydrogen peroxide, it is very easy to detect organisms that produce catalase by simply using a 3% hydrogen peroxide solution that can be purchased at the grocery or drug store.



Figure 10.7: A positive catalase test showing characteristic bubbling as oxygen gas is produced. [No machine-readable author provided. Nase assumed \(based on copyright claims\), CC BY-SA 3.0](#), via Wikimedia Commons

Materials

1. Two phenol red broths for each of the following carbohydrates:
 1. Glucose
 2. Sucrose
 3. Lactose
 4. Mannitol
2. Two MR-VP broths
3. Two Simmon's Citrate slants
4. One starch plate
5. One or two microscope slides
6. Two fresh TSA slants for subculturing your unknowns

Experiment

Day 1 - Inoculations

1. Using your isolated cultures, inoculate each type of media with your gram-positive and your gram-negative organisms (each one gets its own tube!). Use an aseptic technique and be very careful not to contaminate your isolated cultures.
 1. For the phenol red broths, MR-VP broth, and citrate slants use an inoculating loop to inoculate each type of media with your organism. You will do two of each - one for your G+ and one for your G-.
 2. For the starch test, perform a line inoculum with your gram-positive down the very center of the plate. Do not cover the entire plate with your inoculation!
 3. Place all freshly inoculated media in a 35-37 oC incubator, and incubate for 48 hours.
2. Each week during the ID project you should be making fresh subcultures of your organisms and verifying purity by gram staining. Depending on how old your TSA slants are this can be done on Day 1 OR Day 2.

3. If the purity of your newest slants has not been confirmed by Gram-staining, be sure to do that today.

Day 2 - Data Collection

1. Remove all of your cultures from the incubator. Carefully inspect each phenol red broth and citrate slant and note whether the result was positive, negative, or indeterminate.
 1. Sometimes a result is weakly positive - make a note of this!
 2. Use the photos in the introduction to this lab as a reference for what a positive and a negative result should look like.
 3. It can often help to compare your results to an uninoculated control. Your instructor will be happy to get some of those for you to do a comparison.
2. For the MR VP test, place 1 mL of broth into two separate tubes. One tube will be used for the Methyl Red test. The other tube will be used for the Voges-Proskauer test. The tubes do not need to be sterile, and you do not need to use aseptic technique, as we will be disposing of these cultures and the test tubes at the end of this lab.

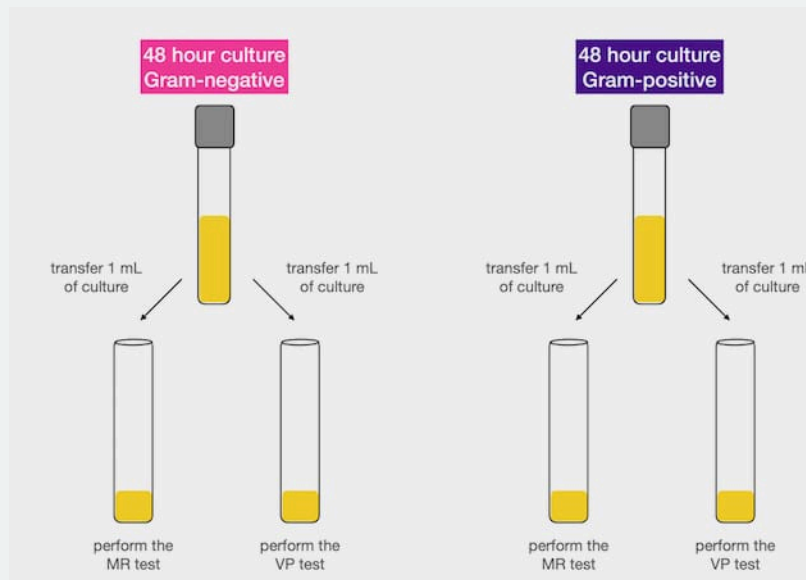


Figure 10.8: Schematic for performing the MR-VP tests.

3. For the Methyl Red test, add three drops of methyl red to the tube and read the result immediately.
4. For the VP test, add 15 drops of reagent A - mix well, but be sure not to splash any. Then add 5 drops of reagent B. Carefully mix well again to oxygenate the medium. Place the VP tubes in a test tube rack and observe for the development of red color at regular intervals starting at 10 minutes for up to 1 hour. Mix again periodically to oxygenate the medium.
5. For the starch test, add several drops of Lugol's iodine solution to the surface of the plate. You need to add enough to saturate the media, but not so much that the culture becomes loosened from the plate surface. Observe if there is clearing around the culture using a lightbox.
6. For the catalase test, place a small amount of growth from your culture onto a clean microscope slide. Add a few drops of H_2O_2 onto the smear. If you want to watch for bubble formation under the microscope you can add a coverslip. A positive result is the rapid production of O_2 gas as evidenced by bubbling. A weak positive will have minor small bubble formation with cell dispersal into the liquid. A negative result is no bubbles or only a few scattered bubbles.
7. Take a photograph and/or draw a picture of EVERY result. Be sure to keep track of which photo is for which media and organism.
 1. It might help to photograph the tubes with a written label that is visible in the photo.
 2. Sometimes student take their photos against a white background such as a clean sheet of paper or the whiteboard.
8. Each week during the ID project you should be making fresh subcultures of your organisms and verifying purity by gram staining. Depending on how old your TSA slants are this can be done on Day 1 OR Day 2.
9. Remember that if your cultures (such as TSA slants) will be incubated for MORE than 48 hours, they need to go in a $30^\circ C$ incubator to prevent cells from entering the "death phase" due to overgrowth.

Data

Questions

Your primary goal as you leave lab today is to have a complete and well-documented record of the results you collected today. Here are a few questions that might help you as you begin to identify your organisms.

1. At this point, we have done two different tests for lactose fermentation. What were they? Did you get the same results for both tests? If not, how will this impact how you use this test result in identifying your bacteria?
2. Name one test where you obtained a clear, unambiguous positive result.
3. Name one test where you obtained a clear, unambiguous negative result.
4. Name one test where your test was inconclusive. Would it make sense to use this test to try to identify which organism you have?
5. Once you have the results of your carbohydrate tests, you may want to consider (or be assigned) the task of beginning to “key out” your organism by creating a dichotomous key.

Creating a Dichotomous Key

Dichotomous keys are used to identify plants, animals, and bacteria based on their unique characteristics. The key leads the user to the correct identification by going through a series of choices. It's a little like playing 20 questions! Each choice either INCLUDES or EXCLUDES the organism as a possible candidate. A dichotomous key always presents you with two choices.

By completing the many tests we've done so far, we've answered a lot of questions about our organisms. For example, we've asked what shape(s) your organisms are (by gram staining and looking at morphology). We've asked if they express citrate permease, and whether or not they ferment a variety of different sugars. This is a good time to use a few of these questions to begin to narrow down which of the unknown organisms you were assigned.

Table 1: Example Test Result Data

Test	Test results for my organism!
Does this organism ferment lactose?	yes
Does this organism reduce nitrate?	no

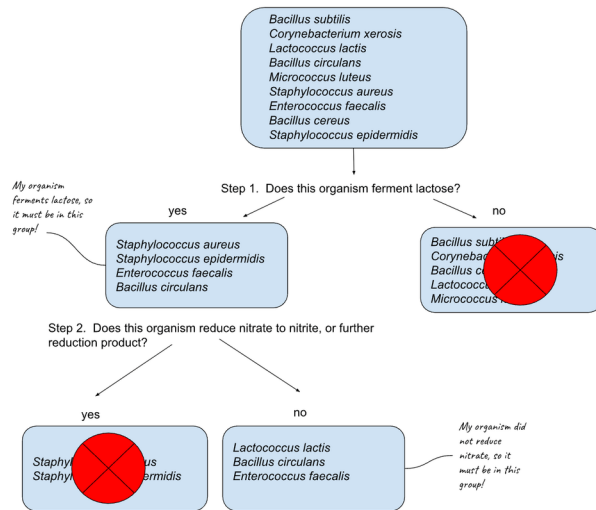


Figure 10.9: Example dichotomous key for the unknown project.

11: Protein Metabolism of Unknowns

Learning Outcomes

By the end of this lab period, you will be able to:

- Describe the pattern of protein metabolism for each of your organisms for the tests that we completed
- Articulate how pH indicators such as phenol red, bromocresol purple, phenol red, etc. are used to track protein utilization.
- Explain how differences in the expression of specific enzymes (such as nitrate reductase, gelatinase, and urease, etc.) can be used to differentiate between bacteria species.

Introduction

In today's lab, we will be continuing with a series of biochemical tests that will help you further narrow down which organisms are your Gram-positive and Gram-negative unknowns. The focus of today's tests is on the metabolism and breakdown of proteins, and the metabolism of nitrogen.

The tests that we will be setting up today are

1. Decarboxylase tests
 1. Lysine
 2. Ornithine
2. Phenylalanine deaminase
3. SIM
 1. Sulfur
 2. Motility
 3. Indole
4. Nitrate reduction
5. Gelatin hydrolysis
6. Urea hydrolysis

Decarboxylase Tests

Amino acids are structurally composed of an amino group (NH_2), a "carboxy" or carboxylic acid group (COOH), and an "R" group, which is the part of the molecule that is variable. Enzymes that remove the carboxylic acid group are called "decarboxylases". Determining whether or not a bacteria makes a specific decarboxylase can be useful in their identification. These tests are useful for helping us ID our Gram-negative bacteria, but not helpful for Gram-positive, so we'll only perform decarboxylase tests on our Gram-negative.

Today, we will be testing for two decarboxylase enzymes, ornithine decarboxylase, and lysine decarboxylase. Decarboxylase broths contain the pH indicator bromocresol purple which turns yellow in acidic conditions, and purple in basic solutions. After inoculation, a layer of mineral oil is overlaid onto the broth, creating anaerobic conditions and inducing the bacteria to ferment glucose. Initially, this will result in the production of acid and the media will turn yellow. However, for those organisms that produce the decarboxylase enzyme, the presence of the amino acid in the media will turn "on" the gene for the enzyme, and result in the production of amines. These amines accumulate and create a basic environment turning the media purple. If no decarboxylase is produced, the medium will remain yellow.

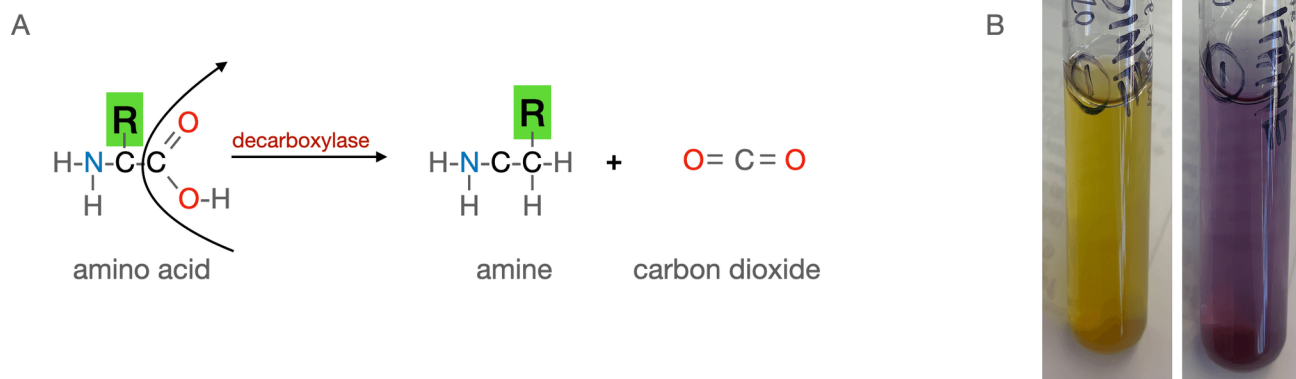


Figure 11.1: The decarboxylase test. A. The decarboxylation reaction showing how the carboxylic acid is removed, forming carbon dioxide. B. Left - a negative decarboxylase result. Right - a positive decarboxylase result. Photo Credit - Celeste Towner, used with permission.

Phenylalanine Deaminase

In addition to enzymes that remove the carboxylic acid group, some bacteria have enzymes that can remove the amino group from specific amino acids. Determining if an organism can remove the amino group from phenylalanine is also a useful test for identifying Gram-negative organisms.

The phenylalanine deaminase test is done by inoculating a phenylalanine slant. If the bacteria make the enzyme, it will react with the phenylalanine and produce phenylpyruvic acid. Phenylpyruvic acid is colorless but will react with ferric chloride (FeCl_3) to produce a forest green color.

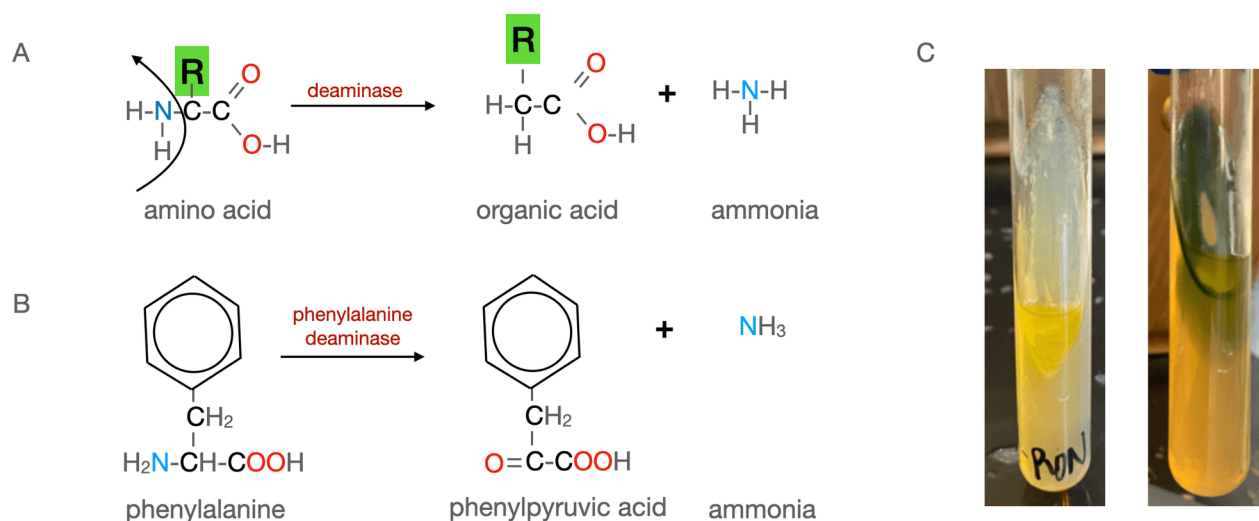


Figure 11.2: Phenylalanine deaminase test. A. General amino acid deamination B. phenylalanine deaminase produces phenylpyruvic acid C. Right - positive deamination reaction. Left - negative result. Photo credits: Ron John Vargas, (negative result), I.H. (positive result), used with permission

SIM Tests

The SIM tests, again only performed on our Gram-negative bacteria, are actually three tests in one tube. In this tube, we will test for sulfur reduction, the production of indole, and bacterial motility. Both sulfur and indole production are the result of amino acid

metabolism. The observation of motility is just a happy “extra” that we can often glean from this test.

SIMs media is a type of “deep” and is inoculated with a needle.

Sulfur reduction

The amino acid cysteine contains a sulfur atom in its “R” group. Some bacteria make an enzyme called cysteine desulfurase, which removes that sulfur atom in the form of hydrogen sulfide gas. The hydrogen sulfide gas will react with ferrous ammonium sulfate (FeSO_4) to produce a black precipitate, ferric sulfide (FeS).

