

MICROBIOLOGY LAB MANUAL SP 25



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College

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Detailed Licensing

Licensing

A detailed breakdown of this resource's licensing can be found in [Back Matter/Detailed Licensing](#).

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 emergency exit and fire blanket. Right - handwashing stations and 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 trash bins. (Figure 1.2)



Figure 1.2: Grey bins (left) are for regular trash. Paper towels are to be placed in the green compost bins (center). Out in the hallway are additional receptacles for trash, compost and recycling (right).

- 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.

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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).

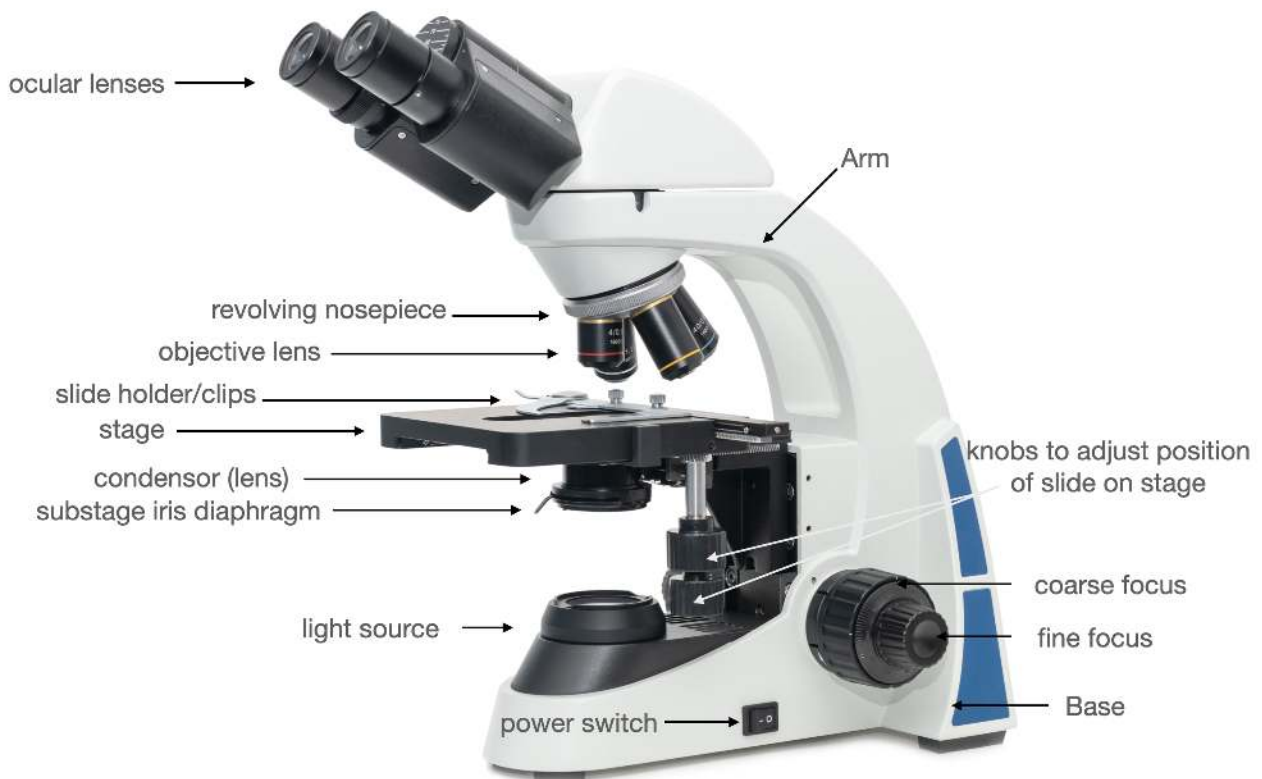


Figure 2.1: The major components of a light microscope. Photo credit iStock.com/kwanchaichaiudom

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.

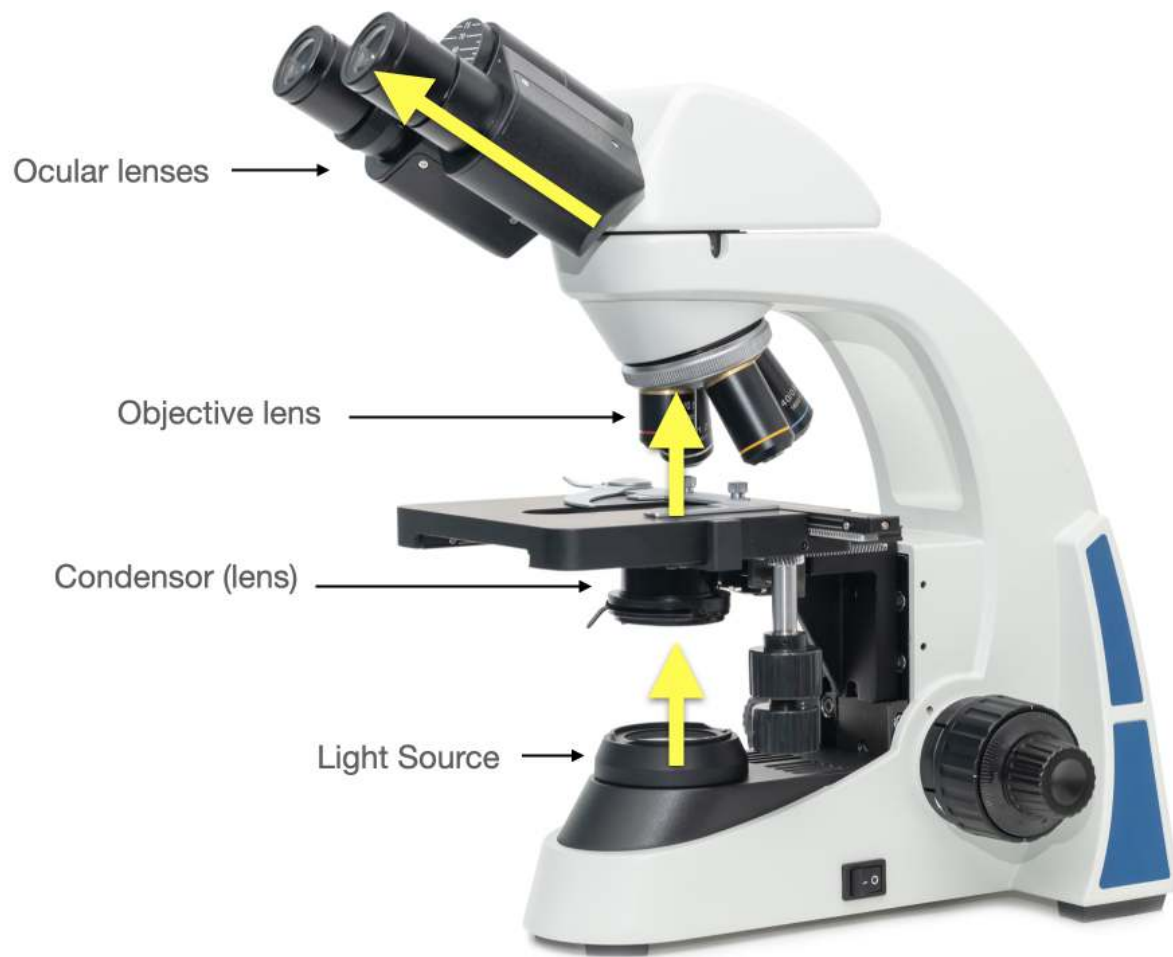


Figure 2.2: The path of light through lenses in a compound light microscope. Photo credit iStock.com/kwanchaichaiudom

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.

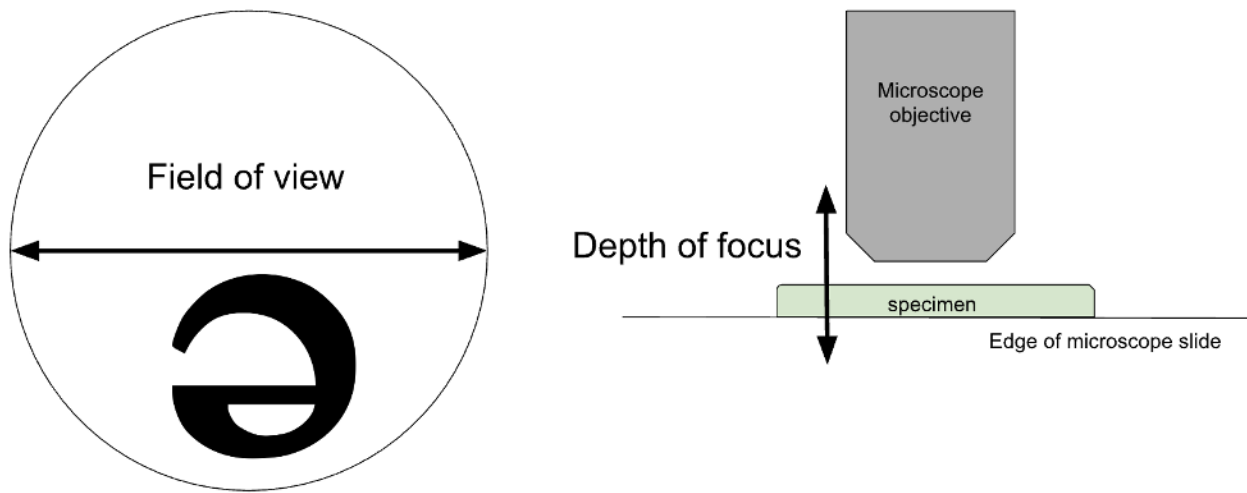


Figure 2.3: Illustrations of the field of view and depth of focus in microscopy.

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.

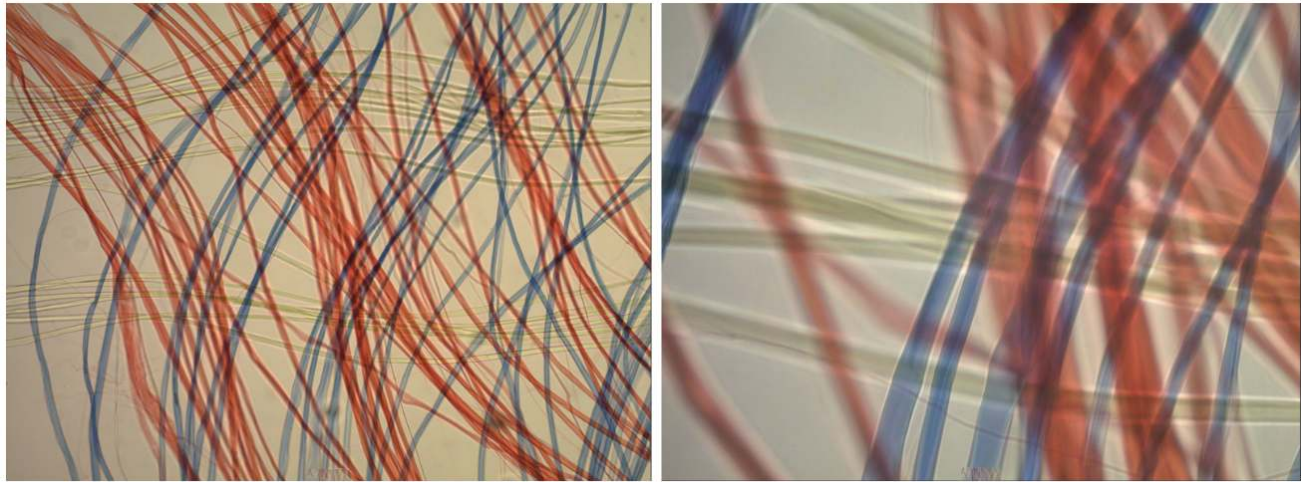


Figure 2.4: Microscope images of crossed silk fibers at 40x (left) and 400x (right) total magnification in a light microscope.

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

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3: The Myth of Spontaneous Generation

Learning Objectives

- Describe spontaneous generation theory and state that this is a theory that was once widely accepted and is an incorrect / false idea.
- Describe biogenesis theory and state that this theory is currently widely accepted.
- Apply the term "turbid" to microbiological cultures and the implications of turbidity.
- Describe Spallanzani's experiment that began to disprove spontaneous generation of microorganisms and why the scientific community did not reject spontaneous generation theory following his experiment.
- Interpret the meaning of Spallanzani's experimental results disproving spontaneous generation of microorganisms.
- Describe Pasteur's experiment that definitively disproved spontaneous generation of microorganisms and why the scientific community accepted the results of this experiment.
- Interpret the meaning of Pasteur's experimental results disproving spontaneous generation of microorganisms.
- Describe the achievements of Tyndall related to sterilization of media.
- Define endospore.
- Explain why endospores prevent sterilization of microbiological media by boiling only.
- Recreate and interpret Spallanzani's experiment disproving spontaneous generation of microorganisms.
- Recreate and interpret Pasteur's experiment disproving spontaneous generation of microorganisms.

The Myth of Spontaneous Generation

Spontaneous generation theory is a myth. It is a false idea that was once a widely accepted belief in the scientific community. Its ideas were so strong that it took multiple experiments by several different scientists to disprove the theory and start to shift the mindset about how living things arise.

Spontaneous generation theory says living things can arise or come from non-living things. In the microbial world, it was believed the microorganisms could be created from a fluid such as a beef broth. This is because a beef broth left out would inevitably begin growing microorganisms that scientists observed using their microscopes. These microbes *did not come from the broth* however as the scientists of the time believed. In reality, *the microorganisms were either in the broth already (in small amounts) or landed in the broth from the air*. Once even one cell was present in the beef broth, the broth could be used by the cells as a source of nutrients they used to be able to grow, divide, and produce a robust population of microorganisms that could then be seen with the microscope.

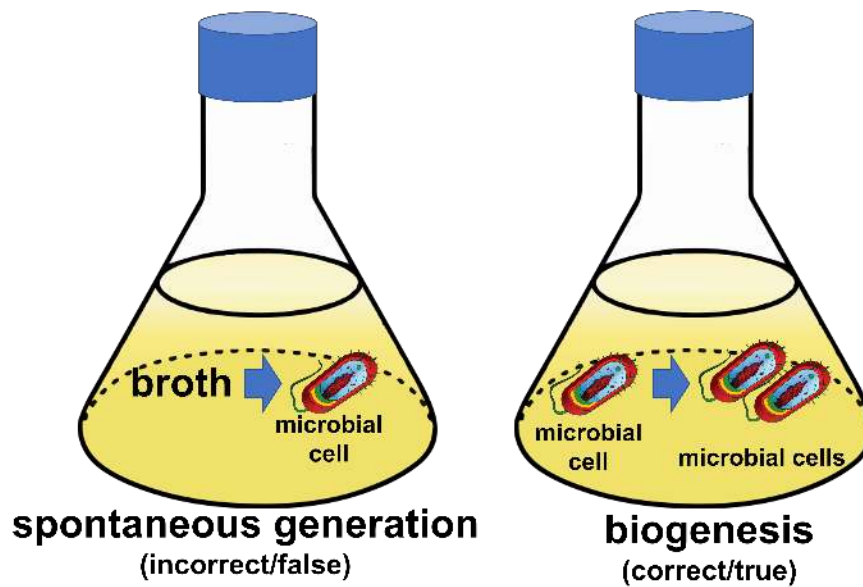


Figure 1: (Left) Spontaneous generation is an incorrect/false theory where scientists of the past believed that a liquid broth could create microbial cells from the broth itself (life coming from non-living matter). (Right) In reality, cells come from other cells (cell theory) or life comes from life (theory of biogenesis). A microbial cell divides to produce other microbial cells. The theory of biogenesis is the correct/true theory currently accepted today.

Instead of the theory of Spontaneous Generation, today the **theory of biogenesis** is currently accepted by the scientific community. This theory says that **life comes from life**. In other words, living things do not arise from non-living matter. A related theory that is also currently accepted is Cell Theory and it says that **cells come from other cells** (among other things).

Historical Experiments Disproving Spontaneous Generation of Microorganisms

Spallanzani's Experiment Disproving Spontaneous Generation Theory of Microorganisms

Lazzaro Spallanzani was an Italian priest who re-examined the spontaneous generation of microorganisms (e.g. bacteria) using a nutrient-rich broth such as a meat broth. He designed and conducted a famous experiment that began to question the validity of spontaneous generation theory.

A nutrient broth that is not sterile will eventually show microbial growth by becoming cloudy unless it is sterilized and microbes are prevented from entering the broth (usually by sealing the container). Cells present in the broth will use the broth as nutrients, divide, and produce a robust population of microorganisms. The broth can be examined using the microscope so that microorganisms growing there can be observed. A broth that begins clear that contains one or more cells (or is exposed to the environment where cells can enter the broth through the air), given time for microbial growth, will become **turbid** (cloudy or thick).