There are other mechanisms by which bacteria can reduce sulfur. The enzyme thiosulfate reductase can reduce sodium thiosulfate to form hydrogen sulfide gas as well. This will also result in the formation of a black precipitate.

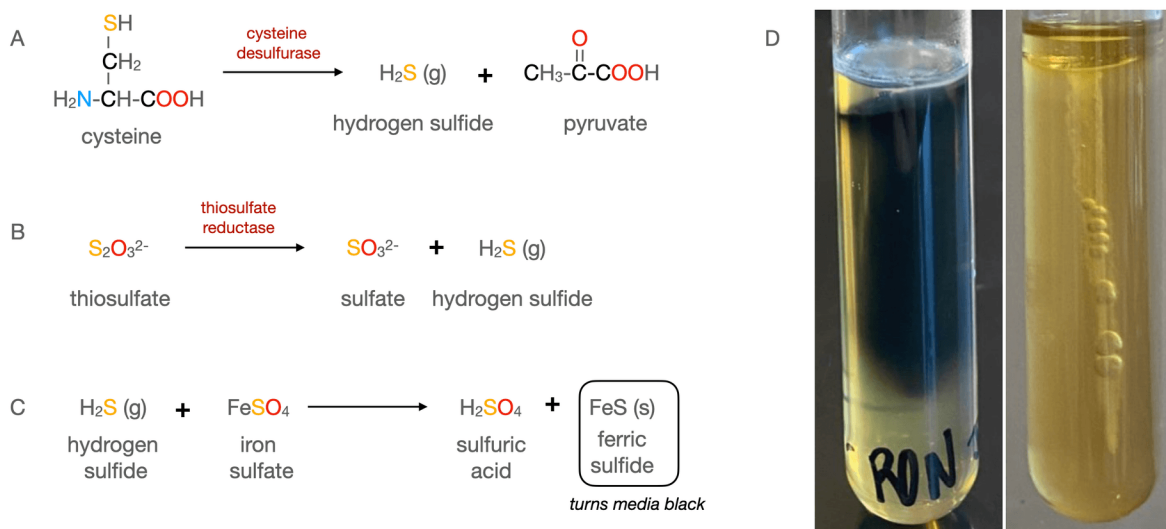


Figure 11.3: SIMs media - detection of H_2S production. A. Production of hydrogen sulfide from cysteine metabolism B. Production of hydrogen sulfide from reduction of thiosulfate C. Reaction of hydrogen sulfide with iron sulfate. D. Reduction of sulfur in SIM media. Right - negative control. Left, positive reaction. Photo credits: Ron John Vargas (positive result) and Maithy Nguyen (negative result), used with permission

Indole

The enzyme tryptophanase hydrolyzes the amino acid tryptophan to pyruvate, ammonia, and indole. Indole will react with p-dimethylamino benzaldehyde (a component of Kovak’s reagent) to form a cherry red color, detected as a layer on top of the deep.

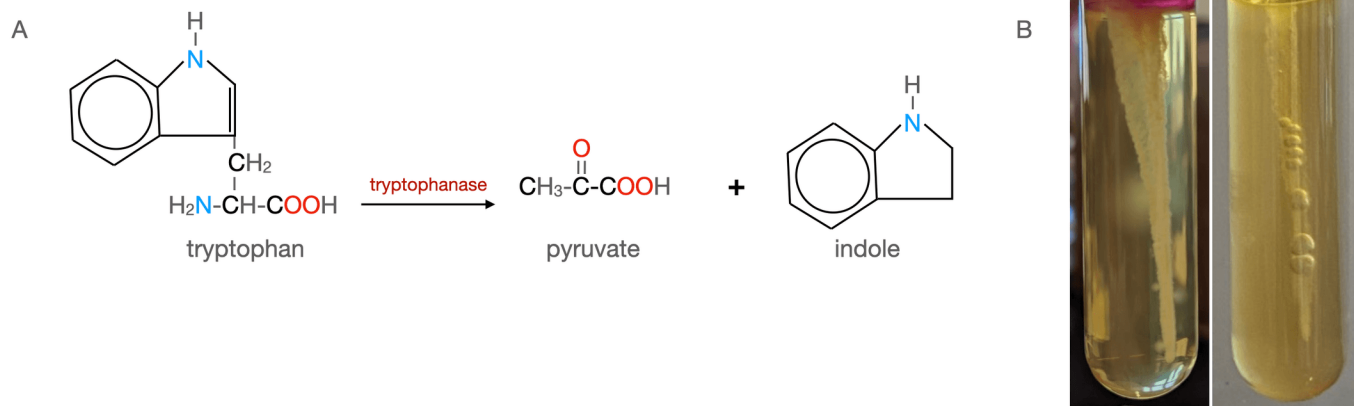


Figure 11.4: A. Tryptophanase catalyzes the breakdown of tryptophan into pyruvate and indole. B. Indole can be detected by adding Kovac's reagent to the SIMs deep, resulting in a cherry red layer on the top. Left - indole positive tube. Right - negative result. Maithy Nguyen (negative result), used with permission

Motility

It is frequently serendipitous that we can determine motility from SIMs media. However, it is only possible to do this when sulfur is not reduced and the tube does not turn black. Because H_2S gas can diffuse rapidly in the media, its presence usually results in a blackening of the entire tube, obscuring any motility observation.

If your organism is negative for sulfur reduction, hold the tube up to the light and look for growth that radiates away from the initial inoculation. This indicates that the bacteria are able to move through the media. If the media in the tube becomes cloudy as compared to an uninoculated control, this indicates that the organism is motile.

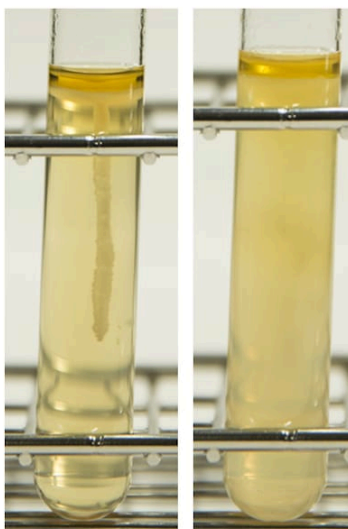


Figure 11.5: Motility in SIMs media. The tube on the right illustrates the cloudy radiating growth typical of a motile organism. The tube on the left is typical of a non-motile organism. A doubt, CC BY-SA 4.0, and A doubt, CC BY-SA 4.0, both via Wikimedia Commons. Both images cropped at top, bottom, and sides.

Nitrate Reduction Test

Many bacteria make an enzyme nitrate reductase that can reduce nitrate (NO_3) to nitrite (NO_2), while other organisms can reduce nitrate (NO_3) further, producing a set of possible intermediates and final products including ammonia (NH_3) and nitrogen gas

(N₂).

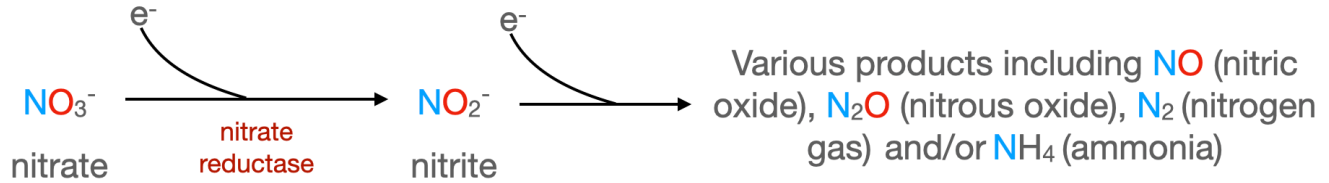


Figure 11.6: Possible results of nitrate reduction. Many organisms “stop” at the production of nitrite, however ammonia, and nitrogen gas are also made by different organisms. Ammonia can be used by bacteria for amino acid synthesis. Denitrifiers, which are important ecologically can reduce nitrate all the way to nitrogen gas.

We identify organisms that have reduced nitrate to nitrite by the addition of two reagents (Nitrate Reagent A, and Nitrate Reagent B). If nitrite has been produced the nitrate broth will turn a deep blood red. That is a positive result and can be recorded.

However, because some organisms further reduce nitrite, it's possible that your organism DID reduce nitrate, but there is simply no nitrite left in the broth because it has all been converted to any of the further endproducts or their intermediates shown in Figure 11.6. So how can you tell whether there was NO nitrate reduction, or that reduction continued past the production of nitrite? We do this by adding some powdered zinc to the nitrate broth. Zinc will react with any remaining nitrate, converting it into nitrite which will then react with Reagents A and B that you added previously. If the tube turns red after the addition of zinc, this is a negative result. If the tube does not turn red, then there was neither nitrate nor nitrite left in the tube, and this would be recorded as positive for nitrate reduction.



Figure 11.7: Schematic diagram showing testing for nitrate reduction and possible outcomes. If after the addition of Nitrate Reagent A and Reagent B, the tube turns red, this is a positive result. Zinc can be used to differentiate between tubes where no reduction has occurred and tubes where nitrate has been further reduced. Photo credit: Ron John Vargas, used with permission

Gelatin Hydrolysis

Earlier in the semester when we learned about agar, we discussed how gelatin was once used (or its use was attempted) to create solid substrates for growing bacteria. We learned that gelatin is a terrible choice because so many bacteria secrete enzymes called gelatinases that break down and liquefies the gelatin. The gelatin hydrolysis test differentiates between organisms that produce gelatinases, and those that do not. The gelatin test is performed in a “deep” and is inoculated with an inoculating needle.

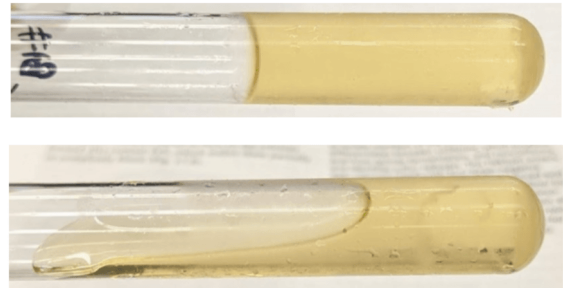
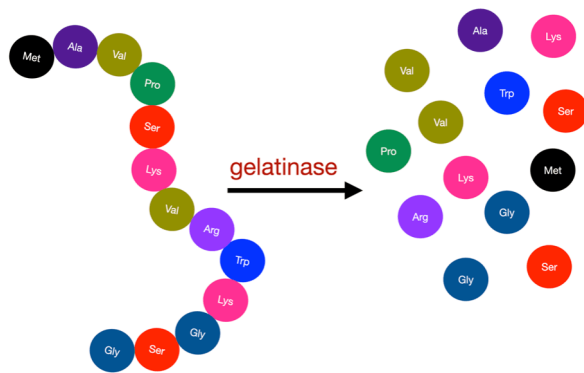
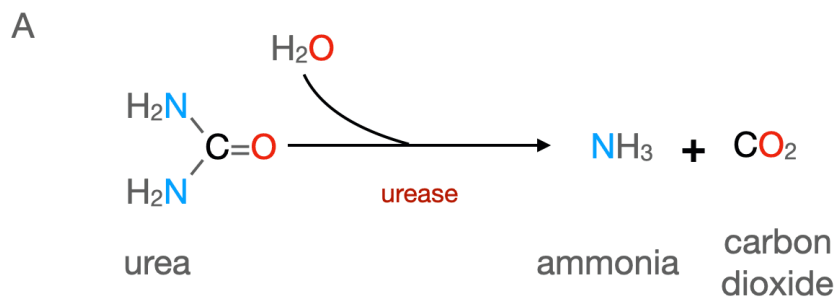


Figure 11.8: Left - Gelatinases break the bonds between individual amino acids in the gelatin protein. Right - The presence of gelatinase will result in liquefaction of the gelatin. Top tube - a negative result. Bottom tube - a positive result.

Urea Hydrolysis

A common byproduct of the decarboxylation of amino acids is the urea molecule. Urea is a metabolic waste product, but the nitrogen in urea can be recycled by some microbes that make the enzyme urease. This enzyme is also a virulence factor in the pathogen *Helicobacter pylori* - it uses this enzyme to generate urea which neutralizes the acid in your stomach where it lives and reproduces.

Urea broth contains only a tiny amount of yeast extract for nutrition and strong buffers that prevent a color change from occurring unless enough ammonia is rapidly produced. Phenol red is the pH indicator, which is pink above pH 8.4. An orange or yellow result is negative.



B

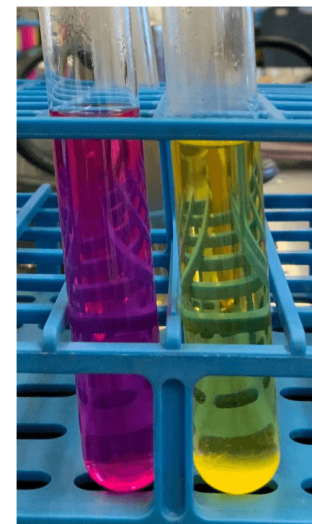


Figure 11.9: A. The hydrolysis of urea results in the production of ammonia, which makes the broth more basic (raises the pH). B. A positive result is shown on the left. A negative result is shown on the right. Photo credit - I.H., used with permission

Materials

Day 1

- One Lysine decarboxylase broth (for Gram-negative only)
- One Ornithine decarboxylase broth (for Gram-negative only)
- Sterile mineral oil in microcentrifuge tubes

- One Phenylalanine deaminase slant (for Gram-negative only)
- One SIM deep (for Gram-negative only)
- Two nitrate broths
- Two nutrient gelatin deeps
- Two urea broths

Day 2

- 12% ferric chloride solution
- Kovac's reagent
- Nitrate test reagents A and B
- Zinc powder
- Ice

Experiment

Day 1

1. Inoculate the lysine decarboxylase and ornithine decarboxylase broths with your Gram-negative organism. Overlay both tubes with 3 to 4 mm sterile mineral oil. Incubate at 37°C for 96 hours (up to one week).
2. Inoculate the phenylalanine deaminase slant with your Gram-negative organism.
3. Use an inoculation needle to inoculate a SIM tube with your Gram-negative organism. 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 insertion line.
4. Inoculate 2 nitrate broths, one with your Gram-positive and one with your Gram-negative organism.
5. Use an inoculation needle to stab inoculate the nutrient gelatin medium.
6. Inoculate 2 urea broths, one with your Gram-positive and one with your Gram-negative organism.
7. Place all freshly inoculated cultures in the 37°C incubator, and incubate for 48 hours. If this is a 5-day incubation, incubate at 30°C to avoid cultures growing past stationary phase into death phase.
8. Each week during the ID project you should be making fresh subcultures of your organisms and verifying purity by gram staining. Depending on how old your TSA slants are this can be done on Day 1 OR Day 2.

Day 2

1. Remove all of your cultures from the incubator. Carefully inspect each decarboxylase broth, and note whether the result was positive (purple), negative (yellow or no change of color), or indeterminate. Compare the colors with the uninoculated control tubes. Sometimes a result is weakly positive - make a note of this! Decarboxylation tests may need to be incubated for up to one week. Return the broth tubes back to the 37°C incubator, and make one more observation in the next lab period if the tube is yellow or indeterminate.
2. For the phenylalanine deaminase test, add a few drops of 12% ferric chloride solution to the slant and observe for color change (green). (Note: this color may fade quickly, so record your results immediately).
3. For the SIM test, examine the tube for spreading from the stab line and formation of black precipitate in the medium. Record any H₂S production and/or motility. Add Kovac's reagent to each tube to a depth of 2 mm - 3 mm. After several minutes, observe the formation of red color in the reagent layer and record the Indole result.
4. For the nitrate reduction test, examine each tube for evidence of gas production. Record your results. Proceed to the addition of reagents to all tubes as follows:
 1. Add 8 drops of reagent A and 8 drops of reagent B to each tube. Mix well, but carefully, and let the tubes stand undisturbed for 10 minutes. Record the tube that has red color as nitrate reduction-positive.
 2. For any tube that did not change color, add a pinch of zinc dust. Mix well, and let the tubes stand for 10 minutes. If the color turns red, record the organism as nitrate reduction-negative. If there is no color change after adding zinc, record the organism as nitrate reduction-positive.
5. For the gelatin hydrolysis test, place all tubes in the ice bath until the control tube is solid. Examine the inoculated medium for gelatin liquefaction. Tip over the tubes to take photos of your test tubes, be careful not to spill liquefied gelatin out the top. Record your results. The gelatin test may need to be incubated for up to one week. Keep the gelatin tubes, and make one more observation in the next lab period.
6. Observe the color of the urease test tubes, and record any tube that has turned pink as urease-positive.

Data

Your primary goal as you leave lab today is to have a complete and well-documented record of the results you collected today. Be sure to update your test table with the new data! Continue to develop your dichotomous keys and identify your unknowns.

This page titled [11: Protein Metabolism of Unknowns](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by [Sheri Miraglia](#).

12: Completion of the ID Project

Learning Outcomes

By the end of this lab period, you will be able to

- Articulate the value of confirmation testing
- Describe the difference between alpha, beta, and gamma hemolysis
- Explain the utility of MSA, bile esculin, EMB, and Hektoen media.
- Finalize the identification of your Gram-positive and your Gram-negative unknowns.

Introduction

At this stage of the ID project, you should have a pretty good idea which unknowns you have, or have narrowed down your Gram-positive and Gram-negative organisms to a few final possibilities. In this lab, you will have the opportunity to perform a few more tests that can help you confirm or exclude those possibilities. All of the tests from today's lab must go into your test table, and be described BUT some of these tests are only completed IF you believe you have certain organisms.

The tests that we will be setting up today are

1. Sheep's Blood Agar - everyone should perform this test on their Gram-positive organism.
2. Mannitol Salt Agar (MSA) - this test can help confirm that you have *Staphylococcus aureus* or *epidermidis*. It can be useful in differentiating between these two organisms and other Gram-positive cocci.
3. Bile esculin - this test can help confirm the presence of *Enterococcus* or *Klebsiella* organisms.
4. Eosin Methylene Blue (EMB) - this media can help confirm the presence of a strong fermenter. In particular, this test can be useful to confirm *E. coli*.
5. Hektoen Enteric Agar - this media is useful for confirming the presence of organisms that produce hydrogen sulfide gas, such as *Salmonella*, and differentiating it from other non-fermenters that are not H₂S producers.
6. Spore-stain - this technique that we learned about in [Lab #7](#), is important for confirming a *Bacillus* species and can be used to differentiate *Bacillus* from other Gram-positive rods.

7. Sheep's Blood Agar

The ability to break down red blood cells (hemolysis) is a virulence factor characteristic of many organisms including Group A *Streptococcus*. These bacteria secrete enzymes called "hemolysins". By breaking down the red blood cells, bacteria are able to release and use the nutrients present in the cells, including heme.

Hemolysis is divided into 3 categories, α (alpha), β (beta), and γ (gamma).

- α -hemolytic organisms break down blood cells completely. They are identified by growing on a blood agar plate, and then noting a complete clearing of blood from around the bacterial growth.
- β -hemolytic organisms partially break down blood cells. Clearing is not observed, but the agar around the bacteria can appear greenish as a result of this partial breakdown.
- γ -hemolytic organisms are NOT hemolytic. No breakdown of RBCs is observed.



Figure 12.1: Examples of α (alpha), β (beta), and γ (gamma) hemolysis. Y tambe, CC BY-SA 3.0, via Wikimedia Commons

Mannitol Salt Agar

Mannitol Salt Agar (MSA) is a highly selective medium that contains 7.5% sodium chloride. This level of salt inhibits the growth of most bacteria, but organisms found on your (salty) skin, are adapted to higher salt, and are able to grow in this medium. This medium is excellent for confirming *Staphylococcus* (or ruling it out, if you have any other Gram-positive cocci).

You should know already whether or not your organism ferments mannitol from your phenol red broths. However, MSA is also differential for mannitol fermentation. The medium contains both mannitol as well as the pH indicator phenol red. Recall that phenol red turns yellow below pH 6.8. Organisms that both grow on MSA (salt tolerant) and ferment mannitol will turn the medium a bright yellow color.

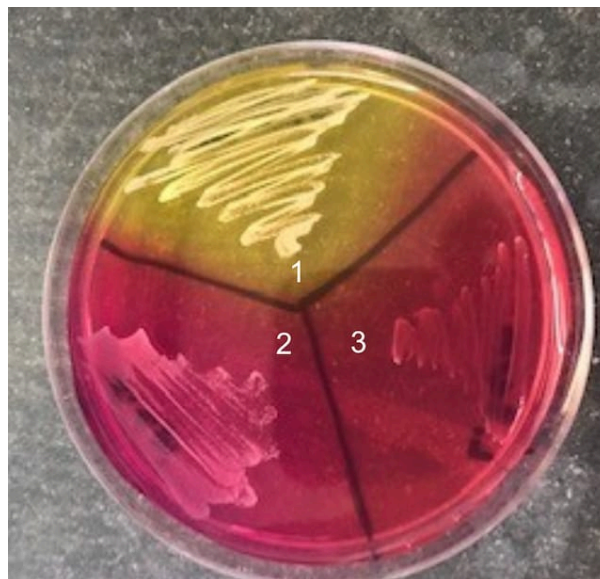


Figure 12.2: Mannitol Salt Agar (MSA) results. 1 - *S. aureus* grows on MSA and ferments mannitol. 2, 3 - *S. epidermidis* and *L. fermentum* both grow on MSA, but neither ferments mannitol. Photo Credit, Ying Liu, used with permission.

Bile Esculin Agar

Bile esculin agar can be used to confirm both *Enterococcus* and *Klebsiella* species, but it's frequently used to differentiate

Enterococcus from *Streptococcus*. Bile esculin agar is selective for Gram-negative bacteria due to the bile, but Gram-positive *Enterococcus* and Group D *Streptococcus* species can still grow on this medium. Esculin is a glucoside that is found in some plants, and glucose is a product of its hydrolysis. The other byproduct of hydrolysis is a molecule called esculetin, which reacts with iron (Fe^{3+}) in the medium to form a dark brown precipitate. Many of our organisms, not just *Enterococcus* and *Klebsiella*, produce esculinase, so it's a good test to do even if you don't believe you have either *Enterococcus* or *Klebsiella*.

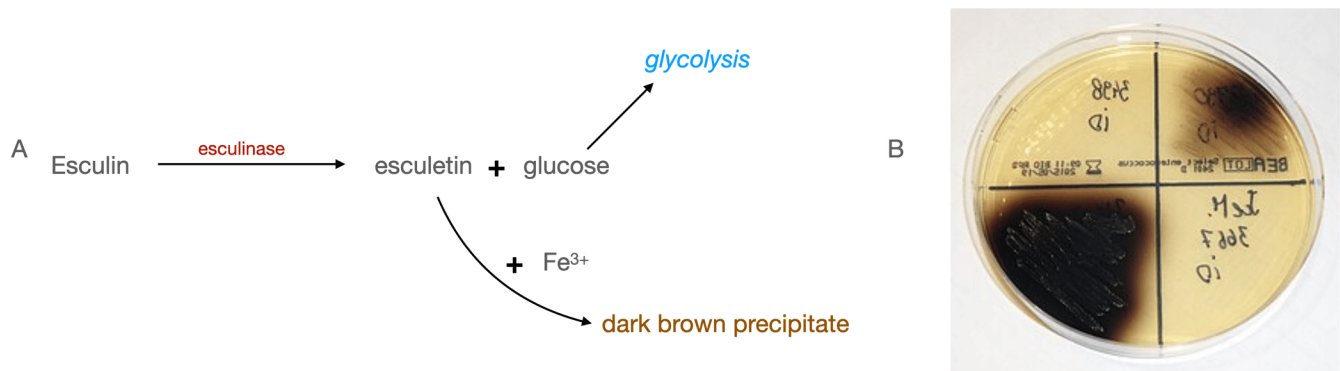


Figure 12.3: A. Esculin can be used by bacteria that make the enzyme esculinase to generate glucose for energy. A bile esculin plate showing the brown precipitate formed when *Enterococcus* is grown on the medium. [Stefan Walkowski](#), CC BY-SA 4.0, via Wikimedia Commons

Eosin-Methylene Blue Agar

EMB (eosin-methylene blue) agar is frequently used to identify fecal coliform bacteria and is both selective and differential. It can be very useful in distinguishing a vigorous fermenter such as *E. coli*, from other fermenters. The dyes present in the medium including Eosin Y and methylene blue, inhibit the growth of Gram-positive bacteria. These dyes are also pH indicators that will form a dark purple precipitate at low pH. EMB plates also contain both sucrose and lactose. Organisms that ferment these sugars, and lower the pH, will turn purple on this medium. However, when the pH drops very low, below 4.9, the Eosin Y and methylene blue will form a complex with a green color. Very vigorous fermenters, such as *E. coli*, will form colonies with a green metallic sheen.

We will be using EMB agar again during our [Food Microbiology lab](#).

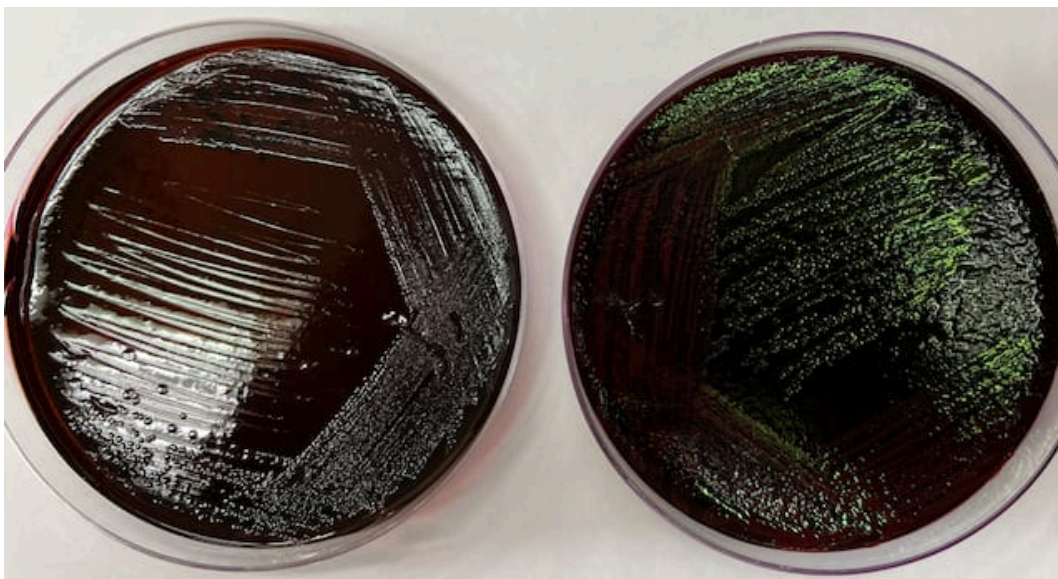


Figure 12.4: Typical growth on EMB. Left - a non-lactose fermenter. Right - a vigorous lactose fermenter such as *E. coli* produces a characteristic green sheen.

Hektoen Enteric Agar

Hektoen enteric agar is both selective and differential and was designed primarily to differentiate between *Salmonella* and *Shigella* in human specimens. It contains bile salts, bromothymol blue, and acid fuchsin, all of which select against the growth of Gram-positive bacteria. It also contains Ferric ammonium citrate or thiosulfate, which will react with hydrogen sulfide (H₂S) to form a black precipitate - similar in principle to the reaction that occurred in the SIMs tubes in [Lab #11](#).

Hektoen also contains lactose, sucrose, and salicin. Organisms such as *E. coli* will ferment these sugars producing acid, which will turn the Bromothymol blue an orange or salmon color. In theory, neither *Salmonella* nor *Shigella* will ferment these sugars, and in fact, they don't in our phenol red broths. However, we often see *Shigella* turning orange on Hektoen in our lab, so don't use an orange colony on Hektoen to rule out *Shigella*.

We will be using Hektoen agar again during our Food Microbiology lab.



Figure 12.5: Left - *Salmonella enterica* produces hydrogen sulfide and turns black on Hektoen. Right - *Shigella sonnei* is not supposed to ferment lactose...but in our lab it always does producing acid and turning the medium orange.

Spore Stains

Gram-positive organisms in the *Bacillus* genera are spore formers. If you believe you have a *Bacillus* as your Gram-positive, or if you have a Gram-positive rod, you should perform a spore stain to confirm or rule out *Bacillus*.

To review spore stains and access the staining protocol, please refer to [Lab #7](#).

Materials

- One sheep's blood agar plate
- One MSA (mannitol salt agar) plate if confirming or ruling out *Staphylococcus*
- One bile esculin plate (everyone should use this to test their G⁺ and G⁻ organisms).
- One EMB(eosin methylene blue) plate if confirming *E. coli*, or other strong fermenter.
- One Hektoen enteric agar plate if confirming an H₂S producer
- Spore staining materials if confirming or ruling out *Bacillus*.

Experiment

Day 1

1. Perform a streak plate on sheep's blood agar.
2. Streak out your Gram-positive and Gram-negative organisms on bile esculin agar. You can perform two line-inoculations on a single plate, or do streak plates if there is enough media available (or if you have an organism that exhibits swarming morphology).
3. If your Gram-negative bacterium is an aggressive fermenter such as *E. coli*, streak it out on EMB to confirm or rule it out.
4. If you suspect your Gram-negative organism to be *Salmonella* or *Shigella*, the HE agar plate is a good confirmation test. Additionally, if there is any ambiguity in the lactose-fermentation status of your Gram-negative bacterium (your MacConkey plate and Lactose phenol red test gave contradictory results), use the HE agar to verify whether you have a coliform or not. Note that any H₂S producer will produce black colonies on Hektoen.
5. If you want to confirm or rule out *Staphylococcus*, streak it out on MSA.
6. If you need to confirm or rule out *Bacillus*, be sure to perform a spore stain (I will be looking for this when I grade your projects if you report *Bacillus*!).
7. If you need to repeat any tests from prior weeks (Lab #10 or Lab #11), you should set those up today.
8. Incubate all newly inoculated media at 37°C for 48 hours or 30°C for 5 days.
9. **Day 2**
10. Observe the hemolysis status of your bacteria from the blood agar plate. Hold the plate up to the light, or observe it over a light box to determine if there are zones of clearing. It is difficult to determine whether or not an organism is alpha or gamma hemolytic. We will record this as a "yes-no" result - your organism is either beta-hemolytic or it's not.
11. Observe your Bile Esculin Agar results for your Gram-Positive and Gram-negative organisms.
12. Observe any results from EMB, MSA, or Hektoen plates that you may have prepared.
13. If you need to confirm or rule out *Bacillus*, be sure to perform a spore stain if you have not done so already.
14. If you need to repeat any tests from prior weeks (Lab #10 or Lab #11), you should set those up today, as this will probably be your last chance.
15. Incubate all newly inoculated media at 37°C for 48 hours or 30°C for 5 days.