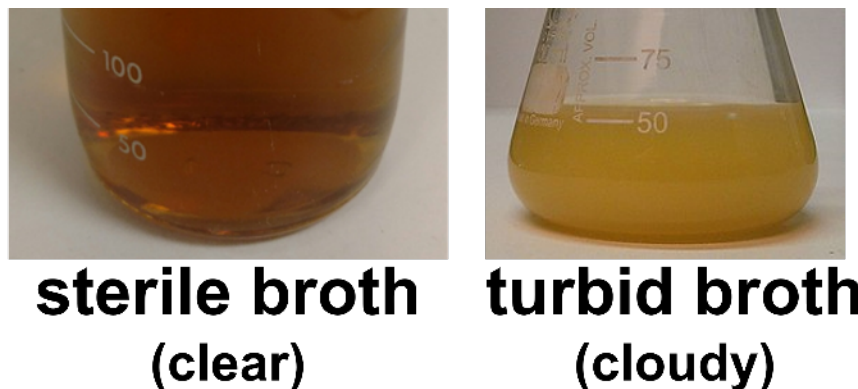


Figure 2: Comparison of sterile microbiology medium (left) that is a clear liquid and a turbid medium (right) that is a cloudy liquid. The sterile medium lacks live microorganisms. The turbid broth contains a dense population of microorganisms.

Spallanzani boiled nutrient broth to kill the microorganisms (sterilization). He compared covered and uncovered boiled nutrient broths to see if the broths would become turbid (cloudy), indicating microbial growth. What Spallanzani observed was the uncovered boiled broth became turbid over time since microorganisms were able to enter the broth from the air. The covered boiled broth however did not become turbid since microorganisms could not enter the sterile broth. This result indicated microorganisms were in the air and would contaminate a broth if exposed.

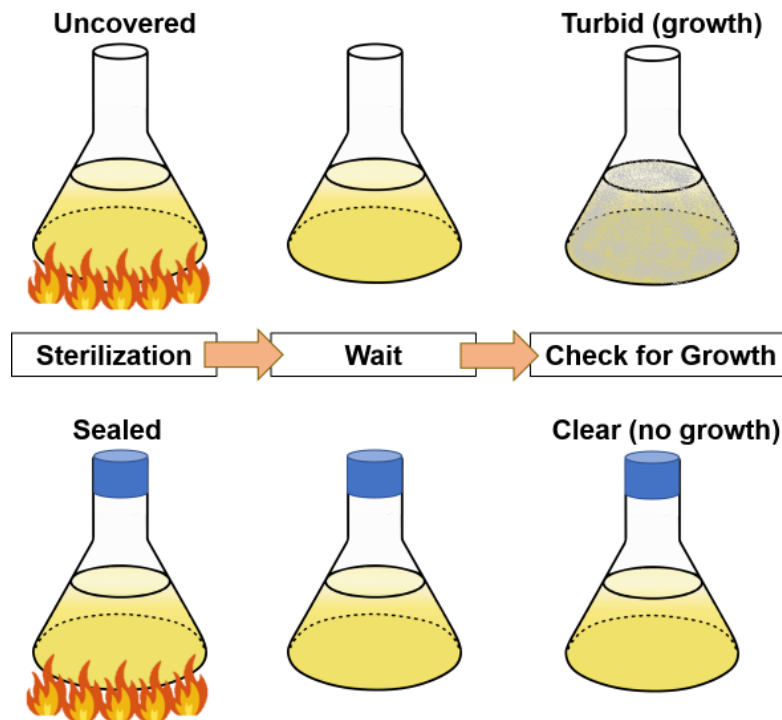


Figure 3: Diagram showing Spallanzani's experimental setup and results. (Top) An uncovered flask containing clear broth is sterilized by boiling. After a period of time (hours/days) the flask is re-examined and found to be turbid. This turbidity is microbial growth in the uncovered flask. Microbes from the air were able to land in the sterile broth and begin growing and dividing to produce a microbial population/community that appears turbid. (Bottom) A covered flask containing clear broth is sterilized by boiling. After a period of time (hours/days) the flask is re-examined and found to be clear. This clear appearance in the sealed flask is an indication that medium remained sterile. Since the sterile medium was sealed, microbes could not land in the medium and begin growing and dividing. As a result, the medium remained sterile.

The scientific community, having believed that spontaneous generation was a fact of nature, still resisted the possibility that spontaneous generation is not possible for microorganisms. The argument was that the sealed flask in Spallanzani's experiment was closed off from an oxygen (O_2). Perhaps microorganisms could not grow in the broth because there was not enough O_2 present in the flask? As a result, spontaneous generation theory persisted.

Pasteur's Swan-Neck Experiment Disproving Spontaneous Generation Theory of Microorganisms

Louis Pasteur designed and conducted an experiment that provided strong evidence disproving spontaneous generation of microorganisms. Pasteur used a flask with a swan-neck that could prevent microbes, including those attached to dust particles in the air, from reaching the sterile nutrient broth while enabling O_2 to pass into the flask. Since Spallanzani's experiment was scrutinized because O_2 could not reach the nutrient broth. The fact that Pasteur developed a flask that allowed O_2 to the broth and not microbes in the air created the conditions the scientific community needed to accept that spontaneous generation of microorganisms does not occur (microbes do not come from non-living matter).

Figure 4: Animation showing Pastures' swan-neck experiment. Two flasks are compared to establish the validity of opposing scientific theories: spontaneous generation theory and biogenesis theory. Two swan-neck flasks are prepared with nutrient broth. Both are sterilized by boiling. One flask the swan-neck is unaltered (experimental) and the other flask the swan-neck is broken off (control). The flask with the swan neck remains clear and sterile. The flask with the broken swan-neck became turbid. This indicated that cells must be introduced into a nutrient broth before microbial growth will occur.

In Pasteur's experiment, a nutrient broth is sterilized in a flask with a swan-neck tube attached. The swan-neck enabled O₂ to reach the broth. The low dip in the swan neck traps dust and microbes and prevents them from reaching the broth. After sterilization, the flask with the swan-neck stayed sterile indefinitely, despite being open to the air. This design effectively trapped microbe-carrying dust particles that could contaminate a sterile growth medium (i.e. introduce microorganisms to the sterile growth medium). If the swan-neck tube was broken off, the result is a direct path for microbes in the air and attached to dust particles to enter the growth medium. Given time, a sterilized flask with the swan-neck broken off became turbid (cloudy), indicating microbial growth. These results indicated that microorganisms come from other microorganisms and that microorganisms do not come from non-living broth. This result conclusively settled the dispute about spontaneous generation: spontaneous generation theory was incorrect and biogenesis theory is correct.

Tyndall's Discovery and Endospores

Pasteur's swan-necked experiment was then challenged when John Tyndall, an Irish scientist, repeated Pasteur's experiment and found some boiled growth media remained sterile while others did not, despite very long boiling times. Through a series of experiments, Tyndall found that nutrient broth can contain heat-resistant microbes (now known as **endospores** – discovered by Ferdinand Cohn the same year as Tyndall's work). Endospores can be produced from certain bacterial species (e.g. *Bacillus* sp. and *Clostridium* sp.) when growth/nutrient conditions are poor. Endospores are analagous to a survival bunker where bacterial cells transfer their DNA into a protective structure to survive the harsh conditions. Endospores can change back into normal bacterial cells (called **vegetative cells** [these are active bacterial cells whereas endospores are an inactive form]) if/when environmental conditions improve and are better for the microbes to grow.