Data

Be sure to photograph and record all of your results from this week's tests and include all of them in your test table. Even if you didn't perform the test, all of them are required to be recorded in your table. Any test not performed can be explained - for example, you might say that you didn't perform a spore stain because your Gram-positive organism was a cocci.

13: Identification of White Blood Cells

Learning Outcomes

By the end of this lab, you will be able to

- Prepare a blood smear
- Perform a Wright's stain
- Identify red blood cells, neutrophils, eosinophils, basophils, platelets, monocytes, and lymphocytes under the microscope
- Perform Wright's stain of a blood smear.
- Conduct a differential blood count to determine the percentage of various leukocytes in blood.

Introduction

Today we will be exploring the white blood cells that are so important to the innate and adaptive immune system. Where will we get the blood? From your finger of course! You will be using a lancet to pierce your finger and collect drops for the preparation of a blood smear. You will then stain the smear using a modified Wright's stain procedure, and then we will be looking at them under the microscope at 1000x.

All of your red and white blood cells originate from a type of stem cell called a hematopoietic stem cell. These cells are found primarily in your bone marrow, but also in your peripheral blood, and throughout your life, they differentiate into any of the red or white blood cells that are needed. Red blood cells have a short life span of about 120 days, so they are constantly being replaced. Many of your white blood cells have even shorter life spans, some of only minutes or days!

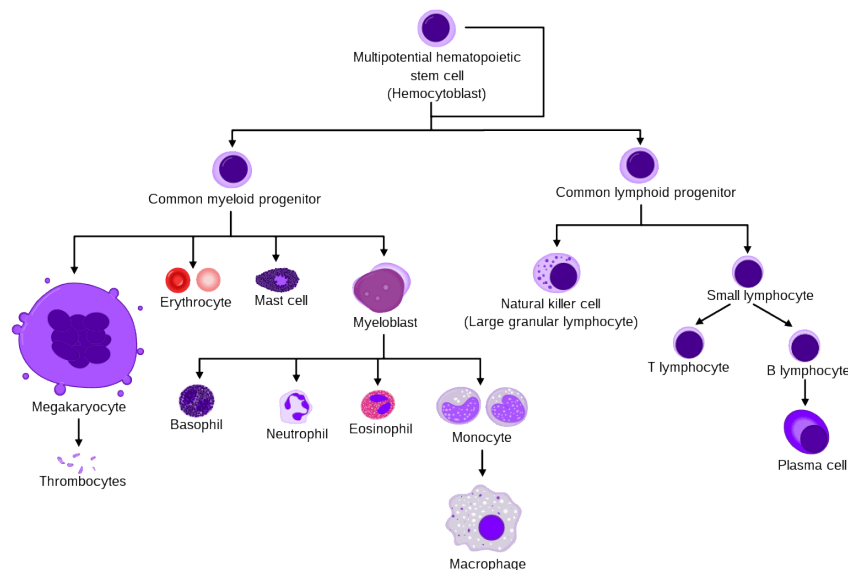
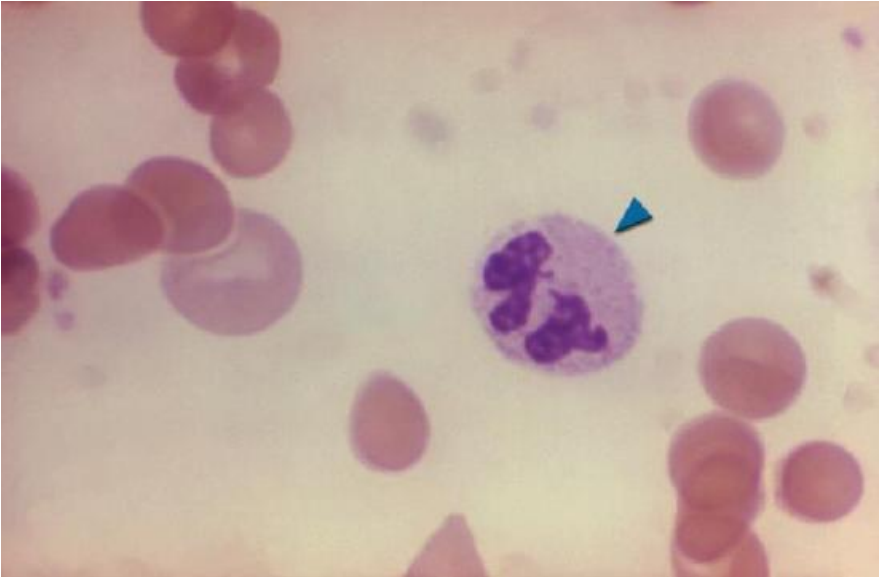
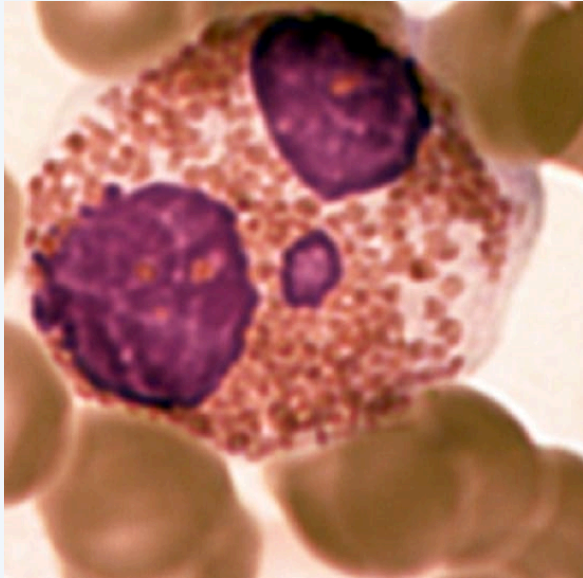
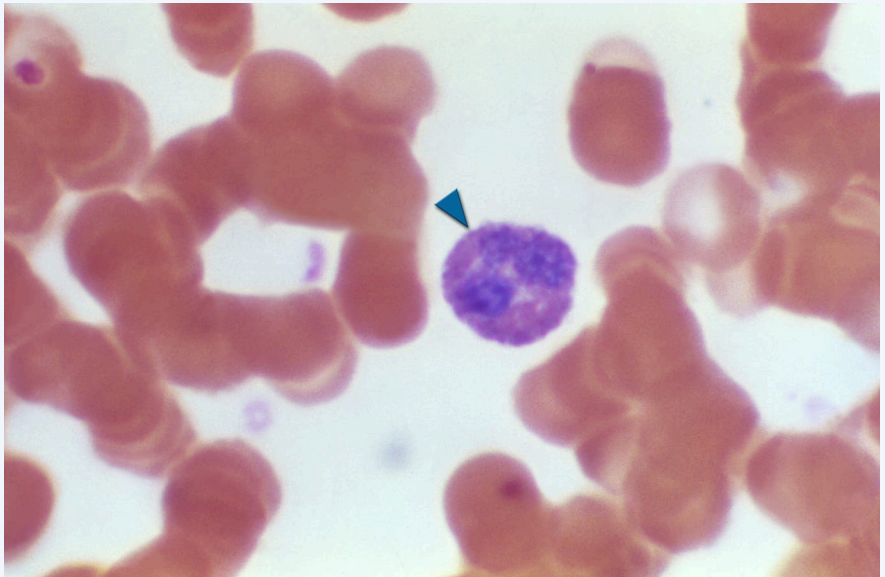


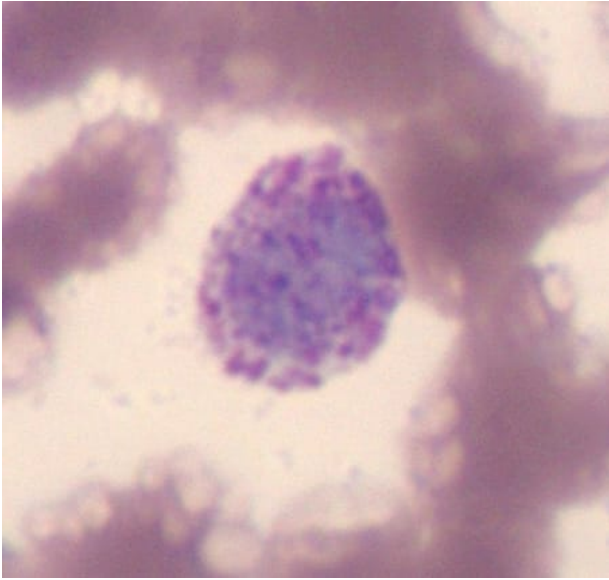
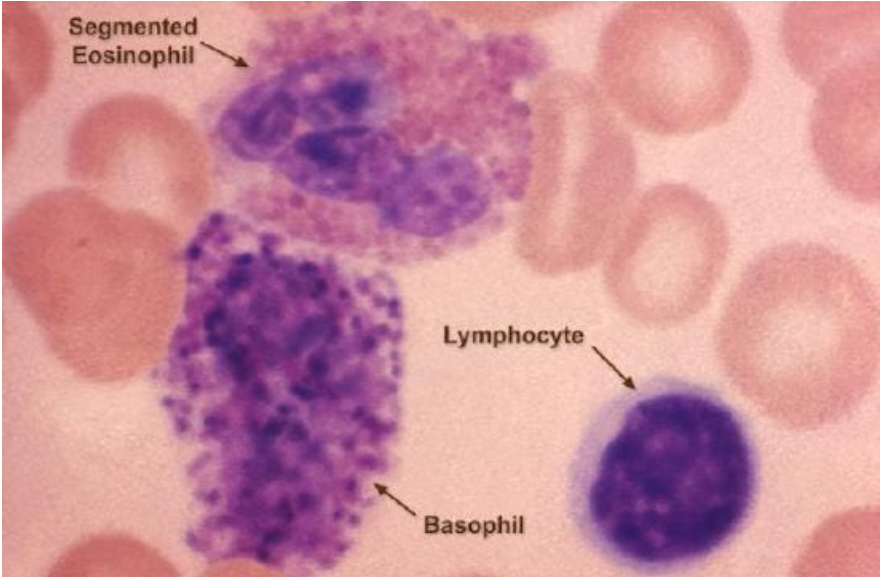
Figure 13.1: Simplified hematopoiesis. By A. Rad and M. Häggström, CC BY-SA 3.0, via Wikimedia Commons

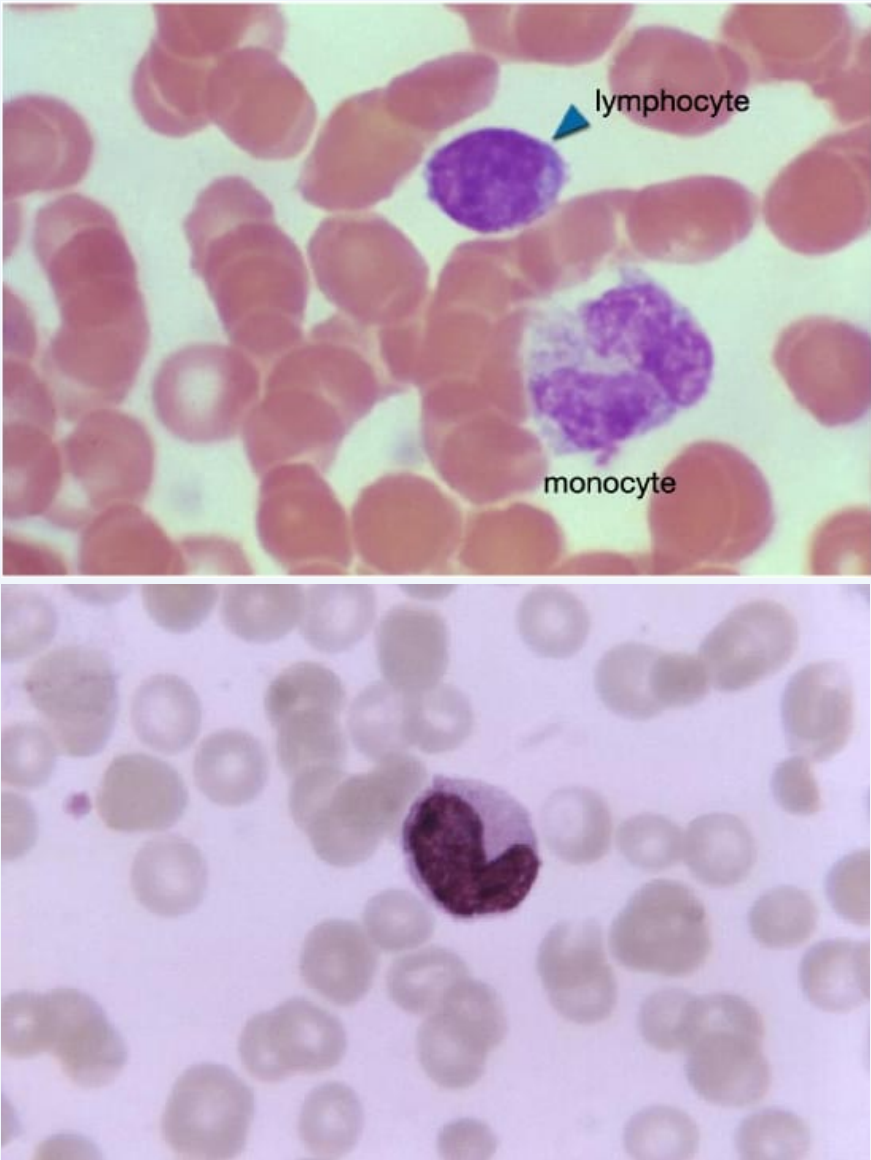
Our Openstax textbook, [Chapter 17.3](#) describes the functions of each of these cells in detail, so these functions are not recapitulated here. However, you will need to be able to recognize them under the microscope. The table below will help you with determining which cells are which.

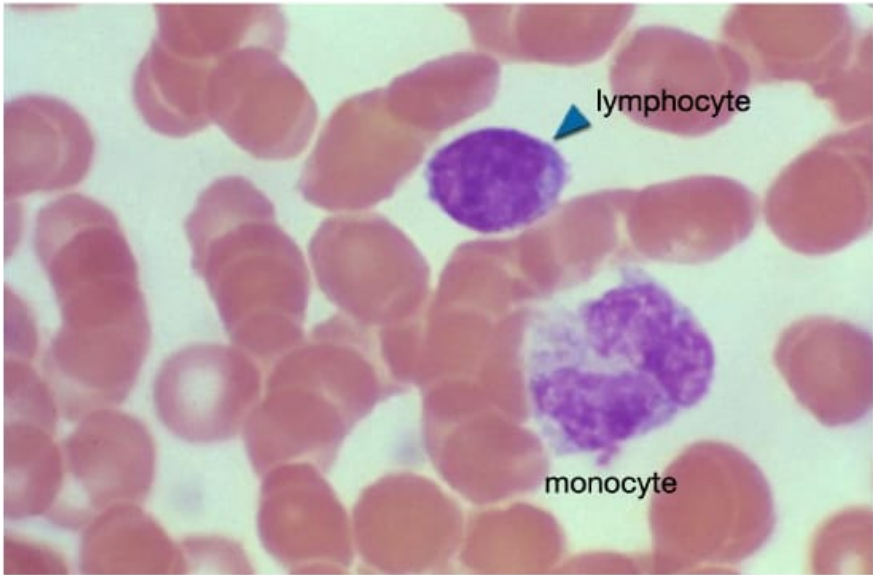
Table 1: Examples of WBC's Seen in Peripheral Blood

Cell type	% found in blood	Images
Neutrophil	55-65%	  User CS99 at German Wikipedia, Public domain, via Wikimedia Commons (left image), CDC Public Health Image Library, Public Domain (right image).

Cell type	% found in blood	Images
Eosinophil	1-2%	  Davidcsaba Dr. David Csaba L., Public domain, via Wikimedia Commons (left), CDC Public Health Image Library, Public Domain (right image).

Cell type	% found in blood	Images
Basophil	0.2%	  CDC Public Health Image Library, Public Domain (right image).

Cell type	% found in blood	Images
Monocytes	3-7%	 CDC Public Health Image Library (left), and CDC Public Health Image Library (right) Public Domain

Cell type	% found in blood	Images
lymphocytes	25-33%	  Bernanke's Crossbow, CC BY-SA 3.0, via Wikimedia Commons (left image), CDC Public Health Image Library, Public Domain (right)

Cell type	% found in blood	Images
platelet	N/A	 CDC Public Health Image Library, Public Domain

Preparing a blood smear

The procedure for making a blood smear is different from making a bacterial smear. Blood smears are prepared by taking a drop of blood and spreading it across the slide vertically using a second slide. Then the blood is further spread horizontally to cover most of the slide surface.

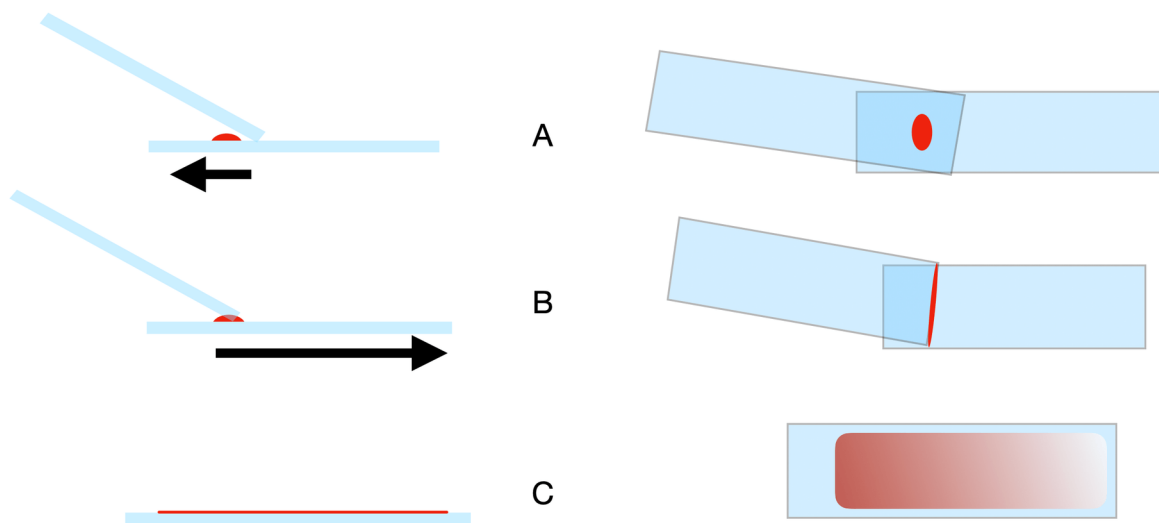


Figure 13.2: Preparing a blood smear. A. After placing a drop of blood on the first slide, hold a second slide at an angle in front of the drop. Pull back until the blood has wicked along the edge of the second slide by capillary action. B. Push the second slide forward as smoothly as you can to the very end of the slide. C. When finished, you should have a nice even layer of blood across the slide's surface.

Wright's Stain

Wright's stain contains the dyes eosin Y and methylene blue. These two stains bind differently to different white blood cell types and allow them to be easily differentiated from one another. Eosin Y stains the cytoplasm of cells a pink or orange color, while methylene blue stains the cellular nuclei a blue color. This makes it fairly straightforward to differentiate the various white blood cells from each other. Additionally, the granules of basophils and eosinophils are stained blue or red, respectively.

We will be using a 1-step Wright's stain in today's lab.

Materials

- Sterile lancets
- Alcohol pad wipes
- Band-aids for finger sticks
- Plastic sharp container
- Wright's stain
- Microscope slides

Experiment

1. Select the finger to puncture, usually the middle or ring finger. Clean the area with an alcohol wipe, and allow it to dry.
2. If the room is cold, you may want to try to warm up your hands first and get some circulation going. Use the sterile lancet to puncture the ball of the finger. Wipe away the first drop of blood if you are bleeding enough. For some of us who struggle to get any drops of blood out, you may want to make a slide from the initial drop and then try to make additional slides from subsequent drops if you can. Touch a drop or two of blood with a clean slide.
3. Hold a second slide at an angle as illustrated in Figure 13.2. While maintaining contact with the bottom slide, pull the top slide back to contact the blood, which will spread by capillary action.
4. Maintain firm contact with the bottom slide and push the top slide in one motion to produce the smear. Your instructor will demonstrate!
5. Let the blood smear air dry. It is not necessary (or desirable) to heat up the slide before staining.
6. Stain the dried smear following the protocol below:
 1. Dip the smear in Wright's stain for 30 seconds
 2. Dip the smear in a beaker of distilled water for 45 seconds
 3. Dip the smear in a second beaker of distilled water for 25 seconds
 4. DO NOT RINSE BETWEEN DIPS!
7. Allow your slide to air dry - do not blot.
8. Scan your smear using your 4x objective and try to find a spot where there is a monolayer of blood cells visible. Systematically scan the slide and count at least 50 white blood cells. Calculate the percentage of neutrophils, lymphocytes, monocytes, eosinophils, and basophils out of the total. Do not count platelets or red blood cells.

Data

Count a total of 50 cells (or more!), and fill out the table below.

Table 2: Student Data Table for Collecting White Blood Cell Counts

	Monocytes	Lymphocytes	Segmented Neutrophils	Eosinophils	Basophils
Number					

	Monocytes	Lymphocytes	Segmented Neutrophils	Eosinophils	Basophils
Percentage					
Expected percentage	3-7%	25-33%	55-65%	1-3%	<0.2%

Questions

1. What is the role of each of the cell types you saw today? Include erythrocytes and platelets in your answer.
2. Which cells that we saw today have primary roles in the innate immune system? What about the adaptive immune system?
3. Which cell type differentiates into innate immune system cells that are “professional” antigen-presenting cells?
4. Can you tell the difference between B- and T-cells under the microscope? If not, what would you have to do to differentiate between them?

14: Antibiotics

Learning Outcomes

By the end of this lab period, you will be able to

- Set up and perform a disk diffusion assay
- Explain the utility of Mueller Hinton media
- Identify and measure zones of inhibition and the location of the minimum inhibitory concentration of each antibiotic on the MH plate.
- Determine if each organism studied is sensitive, intermediate, or resistant to a set of antibiotics.

Introduction

Antibiotics, or antimicrobials are small molecules that inhibit bacterial growth. We have covered the history and mechanisms of antibiotics, as well as resistance to antibiotics in the lecture portion of the course. There is also a great chapter for your review in OpenStax Microbiology ([Chapter 14 - Antimicrobial Drugs](#)).

The mechanisms of antibiotic action are typically divided into five groups

1. Cell wall synthesis inhibitors
2. Protein synthesis inhibitors
3. Nucleic acid (DNA/RNA) synthesis inhibitors
4. Metabolism inhibitors (sulfonamides)
5. Plasma membrane disrupters

Some antibiotics are “broad spectrum” and work against a wide variety of bacteria. However, we know that bacteria as a group are incredibly diverse, and differences in cell walls and metabolic features such as enzyme expression mean that not every antibiotic will be effective against every organism. In order to determine which antibiotic is a good choice as therapeutic, potential antibiotics must be tested against that organism.

Antibiotic Effectiveness Testing

Testing antibiotic effectiveness can be done in several ways. One common method is a dilution assay, where the antibiotic is continually diluted to lower and lower concentrations to determine at which point organisms are able to grow. Dilution assays can be done in test tubes or in microwell plates (Figure 14.1).

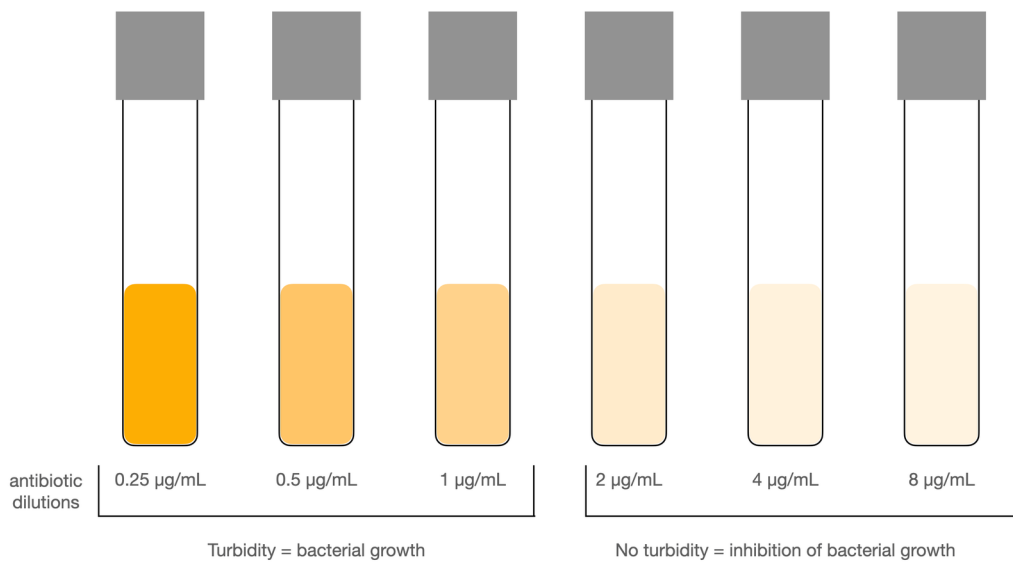


Figure 14.1: Diagram of an antibiotic dilution assay. As the concentration of antibiotic increases, bacterial growth is inhibited.

Today, we will be using a method called a Disk Diffusion Assay, first described by Kirby and Bauer in 1966. The Disk Diffusion Assay uses small antibiotic-impregnated disks which are placed on a newly inoculated Muller-Hinton agar plate. Antibiotics diffuse from the disks outward creating an antibiotic gradient. Where concentrations of antibiotic are high enough, bacterial growth will be inhibited (Figure 14.2).

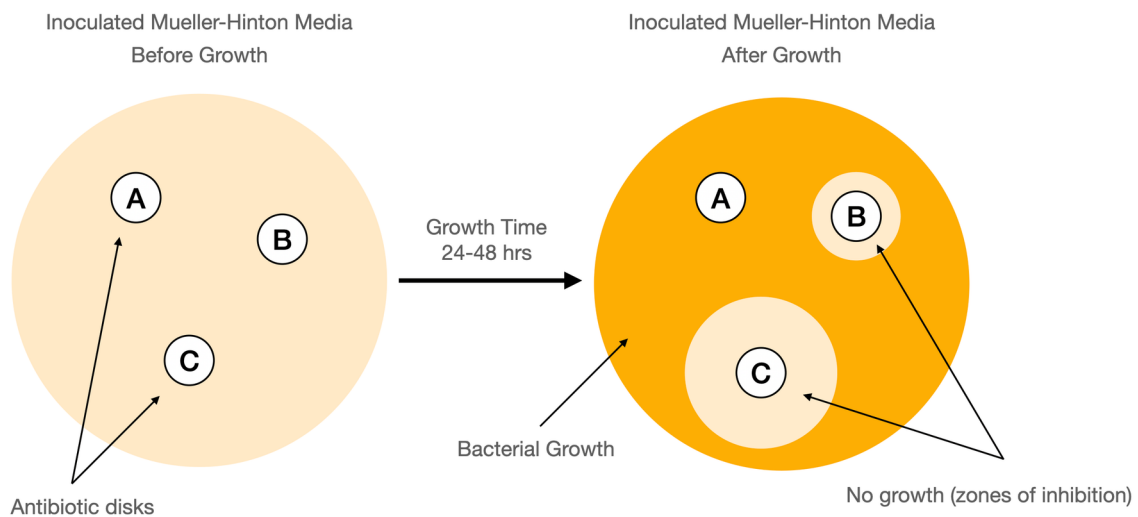


Figure 14.2: Principles of the Disk Diffusion Assay

Mueller Hinton Agar

When the antibiotic disk is placed on inoculated Mueller Hinton (MH) agar, the antibiotic will diffuse outward from the disk. The further from the disk, the lower the antibiotic concentration. In order for these results to be reproducible, MH Agar is a non-selective, non-differential medium that almost all organisms will grow on. It's been designed to be low in molecules that can inhibit antibiotics and it's poured to a precise thickness in order for antibiotic diffusion to be uniform between plates. MH plates also contain starch, which absorbs toxins released from bacteria that might interfere with the antibiotics. Lastly, MH agar is a loose medium that better allows for antibiotic diffusion.

ZOI and MIC

The clearing zone around the antibiotic disk is referred to as the “zone of inhibition” (ZOI). The edge of that zone, where the growth of the microorganism begins, represents the “minimum inhibitory concentration” or MIC. This is the minimum concentration of antibiotic that is required to prevent the bacteria from growing. In order to determine antibiotic effectiveness, the zones of inhibition can be measured in millimeters (mm) and compared to standard values (Figure 14.3).

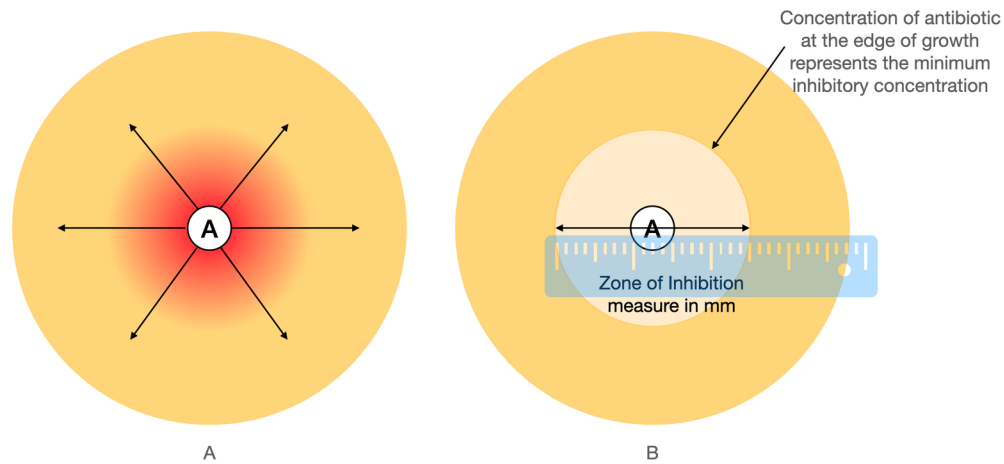


Figure 14.3: A. Antibiotic, shown in red, diffuses outward from the disk. The further from the disk, the lower the concentration. B. The zone of inhibition (ZOI) is measured in millimeters (mm). The minimum inhibitory concentration is the concentration of antibiotic at the very perimeter of growth.



Figure 14.4: Disk diffusion assay performed on *S. aureus*. Note that Polymyxin B (PB 300 disk) does not inhibit *S. aureus* growth.