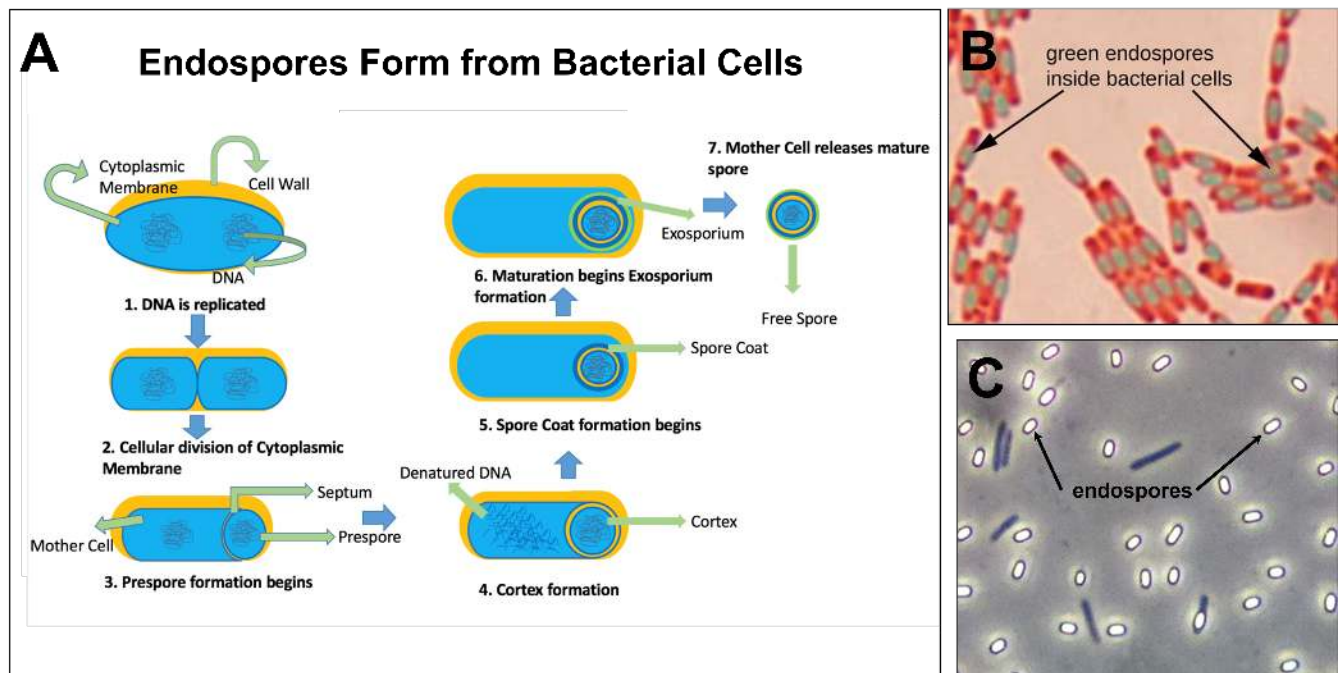


Figure 5: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore coat formation begins, 6. maturation of exosporium formation, 7. mother cell releases mature spore. (B) Microscopic image prepared with an endospore stain shows bacterial cells (pink) with forming spore inside of them (green). (C) Microscopic image showing endospores.

Tyndall developed a process that he found completely sterilized growth media, including killing those containing heat-resistant microbes (endospores). This process is a lengthy series of steps involving repeatedly heating then resting the medium multiple times. This repeated heating and resting process insures sterilization of a medium.



Figure 6: An autoclave. Autoclaves provide a rapid means of sterilization using pressure and heat and they come in different shapes and sizes. This is an example of a free-standing autoclave with media bottles inside ready to be sterilized.

More recently, it has been found endospores can also be killed when medium is heated under pressure. The typical method of sterilization used in laboratories today uses an **autoclave**, a device that places materials to be sterilized under pressure while heating. What used to take a few days to sterilize medium with Tyndall's process now can be sterilized in about an hour.

Attributions

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3.1: Lab Foundations of Modern Cell Theory

Learning Objectives

- Explain the key points of cell theory and the individual contributions of Hooke, Schleiden, Schwann, Remak, and Virchow
- Explain the key points of endosymbiotic theory and cite the evidence that supports this concept
- Explain the contributions of Semmelweis, Snow, Pasteur, Lister, and Koch to the development of germ theory

While some scientists were arguing over the theory of spontaneous generation, other scientists were making discoveries leading to a better understanding of what we now call the cell theory. Modern cell theory has two basic tenets:

- All cells only come from other cells (the principle of biogenesis).
- Cells are the fundamental units of organisms.

Today, these tenets are fundamental to our understanding of life on earth. However, modern cell theory grew out of the collective work of many scientists.

The Origins of Cell Theory

The English scientist Robert Hooke first used the term “cells” in 1665 to describe the small chambers within cork that he observed under a microscope of his own design. To Hooke, thin sections of cork resembled “Honey-comb,” or “small Boxes or Bladders of Air.” He noted that each “Cavern, Bubble, or Cell” was distinct from the others (Figure 3.1.1). At the time, Hooke was not aware that the cork cells were long dead and, therefore, lacked the internal structures found within living cells.

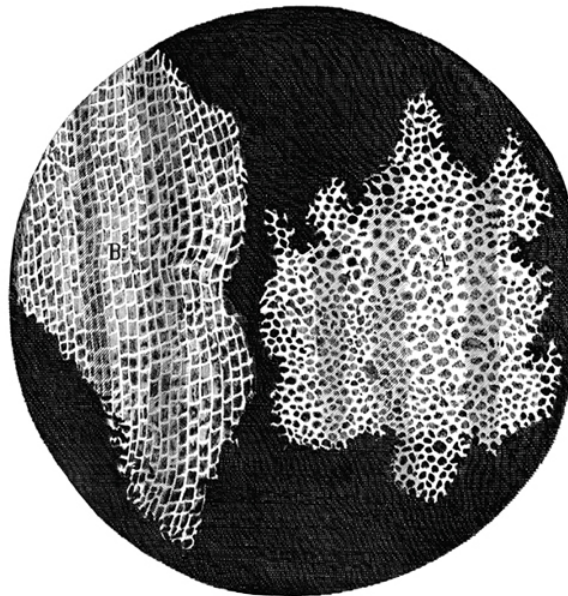


Figure 3.1.1: Robert Hooke (1635–1703) was the first to describe cells based upon his microscopic observations of cork. This illustration was published in his work *Micrographia*.

Despite Hooke’s early description of cells, their significance as the fundamental unit of life was not yet recognized. Nearly 200 years later, in 1838, Matthias Schleiden (1804–1881), a German botanist who made extensive microscopic observations of plant tissues, described them as being composed of cells. Visualizing plant cells was relatively easy because plant cells are clearly separated by their thick cell walls. Schleiden believed that cells formed through crystallization, rather than cell division.

Theodor Schwann (1810–1882), a noted German physiologist, made similar microscopic observations of animal tissue. In 1839, after a conversation with Schleiden, Schwann realized that similarities existed between plant and animal tissues. This laid the foundation for the idea that cells are the fundamental components of plants and animals.

In the 1850s, two Polish scientists living in Germany pushed this idea further, culminating in what we recognize today as the modern cell theory. In 1852, Robert Remak (1815–1865), a prominent neurologist and embryologist, published convincing

evidence that cells are derived from other cells as a result of cell division. However, this idea was questioned by many in the scientific community. Three years later, Rudolf Virchow (1821–1902), a well-respected pathologist, published an editorial essay entitled “Cellular Pathology,” which popularized the concept of cell theory using the Latin phrase *omnis cellula a cellula* (“all cells arise from cells”), which is essentially the second tenet of modern cell theory.¹ Given the similarity of Virchow’s work to Remak’s, there is some controversy as to which scientist should receive credit for articulating cell theory. See the following Eye on Ethics feature for more about this controversy.

Science and Plagiarism

Rudolf Virchow, a prominent, Polish-born, German scientist, is often remembered as the “Father of Pathology.” Well known for innovative approaches, he was one of the first to determine the causes of various diseases by examining their effects on tissues and organs. He was also among the first to use animals in his research and, as a result of his work, he was the first to name numerous diseases and created many other medical terms. Over the course of his career, he published more than 2,000 papers and headed various important medical facilities, including the Charité – Universitätsmedizin Berlin, a prominent Berlin hospital and medical school. But he is, perhaps, best remembered for his 1855 editorial essay titled “Cellular Pathology,” published in *Archiv für Pathologische Anatomie und Physiologie*, a journal that Virchow himself cofounded and still exists today.

Despite his significant scientific legacy, there is some controversy regarding this essay, in which Virchow proposed the central tenet of modern cell theory—that all cells arise from other cells. Robert Remak, a former colleague who worked in the same laboratory as Virchow at the University of Berlin, had published the same idea 3 years before. Though it appears Virchow was familiar with Remak’s work, he neglected to credit Remak’s ideas in his essay. When Remak wrote a letter to Virchow pointing out similarities between Virchow’s ideas and his own, Virchow was dismissive. In 1858, in the preface to one of his books, Virchow wrote that his 1855 publication was just an editorial piece, not a scientific paper, and thus there was no need to cite Remak’s work.



(a)



(b)

Figure 3.1.2: (a) Rudolf Virchow (1821–1902) popularized the cell theory in an 1855 essay entitled “Cellular Pathology.” (b) The idea that all cells originate from other cells was first published in 1852 by his contemporary and former colleague Robert Remak (1815–1865).

By today’s standards, Virchow’s editorial piece would certainly be considered an act of plagiarism, since he presented Remak’s ideas as his own. However, in the 19th century, standards for academic integrity were much less clear. Virchow’s strong reputation, coupled with the fact that Remak was a Jew in a somewhat anti-Semitic political climate, shielded him from any significant repercussions. Today, the process of peer review and the ease of access to the scientific literature help discourage plagiarism. Although scientists are still motivated to publish original ideas that advance scientific knowledge, those who would consider plagiarizing are well aware of the serious consequences.

In academia, plagiarism represents the theft of both individual thought and research—an offense that can destroy reputations and end careers.^{2 3 4 5}

? Exercise 3.1.1

1. What are the key points of the cell theory?
2. What contributions did Rudolf Virchow and Robert Remak make to the development of the cell theory?