Materials

Day 1

- Three Mueller-Hinton agar plates
- Broth cultures of *S. aureus*, *E. coli* and *P. aeruginosa*
- Antibiotic disks representative of the five different mechanisms of antimicrobial action

- Antibiotic disk dispenser or forceps for placement of disks
- Alcohol pads or bath to sterilize forceps
- Cell spreaders, alcohol, and beakers for creating bacterial lawns or sterile cotton swabs

Day 2

- Metric ruler for measuring Zones of Inhibition

Experiment

Day 1

1. Work in pairs. Collect 3 Muller-Hinton agar plates.
2. Label plates with sample type, pair names, date, and lab section
3. Using the three different bacteria provided, pairs will create a bacterial lawn on each Mueller-Hinton plate (one species of bacteria per plate). Utilize sterile cotton swabs or plate-spreading technique as demonstrated by your instructor.
4. Sterilize/disinfect forceps using one of two methods:
 1. Swab with an alcohol pad
 2. Use the alcohol dip and flame technique as demonstrated by your instructor
5. Apply eight (8) different antimicrobial disks on each plate using forceps and gently tap into place. Do not push so hard that you force the disk into the agar. Make sure that all disks are evenly spaced to allow maximum growth of bacteria around them. Use the same set of antibiotics on each plate to allow comparisons of their effects on the three organisms. Make note of which antibiotics you used.
6. Replace lids and set plates in the 37°C incubator upside-down until the next lab meeting. Note: for longer incubations, lower the incubator temperature to 30°C.

Day 2

1. Remove the plates from the incubator. Hold each plate dark background to better visualize the zones of inhibition. The edge of a zone is where no growth is visible to the naked eye.
2. Measure the diameter of each zone of inhibition in millimeters (mm) and record it in your lab notebook.
3. Record your data in Table 2 (or in your lab notebook). Use Table 1 to determine if each organism was sensitive (S), intermediate (I), or resistant to each antibiotic.

Data

Determine if each organism is sensitive, intermediate, or resistant to each of the antibiotics you tested. Your instructor will provide a separate table with standard values for your use.

Table 1 - Interpretation of Zone Diameters

Table 1: Student Data Table

Antibiotic Name/Disk ID	Mechanism of Action	S. aureus		E. coli		P. aeruginosa	
		Zone diameter	S/I/R	Zone diameter	S/I/R	Zone diameter	S/I/R

		<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>		

Questions

- Overall, which organism was most resistant to antibiotics? Consider the environment where this organism is found. Does this result make sense?
- Which antibiotic was the most broad-spectrum in this experiment? What mechanism of action does it represent?
- Which antibiotic was the most narrow spectrum in this experiment? What mechanism of action does it represent?
- Define/describe
 - Mueller Hinton agar
 - Zones of inhibition
 - Minimum inhibitory concentration
- Suppose you perform this test on a hypothetical *Staphylococcus* species with the antibiotics penicillin and chloramphenicol. You record the zone diameters of 25mm for both the chloramphenicol and penicillin disks. Which antibiotic would be more effective against this organism? What does this tell you about comparing zone diameters to each other, and the importance of using the zone diameter interpretive table?

15: Oxygen Requirements

Learning Outcomes

By the end of this lab period, you will be able to

- Classify organisms by their oxygen requirements based on their growth in anaerobic chambers and BHI Shake Tubes
- Determine if the conditions within an anaerobic chamber are truly anaerobic

Introduction

When Earth was formed 4.8 billion years ago, there was no oxygen gas (O_2) in the atmosphere. The first organisms to appear on Earth did not use oxygen and were in fact, poisoned by it. Oxygen gas is a very reactive molecule, prone to forming free radicals, singlet oxygen, and other reactive oxygen species (ROS). These ROS can react with biological molecules and damage them.

When the levels of oxygen gas in the air began to increase as a result of early photosynthesis, most of the organisms on earth, all of them obligate anaerobes, became extinct. This was the first great extinction event on Earth (although it wasn't the last). But evolution is a powerful force and life persisted. Some organisms adapted to this new environment by producing enzymes such as superoxide dismutase and catalase that are capable of detoxification of ROS, allowing organisms to survive. Many of these organisms evolved the ability to use oxygen gas to generate substantially more energy from metabolic pathways such as glycolysis. These organisms survived and thrived in the new atmosphere. There are, however, environments on Earth that remained oxygen-free, and obligate anaerobes continue to live there - think of deep-sea hydrothermal vents, swamps, deep lakes, etc. And, inside larger multicellular organisms such as humans, the bacteria that thrive in our digestive systems are facultative anaerobes, capable of living without oxygen.

In today's lab, we will explore the oxygen requirements of several common microorganisms and use two methodologies to determine whether they are strict anaerobes, strict aerobes, facultative anaerobes, or aerotolerant organisms.

Anaerobic Chambers

One way to determine whether or not an organism can survive in the presence or absence of oxygen is to attempt to grow the organism in an oxygenated or oxygen-free environment. In our lab, our incubators are exposed to normal atmospheric conditions, and thus microorganisms that grow in them can either tolerate, prefer or require oxygen. To create environments that exhibit an absence of oxygen, we place petri plates in a sealable chamber with a "gas pack" that will react with oxygen thus removing it from the chamber. The presence or absence of oxygen is determined by a "test strip" which turns blue when oxygen is present and white when it is not.

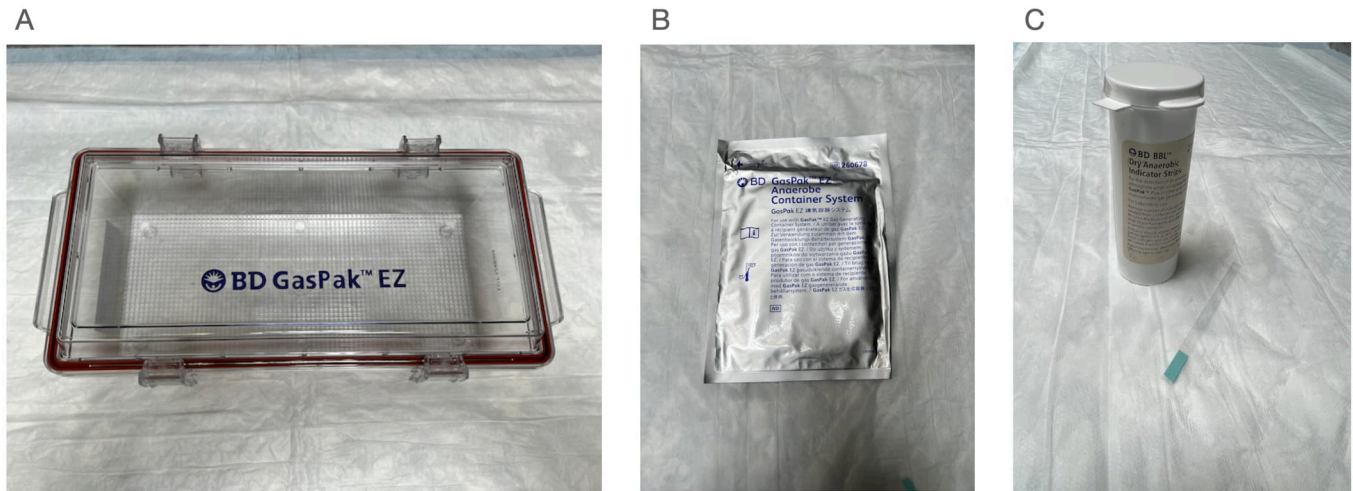


Figure 15.1: A. Anaerobic Chamber for incubating Petri plates in the absence of oxygen. B. Sealed "Gas Pak" for removing oxygen from the chamber. C. Test strips are to be placed in an anaerobic chamber to detect the presence or absence of oxygen. A blue indicator strip means that oxygen is present. When the indicator strip turns white, oxygen has successfully been removed from the container.

Shake Tubes

Brain-heart infusion (BHI) agar deeps can also be used to evaluate the oxygen requirements of an organism. The deeps are first melted, and then the temperature is equilibrated to 60°C or less (warm enough to remain melted, but not so hot that bacteria will be quickly killed). Several drops of liquid culture are dropped into the molten agar and the tube is mixed well ("shake") to evenly distribute the organisms into the media. The tube is then cooled so that the agar solidifies. This process creates an oxygen gradient, where there will be atmospheric concentrations of oxygen at the top of the tube and very little oxygen present at the bottom of the tube. The pattern of growth in the tube can be used to determine what levels of environmental oxygen the organism is adapted to.

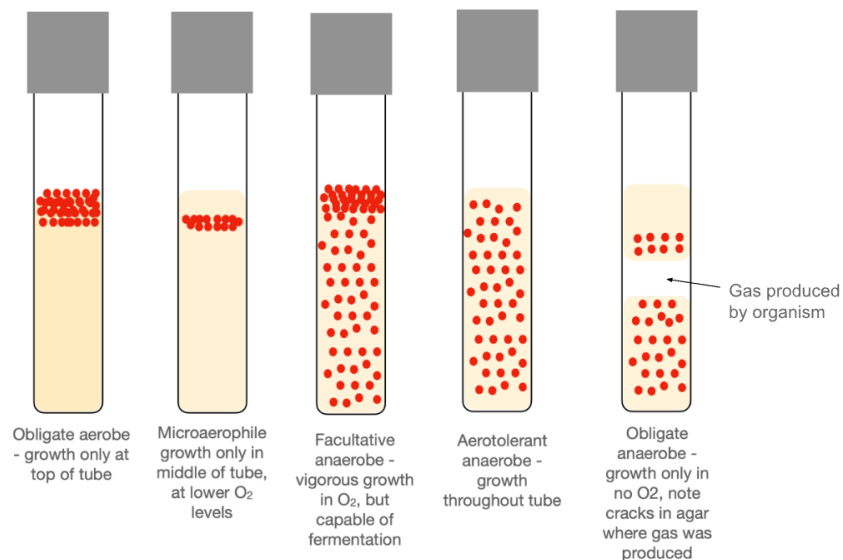


Figure 15.2: Diagram of growth patterns in BHI Shake Tubes of organisms with different oxygen requirements



Figure 15.3: Photograph of BHI shake tubes. Note how growth is only observed at the top of the tube inoculated with an obligate aerobe. The aerotolerant organisms demonstrates uniform growth from top to bottom. For the obligate anaerobe, note the absence of growth at the very top of the tube where oxygen is present. We also typically see large cracks in the agar as a result of gas production by the anaerobic microorganism. Facultative anaerobes grow best in the presence of oxygen as evidenced by the thick layer of growth at the top of the tube, however, they can also grow (more slowly) in the absence of oxygen. Gas can also be produced in these organisms as a result of fermentation.

Materials

- 6 molten tubes of BHI agar equilibrated to 60°C in a dry bath.
- Inoculating loop
- Bunsen burner
- 2 TSA plates
- Anaerobic jar
- Gas packs
- Oxygen indicator strips
- Broth cultures of:
 - *E. coli* (facultative anaerobe)
 - *Staphylococcus aureus* (facultative anaerobe)
 - *Pseudomonas aeruginosa* (obligate aerobe)
 - *Clostridium sporogenes* (obligate anaerobe)
 - *Enterococcus faecalis* (facultative anaerobe)
 - *Lactococcus lactis* (facultative anaerobe)

Experiment

Day 1

BHI Shake Tubes

1. Retrieve 6 molten BHI agar tubes from the dry bath **one at a time** as you inoculate them. The agar will solidify if you take it out of the dry bath and allow it to cool down for too long.
2. Using a sterile transfer pipette, inoculate each tube with 3 drops of one of the six organisms below. Be sure to resuspend the organisms in the broth cultures thoroughly prior to inoculating. After aseptic transfer, mix the bacteria into the molten culture thoroughly (but without inverting the tube!).
 1. *E. coli* (facultative anaerobe)
 2. *Staphylococcus aureus* (facultative anaerobe)
 3. *Pseudomonas aeruginosa* (obligate aerobe)
 4. *Clostridium sporogenes* (obligate anaerobe)
 5. *Enterococcus faecalis* (facultative anaerobe)

6. *Lactococcus lactis* (facultative anaerobe)
3. Cool the tube in ice (or by incubating it on the lab bench after inoculation) to solidify the agar.
4. Incubate tubes at 37 °C for 48 hours or at 30 °C for 5 days.

Anaerobic Chambers

1. For each group of two students, you need two TSA plates. Divide each plate into 6 sections and perform a line inoculation with one organism in each section. After inoculation, you should have two, identical plates (Figure 15.4)

Incubate with Oxygen

Incubate in anaerobic jar



Figure 15.4

1. *E. coli* (facultative anaerobe)
2. *Staphylococcus aureus* (facultative anaerobe)
3. *Pseudomonas aeruginosa* (obligate aerobe)
4. *Clostridium sporogenes* (obligate anaerobe)
5. *Enterococcus faecalis* (facultative anaerobe)
6. *Lactococcus lactis* (facultative anaerobe)
7. Incubate one plate at 37 °C for 48 hours or at 30 °C for 5 days.

Incubate the other plate anaerobically at 37 °C for 48 hours or at 30 °C for 5 days in an anaerobic jar with a palladium catalyst. Use the blue indicator strip to determine if the oxygen is removed from the jar. The blue strip will turn white under anaerobic conditions.

Data

Compare the growth on the plates to the growth in the BHI Shake Tubes. Is the pattern of growth for each organism consistent between the two methodologies? Compile your results into the table below.

Data Table:

Table 1: Student Data Table

Organism	Location of growth in BHI tube	Gas Production?	Growth on Aerobic Plate	Growth on Anaerobic Plate	Aerotolerance category
<i>E. coli</i>					
<i>P. aeruginosa</i>					
<i>S. aureus</i>					
<i>C. sporogenes</i>					
<i>L. lactis</i>					
<i>E. faecalis</i>					

Questions

1. Determine the aerotolerance category for each of the six organisms we looked at based on the data we collected. Now, look each up online or in the Gideon Guide. Do the categories match what you observed?
2. Where in/on the human body would you expect to find an obligate aerobe? What about a facultative anaerobe? Obligate anaerobe?
3. Which one of these two techniques did you find most useful for identifying aerotolerance categories? If you could only use one, which one would you choose?

16: Germicides and UV Light

Learning Outcomes

By the end of this lab period you should be able to:

- Demonstrate familiarity with ways that microbial growth can be controlled in the environment.
- Articulate the limitations of different methods of microbial control.
- Describe features of organisms that are more resistant to control methods.

Introduction

One of the most important means of preventing the transfer of infectious organisms in healthcare (and at home, and in preparing food!) is the control of these organisms in the environment. This can be done by a variety of mechanisms some of which include the application of heat (pasteurization/incineration), chemicals (bleach, alcohol), radiation (UV light, gamma rays), and filtration. Today we are going to explore the effects of UV light as well as a variety of germicides on the growth of microorganisms.

UV Light

We are constantly being exposed to radiation - in fact, the light that you see, and the radiowaves that allow you to text your friends and family with your cell phone are all forms of radiation. This type of long-wavelength radiation is low frequency and low energy and cannot damage your cells (or microorganisms). However, some forms of radiation that have shorter wavelengths are higher energy and can be very damaging to cells. Ultraviolet (UV) light can cause serious burns, and lower wavelength radiation such as X-rays or gamma-rays can ionize molecules (ionizing radiation) resulting in massive cellular damage and death (Figure 16.1).

UV light is frequently used as a way to control microbial growth in the environment. Researchers working with animal cells will often expose their lab benches/hoods to UV light before starting their experiments to reduce the number of bacteria in the environment and reduce the risk of contamination. In hospitals, [UV light is being used to disinfect patient rooms](#) and is [being studied as a way to ensure safer indoor air as well](#).

Today we will expose *E. coli* and *B. subtilis* to UV light to see what effects it has on bacterial growth. How long do you need to expose either of these organisms to UV light in order to kill them? *B. subtilis* makes endospores, does this make a difference in the amount of exposure time required for disinfection?