Endosymbiotic Theory

As scientists were making progress toward understanding the role of cells in plant and animal tissues, others were examining the structures within the cells themselves. In 1831, Scottish botanist Robert Brown (1773–1858) was the first to describe observations of nuclei, which he observed in plant cells. Then, in the early 1880s, German botanist Andreas Schimper (1856–1901) was the first to describe the chloroplasts of plant cells, identifying their role in starch formation during photosynthesis and noting that they divided independent of the nucleus.

Based upon the chloroplasts' ability to reproduce independently, Russian botanist Konstantin Mereshchkowski (1855–1921) suggested in 1905 that chloroplasts may have originated from ancestral photosynthetic bacteria living symbiotically inside a eukaryotic cell. He proposed a similar origin for the nucleus of plant cells. This was the first articulation of the endosymbiotic hypothesis, and would explain how eukaryotic cells evolved from ancestral bacteria.

Mereschkowski's endosymbiotic hypothesis was furthered by American anatomist Ivan Wallin (1883–1969), who began to experimentally examine the similarities between mitochondria, chloroplasts, and bacteria—in other words, to put the endosymbiotic hypothesis to the test using objective investigation. Wallin published a series of papers in the 1920s supporting the endosymbiotic hypothesis, including a 1926 publication co-authored with Mereshchkowski. Wallin claimed he could culture mitochondria outside of their eukaryotic host cells. Many scientists dismissed his cultures of mitochondria as resulting from bacterial contamination. Modern genome sequencing work supports the dissenting scientists by showing that much of the genome of mitochondria had been transferred to the host cell's nucleus, preventing the mitochondria from being able to live on their own.^{6 7}

Wallin's ideas regarding the endosymbiotic hypothesis were largely ignored for the next 50 years because scientists were unaware that these organelles contained their own DNA. However, with the discovery of mitochondrial and chloroplast DNA in the 1960s, the endosymbiotic hypothesis was resurrected. Lynn Margulis (1938–2011), an American geneticist, published her ideas regarding the endosymbiotic hypothesis of the origins of mitochondria and chloroplasts in 1967.⁸ In the decade leading up to her publication, advances in microscopy had allowed scientists to differentiate prokaryotic cells from eukaryotic cells. In her publication, Margulis reviewed the literature and argued that the eukaryotic organelles such as mitochondria and chloroplasts are of prokaryotic origin. She presented a growing body of microscopic, genetic, molecular biology, fossil, and geological data to support her claims.

Again, this hypothesis was not initially popular, but mounting genetic evidence due to the advent of DNA sequencing supported the endosymbiotic theory, which is now defined as the theory that mitochondria and chloroplasts arose as a result of prokaryotic cells establishing a symbiotic relationship within a eukaryotic host (Figure 3.1.3). With Margulis' initial endosymbiotic theory gaining wide acceptance, she expanded on the theory in her 1981 book *Symbiosis in Cell Evolution*. In it, she explains how endosymbiosis is a major driving factor in the evolution of organisms. More recent genetic sequencing and phylogenetic analysis show that mitochondrial DNA and chloroplast DNA are highly related to their bacterial counterparts, both in DNA sequence and chromosome structure. However, mitochondrial DNA and chloroplast DNA are reduced compared with nuclear DNA because many of the genes have moved from the organelles into the host cell's nucleus. Additionally, mitochondrial and chloroplast ribosomes are structurally similar to bacterial ribosomes, rather than to the eukaryotic ribosomes of their hosts. Last, the binary fission of these organelles strongly resembles the binary fission of bacteria, as compared with mitosis performed by eukaryotic cells. Since Margulis' original proposal, scientists have observed several examples of bacterial endosymbionts in modern-day eukaryotic cells. Examples include the endosymbiotic bacteria found within the guts of certain insects, such as cockroaches,⁹ and photosynthetic bacteria-like organelles found in protists.¹⁰

The Endosymbiotic Theory

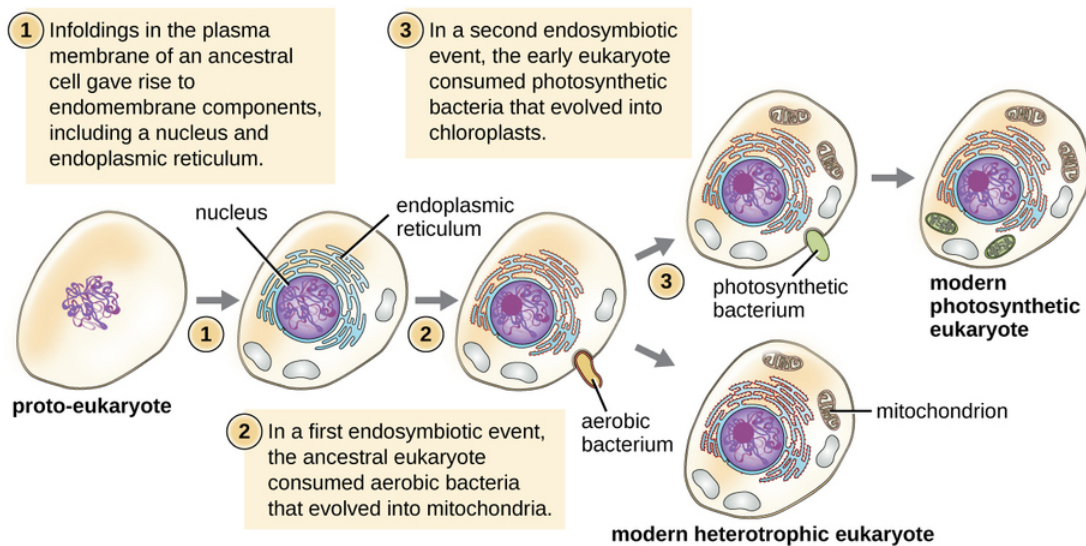


Figure 3.1.3: According to the endosymbiotic theory, mitochondria and chloroplasts are each derived from the uptake of bacteria. These bacteria established a symbiotic relationship with their host cell that eventually led to the bacteria evolving into mitochondria and chloroplasts.

? Exercise 3.1.2

1. What does the modern endosymbiotic theory state?
2. What evidence supports the endosymbiotic theory?

The Germ Theory of Disease

Prior to the discovery of microbes during the 17th century, other theories circulated about the origins of disease. For example, the ancient Greeks proposed the miasma theory, which held that disease originated from particles emanating from decomposing matter, such as that in sewage or cesspits. Such particles infected humans in close proximity to the rotting material. Diseases including the Black Death, which ravaged Europe's population during the Middle Ages, were thought to have originated in this way.

In 1546, Italian physician Girolamo Fracastoro proposed, in his essay *De Contagione et Contagiosis Morbis*, that seed-like spores may be transferred between individuals through direct contact, exposure to contaminated clothing, or through the air. We now recognize Fracastoro as an early proponent of the germ theory of disease, which states that diseases may result from microbial infection. However, in the 16th century, Fracastoro's ideas were not widely accepted and would be largely forgotten until the 19th century.

In 1847, Hungarian obstetrician Ignaz Semmelweis (Figure 3.1.4) observed that mothers who gave birth in hospital wards staffed by physicians and medical students were more likely to suffer and die from puerperal fever after childbirth (10%–20% mortality rate) than were mothers in wards staffed by midwives (1% mortality rate). Semmelweis observed medical students performing autopsies and then subsequently carrying out vaginal examinations on living patients without washing their hands in between. He suspected that the students carried disease from the autopsies to the patients they examined. His suspicions were supported by the untimely death of a friend, a physician who contracted a fatal wound infection after a postmortem examination of a woman who had died of a puerperal infection. The dead physician's wound had been caused by a scalpel used during the examination, and his subsequent illness and death closely paralleled that of the dead patient.

Although Semmelweis did not know the true cause of puerperal fever, he proposed that physicians were somehow transferring the causative agent to their patients. He suggested that the number of puerperal fever cases could be reduced if physicians and medical students simply washed their hands with chlorinated lime water before and after examining every patient. When this practice was implemented, the maternal mortality rate in mothers cared for by physicians dropped to the same 1% mortality rate observed among mothers cared for by midwives. This demonstrated that handwashing was a very effective method for preventing disease transmission. Despite this great success, many discounted Semmelweis's work at the time, and physicians were slow to adopt the

simple procedure of handwashing to prevent infections in their patients because it contradicted established norms for that time period.

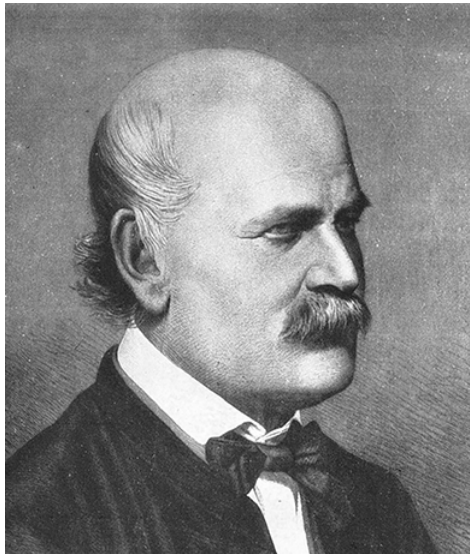


Figure 3.1.4: Ignaz Semmelweis (1818–1865) was a proponent of the importance of handwashing to prevent transfer of disease between patients by physicians.

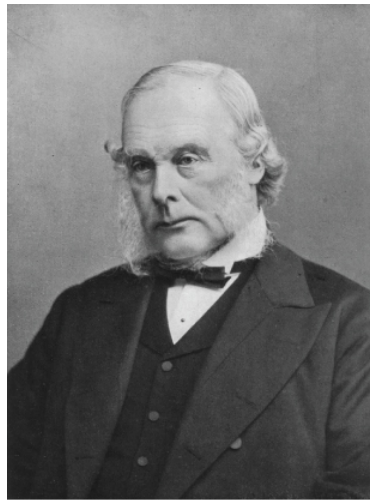
Around the same time Semmelweis was promoting handwashing, in 1848, British physician John Snow conducted studies to track the source of cholera outbreaks in London. By tracing the outbreaks to two specific water sources, both of which were contaminated by sewage, Snow ultimately demonstrated that cholera bacteria were transmitted via drinking water. Snow's work is influential in that it represents the first known epidemiological study, and it resulted in the first known public health response to an epidemic. The work of both Semmelweis and Snow clearly refuted the prevailing miasma theory of the day, showing that disease is not only transmitted through the air but also through contaminated items.

Although the work of Semmelweis and Snow successfully showed the role of sanitation in preventing infectious disease, the cause of disease was not fully understood. The subsequent work of Louis Pasteur, Robert Koch, and Joseph Lister would further substantiate the germ theory of disease.

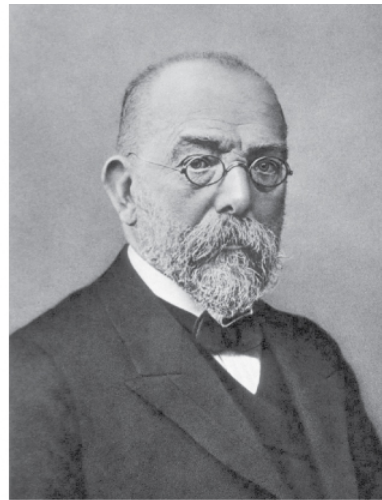
While studying the causes of beer and wine spoilage in 1856, Pasteur discovered properties of fermentation by microorganisms. He had demonstrated with his swan-neck flask experiments ([link](#)) that airborne microbes, not spontaneous generation, were the cause of food spoilage, and he suggested that if microbes were responsible for food spoilage and fermentation, they could also be responsible for causing infection. This was the foundation for the germ theory of disease.

Meanwhile, British surgeon Joseph Lister (Figure 3.1.5) was trying to determine the causes of postsurgical infections. Many physicians did not give credence to the idea that microbes on their hands, on their clothes, or in the air could infect patients' surgical wounds, despite the fact that 50% of surgical patients, on average, were dying of postsurgical infections.¹¹ Lister, however, was familiar with the work of Semmelweis and Pasteur; therefore, he insisted on handwashing and extreme cleanliness during surgery. In 1867, to further decrease the incidence of postsurgical wound infections, Lister began using carbolic acid (phenol) spray disinfectant/antiseptic during surgery. His extremely successful efforts to reduce postsurgical infection caused his techniques to become a standard medical practice.

A few years later, Robert Koch (Figure 3.1.5) proposed a series of postulates (Koch's postulates) based on the idea that the cause of a specific disease could be attributed to a specific microbe. Using these postulates, Koch and his colleagues were able to definitively identify the causative pathogens of specific diseases, including anthrax, tuberculosis, and cholera. Koch's "one microbe, one disease" concept was the culmination of the 19th century's paradigm shift away from miasma theory and toward the germ theory of disease. Koch's postulates are discussed more thoroughly in [How Pathogens Cause Disease](#).



(a)



(b)

Figure 3.1.5: (a) Joseph Lister developed procedures for the proper care of surgical wounds and the sterilization of surgical equipment. (b) Robert Koch established a protocol to determine the cause of infectious disease. Both scientists contributed significantly to the acceptance of the germ theory of disease.

? Exercise 3.1.3

1. Compare and contrast the miasma theory of disease with the germ theory of disease.
2. How did Joseph Lister's work contribute to the debate between the miasma theory and germ theory and how did this increase the success of medical procedures?

📌 Clinical Focus: Part 2

After suffering a fever, congestion, cough, and increasing aches and pains for several days, Barbara suspects that she has a case of the flu. She decides to visit the health center at her university. The PA tells Barbara that her symptoms could be due to a range of diseases, such as influenza, bronchitis, pneumonia, or tuberculosis.

During her physical examination, the PA notes that Barbara's heart rate is slightly elevated. Using a pulse oximeter, a small device that clips on her finger, he finds that Barbara has hypoxemia—a lower-than-normal level of oxygen in the blood. Using a stethoscope, the PA listens for abnormal sounds made by Barbara's heart, lungs, and digestive system. As Barbara breathes, the PA hears a crackling sound and notes a slight shortness of breath. He collects a sputum sample, noting the greenish color of the mucus, and orders a chest radiograph, which shows a "shadow" in the left lung. All of these signs are suggestive of pneumonia, a condition in which the lungs fill with mucus (Figure 3.1.6).



lung infiltrated, suggestive of pneumonia



normal lungs

Figure 3.1.6: This is a chest radiograph typical of pneumonia. Because X-ray images are negative images, a “shadow” is seen as a white area within the lung that should otherwise be black. In this case, the left lung shows a shadow as a result of pockets in the lung that have become filled with fluid. (credit left: modification of work by “Christaras A”/Wikimedia Commons)

Exercise 3.1.4

What kinds of infectious agents are known to cause pneumonia?

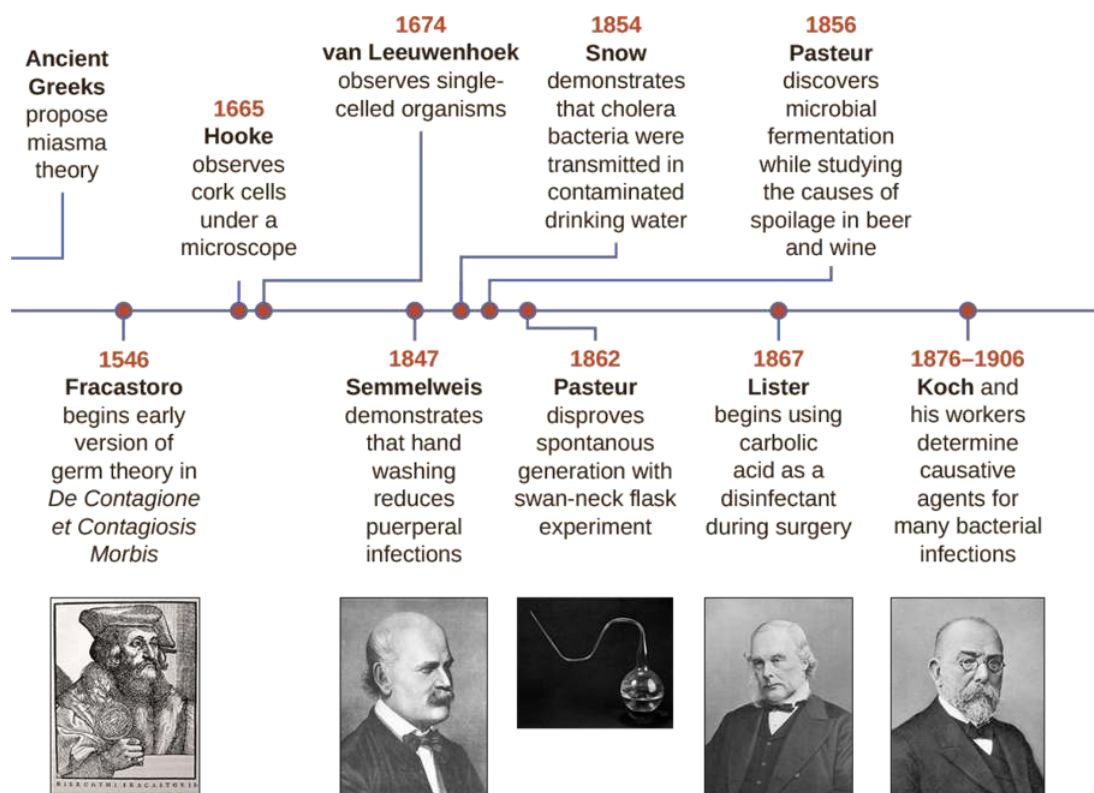


Figure 3.1.7: (credit “swan-neck flask”: modification of work by Wellcome Images)

Lab: The Cell Theory Journal

BACKGROUND INFORMATION

Imagine life before the discovery of cells, when diseases attacked the human population, unseen and undetected by doctors and scientists of the time. Diseases like the Black Plague, caused by a bacterium, brought about one of the greatest pandemics in human history. The Irish Potato Famine was caused by a unicellular fungus that infected potatoes, thereby destroying one of the country's main food sources and resulting in mass starvation.