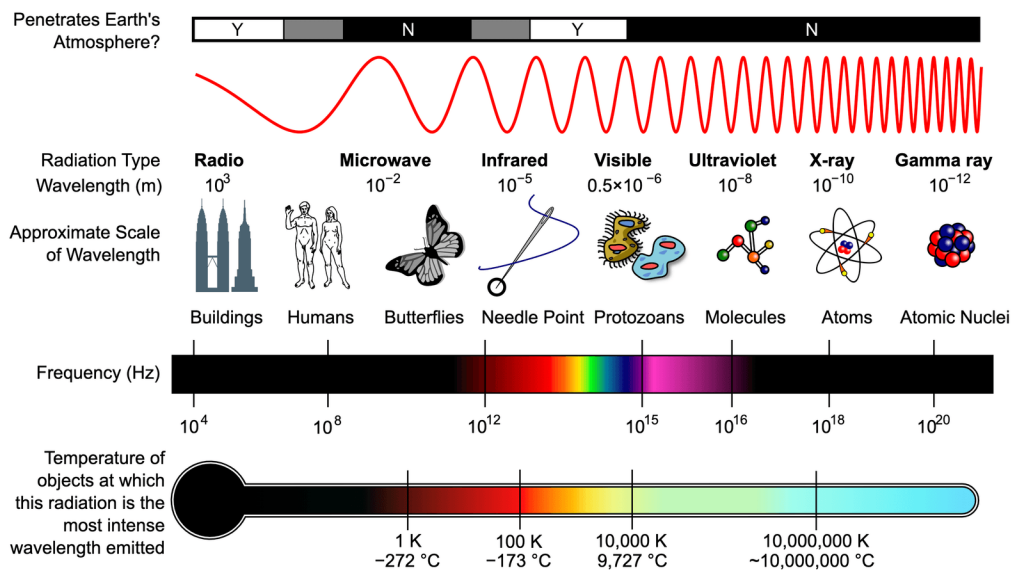


Figure 16.1: The electromagnetic spectrum. [Inductiveload](#), [NASA](#), [CC BY-SA 3.0](#), via Wikimedia Commons

Germicides

You encounter chemical germicides on a daily basis. Each time you wash your hands with soap, you are applying a low-level germicide to your skin. Do you use hand sanitizers? They contain alcohol, another chemical germicide. Do you add bleach to a load of clothes? Bleach is a strong disinfectant classified as a high-level germicide. Germicides are designed to reduce the levels of microorganisms on skin or surfaces to safe levels that will not cause disease. Some germicides are so strong that they can be used to sterilize surfaces or plasticware.

Germicides work by damaging microorganisms. They are frequently made from molecules that react with proteins and DNA, damaging them beyond repair and killing the cell. Some, like alcohol or soap, disrupt cellular plasma membranes. The effectiveness of a germicide is based on its mechanism of action, its concentration, and the time that the surface is exposed to the germicide. Other factors are also at play - including the number of microorganisms that are present, and the environment itself. It's faster to disinfect a surface clean of visible debris than to disinfect a surface covered with vomit or feces (not to be too graphic!).

Today we will test four different germicides, each at three different concentrations against three different organisms. We will first coat small ceramic beads with a bacterial culture. We will then disinfect the beads by soaking them in the disinfectant solution. Then we will place the beads in fresh media to determine if any bacteria were still alive on the beads and able to grow (Figure 16.2).

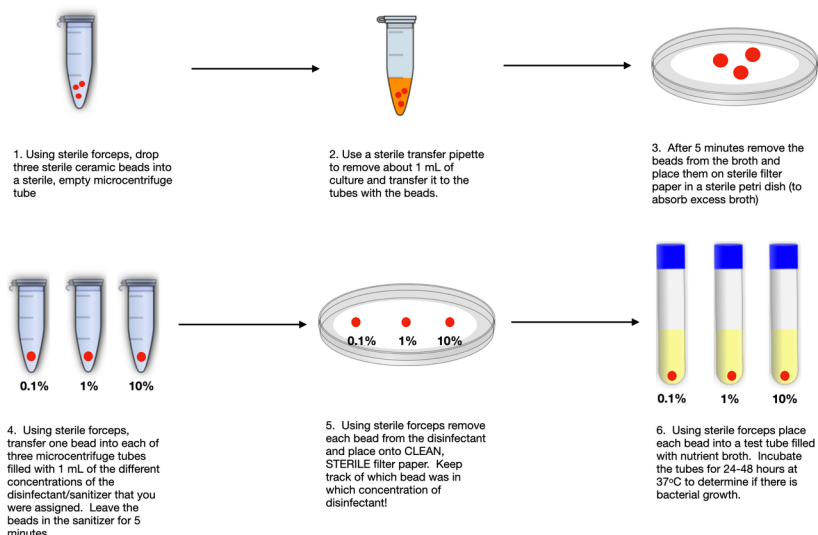


Figure 16.2: Schematic for setting up the germicides experiment.

Materials

UV light experiment

- UV safety glasses
- Short wavelength UV light source
- Cell spreaders and alcohol for flaming and sterile transfer pipettes
- Cardboard or black paper for shielding
- NA plates (two per lab partner pair)
- *Bacillus subtilis* 48-hour (or longer) broth culture
- *E. coli* 24-48 hour broth culture

Germicides experiment

- Bleach (0.01%, 1%, and 10%)
- Lysol® Brand II (0.01%, 1% and 10%)
- Hydrogen peroxide (0.01%, 1%, and 10%)
- Isopropyl (rubbing) alcohol (10%, 50%, 70%)
- Sterile ceramic beads
- Sterile 60 mm Petri dishes
- Sterile filter paper
- Sterile transfer pipettes
- Three tubes of nutrient broth per student pair
- Needle-nose forceps for manipulating the beads
- Alcohol wipes for sanitizing the forceps (or can be sanitized by dipping in alcohol and flaming)
- *S. aureus* 24-48 hour broth culture
- *P. aeruginosa* 24-48 hour broth culture
- *E. coli* 24-48 hour broth culture

Experiment

UV Light Day 1

1. Obtain two NA or TSA plates. Label the bottom of each with the name of the organism, your name, and the amount of time the organism will be exposed to UV light.
2. Use a sterile transfer pipette to place 2-3 drops of culture onto the plate and use the spread plate method to create a uniform inoculation. When incubated this should create a bacterial lawn.

3. Repeat with the second organism (*E. coli*).
4. Bring your plates to the UV light source and turn the source on using the SHORTEST wavelength option. Remove the lid (plastic absorbs UV light!) and cover ½ of the plate with black paper or cardboard. Expose the other half of the plate by leaving it directly under the light. Set the timer for the amount of exposure you were assigned.
5. When the timer goes off, remove the plate from the UV light, replace the lid, and incubate at 37°C for 48 hours or 30°C for 5 days.

UV light Day 2

1. Remove your plates from the incubator. Observe for growth. You should have confluent growth (a lawn) on the side NOT exposed to UV light).
2. On the other side, note the amount of growth. Your instructor may ask you to estimate the percentage of growth on the exposed side vs. the unexposed side. Alternatively, you could express the growth as “confluent growth”, “individual colonies” or “no growth”.
3. After recording your data, dispose of plates in the biohazard waste.

Germicides Day 1

1. Using sterile forceps (or forceps sanitized with alcohol wipes), place three sterile beads into a sterile microcentrifuge tube.
2. Use a sterile plastic transfer pipette to transfer 1 mL of a 24-48 hour culture of bacteria to the tube with the beads (your instructor will assign your group an organism to work with).
3. Allow the bacteria to thoroughly coat the beads (incubate for about 5 minutes).
4. Place a piece of sterile filter or bibulous paper into a sterile petri dish. Remove the beads using sanitized forceps or by decanting the tube. The filter paper should absorb excess bacteria.
5. Fill three new sterile microcentrifuge tubes with three different concentrations of disinfectant. You will be assigned ONE of the disinfectants to work with.

Table 1: Concentrations of Different Disinfectants for Testing

Disinfectant	Concentration 1	Concentration 2	Concentration 3
Bleach	0.1%	1%	10%
Lysol	0.1%	1%	10%
Hydrogen Peroxide	0.1%	1%	10%
Isopropyl (rubbing) alcohol	10%	50%	70%

6. Place one bead into each tube and allow the bead to be disinfected/sanitized for 5 minutes.
7. Remove the beads using forceps or by decanting onto a new, clean, and sterile piece of filter paper in a new, sterile petri dish.
8. Place each bead into a culture tube containing nutrient broth and incubate for 48 hours at 37°C.

Germicides Day 2

Compare each of your incubated tubes to an uninoculated control. Note where tubes are cloudy, indicating growth, and where they remain clear, indicating no growth. Record your results and share them with the class.

Data

UV Light

Compare your results with the class data. Your instructor may ask you to estimate the percentage of growth on the exposed side vs. the unexposed side. Alternatively, you could express the growth as “confluent growth”, “individual colonies” or “no growth”.

Table 2: Student Data Table for Collecting Class UV Light Results

B. subtilis			E. coli	
Exposure Time (minutes)	Unexposed	Exposed	Unexposed	Exposed
T=0				
T=				
T=				
T=				
T=				
T=				

Questions - UV Light

1. What was the purpose of the “unexposed” half of the plate?
2. This is not a quantitative exercise. Keeping this in mind, what is the general trend between bacterial death and UV exposure time?
3. Which organism survived the longest and why?
4. Why were you told to remove the plastic Petri plate lids before exposing the bacteria?

Germicides

Compare your results with the class data. Where there was growth, put a “+” or “G” symbol in the table. Where there was no growth, put a “-” or “NG” symbol in the table.

Table 3: Student Data Table for Collecting Class Germicides Data

Organism	Bleach			Hydrogen Peroxide			Lysol		Isopropyl Alcohol			
	0.1%	1.0%	10%	0.1%	1.0%	10%	0.1%	1.0%	10%	10 %	50%	70%

	Bleach			Hydrogen Peroxide			Lysol		Isopropyl Alcohol			
P. aeruginosa												
S. aureus												
E. coli												

Questions - Germicides

1. Which organism seemed to withstand disinfection best? Does this make sense based on what you know about the environment in which the organisms is typically found?
2. Which disinfectant performed the best overall?
3. Did any of the data not make sense? If so, what experiment would you design to better understand this data?

17: Food Microbiology

Learning Outcomes

By the end of this laboratory period, you should be able to

- Calculate the number of colony-forming units (CFU) in 1 gram of food using serial dilutions and plate counts.
- Explain how the data obtained from a standard plate count and a Hektoen or EMB plate are different and why they are both useful
- Describe situations where microbes in food are dangerous, and compare them to situations where the presence of microbes in food is deliberate.

Introduction

Every time you take a bite of food, microorganisms - sometimes millions of them - enter your body with the food. If the food is fermented, like yogurt or kimchi, the organisms were added on purpose to transform the food into a fermented product. However, even a bite or two of salad is usually accompanied by an abundance of microorganisms. This is not a problem...usually. Unfortunately, there are a few pathogenic microorganisms that utilize your gastrointestinal tract as a portal of entry. These organisms including Norovirus, *Salmonella*, *E. coli* O157:H7, and others, can wreak havoc on your body causing misery, serious illness, and occasionally death.

As a result of this, there are methods to determine how many microbes are in food, and what kinds of microbes are there. The “who” is usually more important than the “how many” since a few dangerous organisms are far riskier to health than an abundance of non-pathogenic bacteria.

Today, we will explore six different kinds of food, and ask the questions:

1. How many organisms (colony-forming units or “CFU”) are present per gram?
2. Do the organisms found in the food share metabolic features such as hydrogen sulfide formation with organisms like *Salmonella*? Are there an abundance of facultative anaerobes consistent with the kinds of organisms found in sewage?

The “How Many” Question - Serial Dilutions and Standard Plate Counts

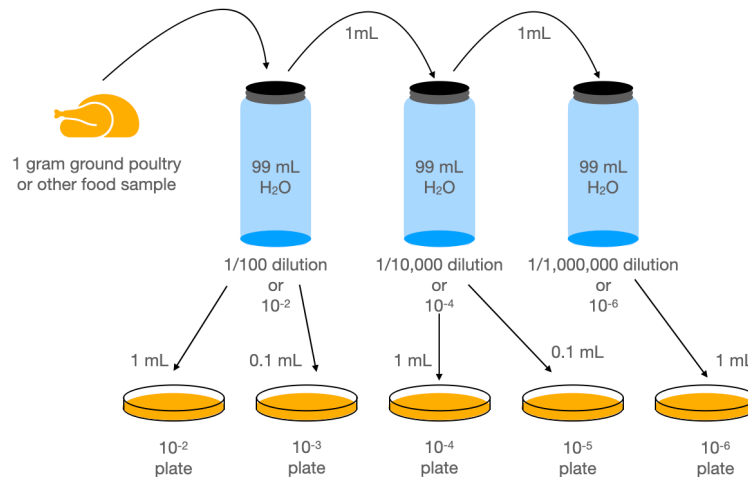
Serial Dilutions

The first thing we are going to do is perform a serial dilution of the organisms in/on the food. This is accomplished by weighing out a specific amount of the food and diluting it in water several times to yield a range of concentrations. In our case, we will weigh out 1 gram of each type of food and dilute it three times, each by 100x. This gives us three different dilutions:

- 1/100 dilution (the original sample is diluted one hundred times)
- 1/10,000 dilution (the original sample diluted ten thousand times)
- 1/1,000,000 dilution (the original sample diluted one million times)

The next step is to determine how many organisms or “CFU” are present in 1 gram of each dilution. Not every organism on the food is viable or will be able to grow under the standard laboratory conditions that we use. So a “CFU” by definition means it’s an organism that is capable of forming a colony under our specific media and incubation conditions.

To determine how many CFU are present, we will take 1 mL or 0.1 mL of each dilution above and grow any viable organisms in



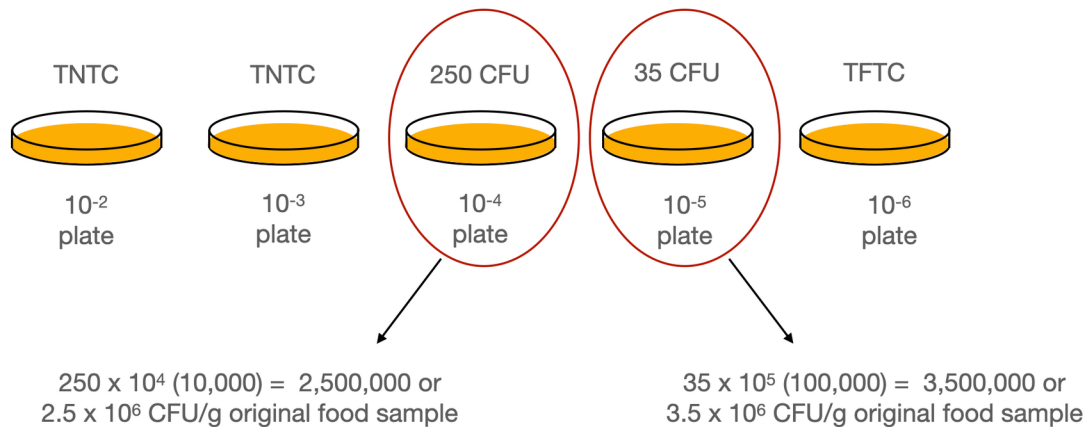
TSA agar.