The invention of the microscope allowed for the discovery of cells and unicellular organisms, opening scientists' eyes to a whole other world - the world of microorganisms. The study of microorganisms is known as microbiology. The microscope opened the doors to many modern fields of science, including:

Microscopy - the study and use of microscopes

Bacteriology - the study of bacteria **Mycology** - the study of fungi **Parasitology** - the study of parasites **Virology** - the study of viruses **Botany** - the study of plants **Cytology** - the study of cells

In the nearly four hundred years since Robert Hooke identified the cell, the work of other scientists led to the development of the **Cell Theory**.

The Cell Theory states three points:

1. *All living things are made up of cells.*
2. *Cells are the basic unit of structure and function in living things.*
3. *All cells come from pre-existing cells.*

By studying these early scientists and their discoveries, you will be taking a scientific journey back in time. As you view each slide, imagine that what you see is what each of the scientists saw through the ocular lenses of their primitive microscopes!

This activity consists of 8 stations. Find the following materials at each station:

MATERIALS:

- Microscope
- Slide

PROCEDURE:

1. Go to the station for your group, as designated by the instructor.
2. Once at the station, find the write-up for that station number on your Lab Report.

At each station:

1. The historian should read the narrative for that station. Take turns reading so that each member of your group is the historian at least twice.
2. Listen carefully to the narrative, as it sets the stage for that scientist's work.
3. Write the scientist's name on the blank line, next to the date of the discovery.
4. Have each person in your group take a turn looking through the microscope at the station. When it is your turn, look through the microscope and then, in the circle for the appropriate station number on the worksheet, sketch what you see. In the space below the circle, record the magnification used.
5. In your own words, describe what you see, under 'written observations'. In your description, be sure to note the name of the slide you are observing.

Your instructor will give you a designated amount of time at each station. When time is up, proceed to the next numbered station. For example, if your group began at station #6, your group should now proceed to station #7. The group at station #8 should proceed to station #1

Use this format for completing the write ups:

1978	Scientist: Their name	Magnification: 40x, 100x, or 400x
Written Observations:	Describe what you see here and be as detailed as possible	Draw your slide here: Label as needed

Station #1:

Narrative: An English scientist named made a primitive microscope and used it to look at a thin piece of cork (a piece of bark from a cork oak tree). He described the structures that he saw as cells, coming from the Latin word 'cella', meaning "small rooms". He wrote about what he saw in a book called *Micrographia*, published in 1665. In your sketch of the cork cells that you observe at this station, label one of the "small rooms" that you see "cell".

1665	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #2:

Narrative: was a fabric merchant from Holland. He was interested in lenses and used them to count threads in woven cloth. He became so interested in lenses that he learned how to make his own

by grinding and polishing pieces of glass. He noticed that if he made smaller lenses with greater arcs, the lenses produced greater magnification. Although he did not invent the microscope, he improved the microscopes of his day by increasing the magnification. His microscopes were able to magnify over 200 times what the unaided eye could observe and Leeuwenhoek was able to see things that no one had seen before. In 1674, he observed algae and many swimming 'animalcules' (tiny animals or as we know them, protists) in a drop of water. Because of his discoveries, he is often referred to as the father of microbiology. The long string of cells you will view at this station are called *Spirogyra*. *Spirogyra* are green algae that are often found in pond water. Count how many cells appear in your field of view and note that number in your observations. *Hint: The individual cells may be easier to see in some strings than in others.*

1674	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #3:

Narrative: was a French botanist (a scientist who studies plants). In 1809, he observed that plants are made up of cells that form a continuous membrane. For this and other observations concerning plant and animal cells, he is known as the founder of cytology (the study of cells). The cells you will view at this station are generalized plant cells. Because these are plant cells, you will see a box- like structure surrounding each cell. This structure is known as the cell wall. In your sketch, label the "cell wall".

1809	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #4:

Narrative: was a French soldier and professor. Lamarck observed that all living things (plant or animal) are made up of cellular tissue. Lamarck is much better known for his work in evolution, but is no doubt a contributor to the Cell Theory by making the above observation. In the slide of a general animal cell that you will observe at this station, notice that all of the cells that make up this tissue are very similar. In your sketch, label one of these cells as an "animal cell".

1810	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #5:

Narrative: was a Scottish botanist. He named the cell **nucleus** while viewing orchid epidermal cells. The word "nucleus" is Latin for "kernel" or "little nut." He also discovered the movement of certain cells suspended in a liquid. This motion is called Brownian movement, named after him. In the *allium* or onion epidermal cells that you will view at this station, notice the small dark structures near the center of each cell. This is the nucleus of the cell. In your sketch, label one of these structures "nucleus".

1831	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #6:

Narrative: was a German botanist. During his studies, he began to formulate the Cell Theory for plants. In his published paper of 1838, Schleiden proposed that all plants are composed of cells and that the cell is the most basic unit of plant life. He also forecasted that plant growth takes place by the generation of new cells somehow involving the nucleus. Although his idea of how cell division took place was not correct, he was definitely on the right track! In the slide of *tilia* (basswood) root cells that you will observe at this station, notice the cell wall surrounding each cell. In your sketch, label one of the cells "plant cell of plant tissue".

1838	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #7:

Narrative: was a German physiologist (a scientist who studies living organisms). He discovered cells that surround and protect the axons of nerve cells. (An axon is the part of a nerve cell where impulses travel away from the cell body.) Today these cells are named after him. He took the work of Matthias Schleiden one step further and applied the Cell Theory to plants and animals. In his publication of 1839, he stated that cells are organisms, all organisms consist of one or more cells, and the cell is the basic unit of structure for all organisms (plants and animals). He further discussed cell origin, organization, and differentiation. At this station, you will be viewing a slide of frog skin. Notice that the skin is made of many similar epidermal cells. In your sketch, label one of the cells "animal cell of animal tissue".

1839	Scientist:	Magnification:
Written Observations:		Draw your slide here:

Station #8:

Narrative: was a German doctor. His many discoveries include being the first to recognize leukemia. He also recognized the cell as the basic unit of structure and function in all living things and is known for his theory of 1858, "Omnis cellula e cellula" (Latin for "every cell originates from another existing cell like it"). The slide that you will view at this station is an *allium* or onion root tip undergoing cell division. You may be able to distinguish other stages of the cell cycle. In your sketch, find an area where one cell is clearly dividing into two and label that area "cell division".

1858	Scientist:	Magnification:
Written Observations:		Draw your slide here:

CONCLUSIONS:

Referring to the instructions online, list the three points of the **Cell Theory**, below.

- 1.
- 2.
- 3.

After the name of each scientist, note which point(s) of the Cell Theory (1, 2 and or 3) is/are supported by that scientist's work.

Robert Hooke	
Antony Leeuwenhoek	
Charles-Francois Brisseau de Mirbel	
Jean-Baptiste Lamarck	
Robert Brown	
Matthias Schleiden	
Theodor Schwann	
Rudolf Virchow	

EXTENSION ACTIVITY:

Create a time line to summarize the development of the Cell Theory.

Key Concepts and Summary

- Although cells were first observed in the 1660s by Robert Hooke, **cell theory** was not well accepted for another 200 years. The work of scientists such as Schleiden, Schwann, Remak, and Virchow contributed to its acceptance.
- Endosymbiotic theory** states that mitochondria and chloroplasts, organelles found in many types of organisms, have their origins in bacteria. Significant structural and genetic information support this theory.
- The **miasma theory of disease** was widely accepted until the 19th century, when it was replaced by the **germ theory of disease** thanks to the work of Semmelweis, Snow, Pasteur, Lister, and Koch, and others.