Figure 17.1: Schematic diagram of how to set up serial dilutions and plates to determine CFU/gram of each type of food

Referring to Figure 17.1 - you can see how 1 gram of food is diluted into 99 mL of water. Water weighs 1g/mL, so that's the same as 99 grams of water or a 1/100 (1 g food/100 g total) dilution. If you take 1 mL of that dilution and prepare a TSA plate with that sample, the number of colonies (CFU) on that plate will be 1/100th the number of CFU per original gram of food. The same calculation can be made for the 10⁻⁴ and 10⁻⁶ dilutions. Although we are not preparing 1/1000 (10⁻³) or 1/100,000 (10⁻⁵) dilutions, we can easily prepare plates representing these dilutions by plating only 0.1 mL (one-tenth of a mL) of the 10⁻² and 10⁻⁴ dilutions respectively.

After incubation for 24-48 hours, visible colonies will appear on the plates. The challenge now is to count them! Each plate should contain approximately 10x fewer colonies than the plate before. If your 10⁻² plate contained 1000 colonies, you would expect your 10⁻³ plate to contain only 100 colonies, because you added 1/10th the amount of volume to that plate. This is true for each successive plate prepared in this experiment.

It's impossible to count a plate with 1000 colonies. In fact, it's not really possible to accurately count a plate with more than 300 colonies - we consider a plate with more than 300 colonies "too numerous to count" or "TNTC". By the same token, a plate with fewer than 30 colonies does not have a statistically significant number of organisms present to provide a reliable count, and these plates are considered "too few to count" or TFTC. Therefore, in the 5 plates that we prepare, we are hoping that one or two of them contains between 30-300 colonies so that we can use them to calculate the number of CFU/gram in our original food sample.



The original food sample contained approximately 2-4 million CFU/gram

Figure 17.2: Example data set. To calculate the number of CFU in the original sample, you would take 250 CFU and multiply it by ten thousand, or 35 and multiply it by a million. Together, this gives you a final answer of around 2-4 million CFU per gram of food.

The “Who” Question

In [Lab 12](#), we learned about two types of differential media that are both commonly used in food microbiology and wastewater sampling - Hektoen and EMB. We will be using this media again today for a qualitative assessment of the kinds of organisms that are present in each type of food.

Eosin-Methylene Blue Agar

EMB (eosin-methylene blue) agar is frequently used to identify fecal coliform bacteria and is both selective and differential. It can be very useful in distinguishing a vigorous fermenter such as *E. coli*, from other fermenters. The dyes present in the medium including Eosin Y and methylene blue, inhibit the growth of Gram-positive bacteria. These dyes are also pH indicators that will form a dark purple precipitate at low pH. EMB plates also contain both sucrose and lactose. Organisms that ferment these sugars, and lower the pH, will turn purple on this medium. However, when the pH drops very low, below 4.9, the Eosin Y and methylene blue will form a complex with a green color. Very vigorous fermenters, such as *E. coli*, will form colonies with a green metallic sheen.

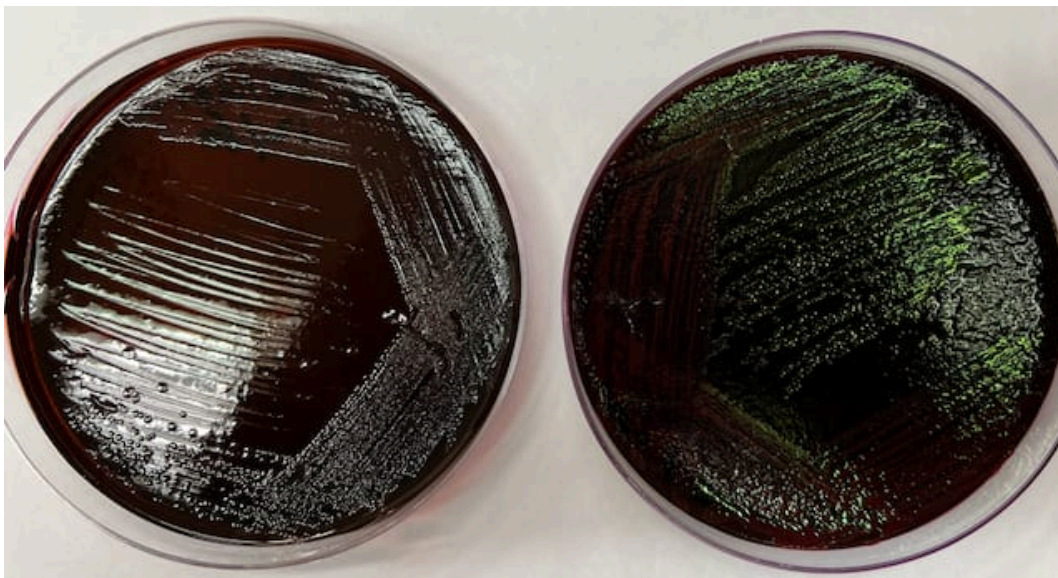


Figure 17.3: Typical growth on EMB. Left - a non-lactose fermentor. Right - a vigorous lactose fermenter such as *E. coli* produces a characteristic green sheen.

Hektoen Enteric Agar

Hektoen enteric agar is both selective and differential and was designed primarily to differentiate between *Salmonella* and *Shigella* in human specimens. It contains bile salts, Bromothymol blue, and acid fuchsin, all of which select against the growth of Gram-positive bacteria. It also contains Ferric ammonium citrate or thiosulfate, which will react with hydrogen sulfide (H_2S) to form a black precipitate - similar in principle to the reaction that occurred in the SIMs tubes in [Lab #11](#).

Hektoen also contains lactose, sucrose, and salicin. Organisms such as *E. coli* will ferment these sugars producing acid, which will turn the Bromothymol blue an orange or salmon color. In theory, neither *Salmonella* nor *Shigella* will ferment these sugars, and in fact, they don't in our phenol red broths. However, we often see *Shigella* turning orange on Hektoen in our lab, so don't use an orange colony on Hektoen to rule out *Shigella*.



Figure 17.4: Left - *Salmonella enterica* produces hydrogen sulfide and turns black on Hektoen. Right - *Shigella sonnei* is not supposed to ferment lactose...but in our lab it always does producing acid and turning the medium orange.

Materials

Per Student Group:

- Food sample (ground beef, ground poultry, fresh spinach, fresh lettuce, yogurt, or kombucha).
- Plastic weigh boat
- Digital Scale
- Alcohol Wipes
- Forceps
- Pipette pump
- 3 x Glass or plastic mL Pipettes (Do not obtain until you are ready to use each one so that they remain sterile. Use plastic pipettes for ground beef, yogurt, and ground poultry.
- 3 99 mL sterile water blanks
- 5 sterile Petri dishes (empty)
- 5 molten TSA deeps equilibrated to 60°C
- Cell spreaders
- EMB agar plate
- Hektoen agar plate

Experiment

Day 1

For steps 1-10, refer to Figure 17.1.

Prepare Dilution blanks

1. Collect three 99 mL water blanks and label them 10⁻², 10⁻⁴, and 10⁻⁶.
2. Weigh out 1 gram of the food sample you've been assigned. Try to avoid adding any organisms to the food! Wipe out the weigh boat with an alcohol wipe, dip forceps into alcohol, and flame before using them to handle or tear food. Wear gloves! Do not touch the food with your bare hands.
3. Place the food (1g) into the 10⁻² dilution blank. Mix thoroughly.
4. Using a 1 mL glass or plastic pipette, transfer 1 mL of the 10⁻² dilution blank into the 10⁻⁴ dilution blank. Mix thoroughly.
5. Using a fresh 1 mL glass or plastic pipette, transfer 1 mL of the 10⁻⁴ dilution blank into the 10⁻⁶ dilution blank. Mix thoroughly.

Prepare Plates

1. Collect 5 empty Petri dishes, and label them with your names, dates, and the dilution they represent (10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶).
2. Ensure that each dilution blank is well-mixed. Using correct aseptic technique, pipette
 - 1.0 mL of the 10⁻² dilution into the 10⁻² Petri plate
 - 0.1 mL of the 10⁻² dilution into the 10⁻³ Petri plate
 - 1.0 mL of the 10⁻⁴ dilution into the 10⁻⁴ Petri plate
 - 0.1 mL of the 10⁻⁴ dilution into the 10⁻⁵ Petri plate
 - 1.0 mL of the 10⁻⁶ dilution into the 10⁻⁶ Petri plate
3. Retrieve molten TSA deeps one at a time from the dry bath. Pour one deep into each plate, swirling gently (make a Figure 17.8 with the plate) to mix the dilutions with the agar. Do not swirl so hard that the agar touches the plate lid.
4. Allow the agar to cool and solidify on the lab bench.
5. Stack the five plates and secure them with a rubber band. Incubate at 37 °C for 48 hours or at 30 °C for 5 days.
6. **Prepare Hektoen and EMB Plates**

Pipette 0.1 mL of your 10⁻² dilution onto one Hektoen and one EMB plate. Use the cell spreading technique to evenly distribute any bacteria across the plate. Stack the two plates and secure them with a rubber band. Incubate at 37 °C for 48 hours or at 30 °C for 5 days.

Day 2

Inspect your five dilution plates. Determine which ones have between 30 and 300 colonies, and count the number of colonies present on those plates. Complete the table below. Remember that if the plate contains more than 300 colonies, you will record "TNTC". If there are fewer than 30, record "TFTC".

Data

Calculate the number of CFU per gram in the original sample:

$$CFU \text{ per plate} \times \text{dilution factor} = CFU \text{ per 1 gram original food sample}$$

Your data

Food Sample _____

Table 1: Student Data From Standard Plate Counts

	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
CFU/plate					
CFU per original 1 g food					

EMB/Hektoen Plate Results:

Table 2: Student Data from Hektoen and EMB Plates

Bacteria Types	Plate type used for determination	Present	Absent
<i>E. coli</i>			
Lactose Fermenters			
<i>Salmonella spp.</i>			

Class Data:

Record as CFU/gram of the original food sample

Table 3: Class Data from Standard Plate Counts

Plates					
Food Type	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶

Questions

1. Based on the class data, which type of food had the highest CFU per gram? Does this make sense based on the type of food?
2. Based on the class data, which type of food had the lowest CFU per gram? Does this make sense based on the type of food?
3. Did any food contain fermenters? Would you expect this type of food to contain microorganisms that ferment sugars?
4. Did any food contain putative *E. coli* or *Salmonella* based on EMB and Hektoen data?
5. Do you consider any of these foods to be unsafe to eat? Explain your answer.

INDEX

G

gram staining

6: Gram Stains

GLOSSARY

S

Sample Word 1 | Sample Definition 1

This page titled [Glossary](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by [Sheri Miraglia](#).

DETAILED LICENSING

Overview

Title: Microbiology Lab Manual (Miraglia)

Webpages: 29

All licenses found:

- [CC BY-SA 4.0](#): 93.1% (27 pages)
- [Undeclared](#): 6.9% (2 pages)

By Page

- Microbiology Lab Manual (Miraglia) - [CC BY-SA 4.0](#)
 - Front Matter - [CC BY-SA 4.0](#)
 - TitlePage - [CC BY-SA 4.0](#)
 - InfoPage - [CC BY-SA 4.0](#)
 - Table of Contents - [Undeclared](#)
 - Licensing - [CC BY-SA 4.0](#)
 - Experiments - [CC BY-SA 4.0](#)
 - 1: Introduction to the Microbiology Laboratory - [CC BY-SA 4.0](#)
 - 2: The Microscope - [CC BY-SA 4.0](#)
 - 3: Examination of Living Microbes and Environmental Plates - [CC BY-SA 4.0](#)
 - 4: Colony and Cell Morphology; Simple Stains - [CC BY-SA 4.0](#)
 - 5: Aseptic Technique and Streak Plates - [CC BY-SA 4.0](#)
 - 6: Gram Stains - [CC BY-SA 4.0](#)
 - 7: Acid Fast and Endospore Stains - [CC BY-SA 4.0](#)
 - 8: Gram Stain Unknown - [CC BY-SA 4.0](#)
 - 9: Introduction to the Bacterial ID Project - [CC BY-SA 4.0](#)
 - 10: Carbohydrate Metabolism of Unknowns - [CC BY-SA 4.0](#)
 - 11: Protein Metabolism of Unknowns - [CC BY-SA 4.0](#)
 - 12: Completion of the ID Project - [CC BY-SA 4.0](#)
 - 13: Identification of White Blood Cells - [CC BY-SA 4.0](#)
 - 14: Antibiotics - [CC BY-SA 4.0](#)
 - 15: Oxygen Requirements - [CC BY-SA 4.0](#)
 - 16: Germicides and UV Light - [CC BY-SA 4.0](#)
 - 17: Food Microbiology - [CC BY-SA 4.0](#)
 - Back Matter - [CC BY-SA 4.0](#)
 - Index - [CC BY-SA 4.0](#)
 - Glossary - [CC BY-SA 4.0](#)
 - Detailed Licensing - [CC BY-SA 4.0](#)
 - Detailed Licensing - [Undeclared](#)

This page titled [Microbiology Lab Manual \(Miraglia\)](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by Sheri Miraglia.

DETAILED LICENSING

Overview

Title: Microbiology Lab Manual (Miraglia)

Webpages: 29

All licenses found:

- [CC BY-SA 4.0](#): 93.1% (27 pages)
- [Undeclared](#): 6.9% (2 pages)

By Page

- Microbiology Lab Manual (Miraglia) - [CC BY-SA 4.0](#)
 - Front Matter - [CC BY-SA 4.0](#)
 - TitlePage - [CC BY-SA 4.0](#)
 - InfoPage - [CC BY-SA 4.0](#)
 - Table of Contents - [Undeclared](#)
 - Licensing - [CC BY-SA 4.0](#)
 - Experiments - [CC BY-SA 4.0](#)
 - 1: Introduction to the Microbiology Laboratory - [CC BY-SA 4.0](#)
 - 2: The Microscope - [CC BY-SA 4.0](#)
 - 3: Examination of Living Microbes and Environmental Plates - [CC BY-SA 4.0](#)
 - 4: Colony and Cell Morphology; Simple Stains - [CC BY-SA 4.0](#)
 - 5: Aseptic Technique and Streak Plates - [CC BY-SA 4.0](#)
 - 6: Gram Stains - [CC BY-SA 4.0](#)
 - 7: Acid Fast and Endospore Stains - [CC BY-SA 4.0](#)
 - 8: Gram Stain Unknown - [CC BY-SA 4.0](#)
 - 9: Introduction to the Bacterial ID Project - [CC BY-SA 4.0](#)
 - 10: Carbohydrate Metabolism of Unknowns - [CC BY-SA 4.0](#)
 - 11: Protein Metabolism of Unknowns - [CC BY-SA 4.0](#)
 - 12: Completion of the ID Project - [CC BY-SA 4.0](#)
 - 13: Identification of White Blood Cells - [CC BY-SA 4.0](#)
 - 14: Antibiotics - [CC BY-SA 4.0](#)
 - 15: Oxygen Requirements - [CC BY-SA 4.0](#)
 - 16: Germicides and UV Light - [CC BY-SA 4.0](#)
 - 17: Food Microbiology - [CC BY-SA 4.0](#)
 - Back Matter - [CC BY-SA 4.0](#)
 - Index - [CC BY-SA 4.0](#)
 - Glossary - [CC BY-SA 4.0](#)
 - Detailed Licensing - [CC BY-SA 4.0](#)
 - Detailed Licensing - [Undeclared](#)

This page titled [Microbiology Lab Manual \(Miraglia\)](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by Sheri Miraglia.