Footnotes

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- 2 B. Kisch. "Forgotten Leaders in Modern Medicine, Valentin, Gouby, Remak, Auerbach." *Transactions of the American Philosophical Society* 44 (1954):139–317.
- 3 H. Harris. *The Birth of the Cell*. New Haven, CT: Yale University Press, 2000:133.
- 4 C. Webster (ed.). *Biology, Medicine and Society 1840-1940*. Cambridge, UK; Cambridge University Press, 1981:118–119.
- 5 C. Zuchora-Walske. *Key Discoveries in Life Science*. Minneapolis, MN: Lerner Publishing, 2015:12–13.
- 6 T. Embley, W. Martin. "Eukaryotic Evolution, Changes, and Challenges." *Nature* Vol. 440 (2006):623–630.
- 7 O.G. Berg, C.G. Kurland. "Why Mitochondrial Genes Are Most Often Found in Nuclei." *Molecular Biology and Evolution* 17 no. 6 (2000):951–961.
- 8 L. Sagan. "On the Origin of Mitosing Cells." *Journal of Theoretical Biology* 14 no. 3 (1967):225–274.
- 9 A.E. Douglas. "The Microbial Dimension in Insect Nutritional Ecology." *Functional Ecology* 23 (2009):38–47.
- 10 J.M. Jaynes, L.P. Vernon. "The Cyanelle of *Cyanophora paradoxa*: Almost a Cyanobacterial Chloroplast." *Trends in Biochemical Sciences* 7 no. 1 (1982):22–24.
- 11 Alexander, J. Wesley. "The Contributions of Infection Control to a Century of Progress" *Annals of Surgery* 201:423-428, 1985.

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4: 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!

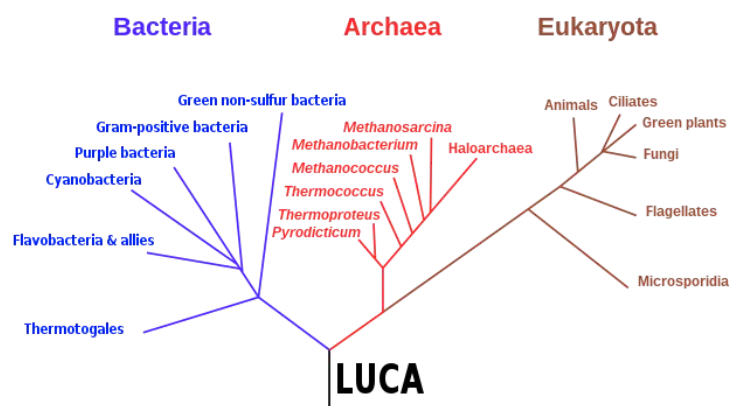
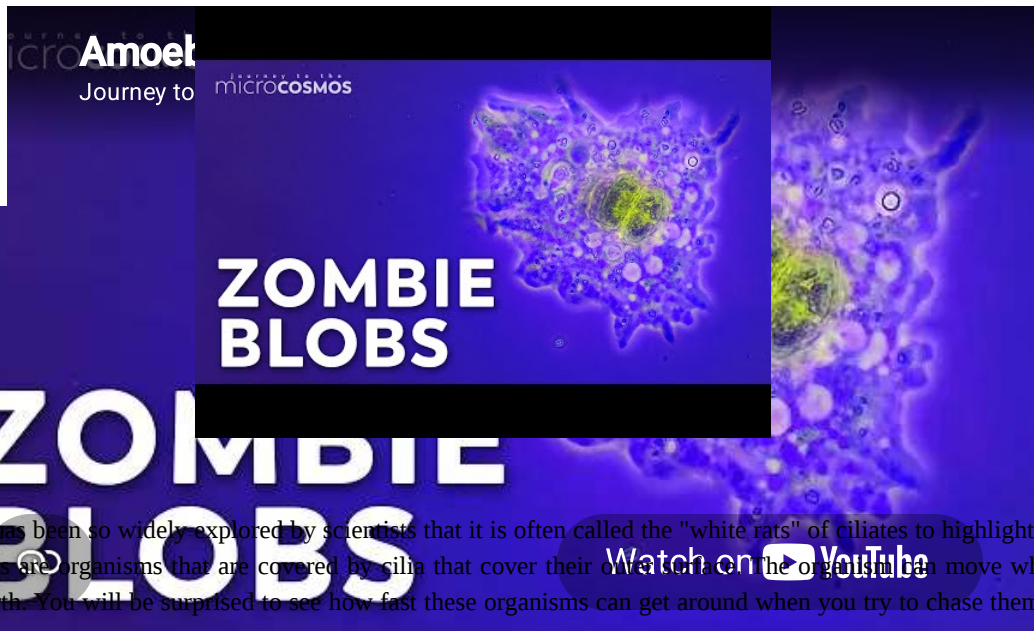


Figure 4.1: The three domains of life. Single-celled eukaryotic organisms such as protozoa and fungi are in the domain Eukaryota. Chiswick Chap, CC BY-SA 4.0, via Wikimedia Commons

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.



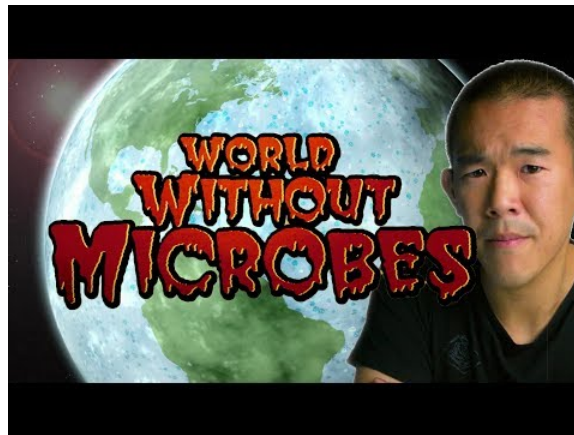
Trichonympha

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 4.2).
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.

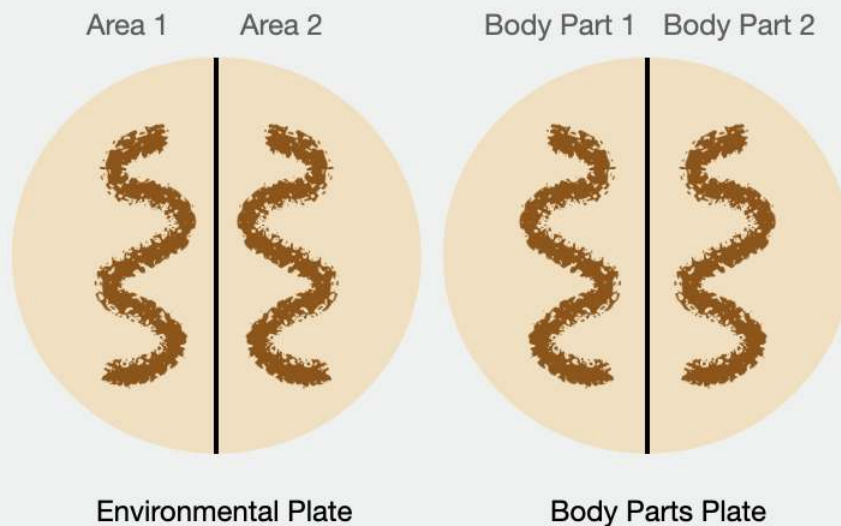


Figure 4.2: Diagram of how to prepare your environmental and body part plates

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?

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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.

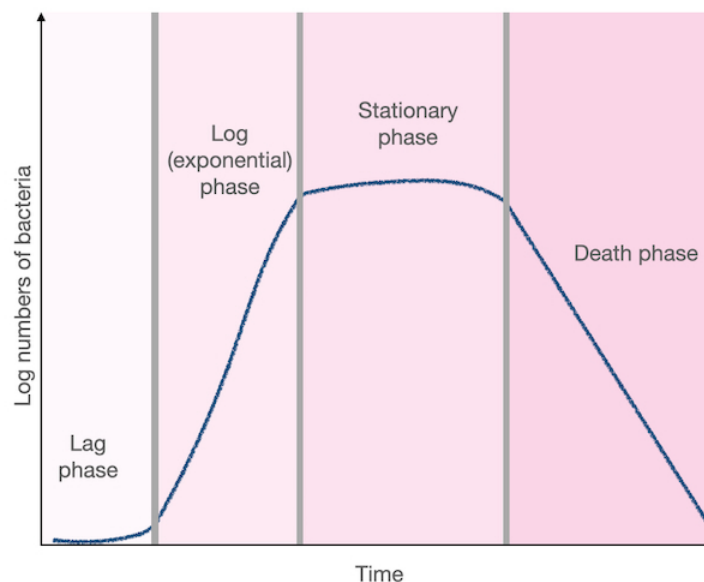


Figure 5.1: Bacterial growth curve showing the stages of growth over time. Eventually, all of the bacteria in a culture will die without subculturing into fresh media.

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.

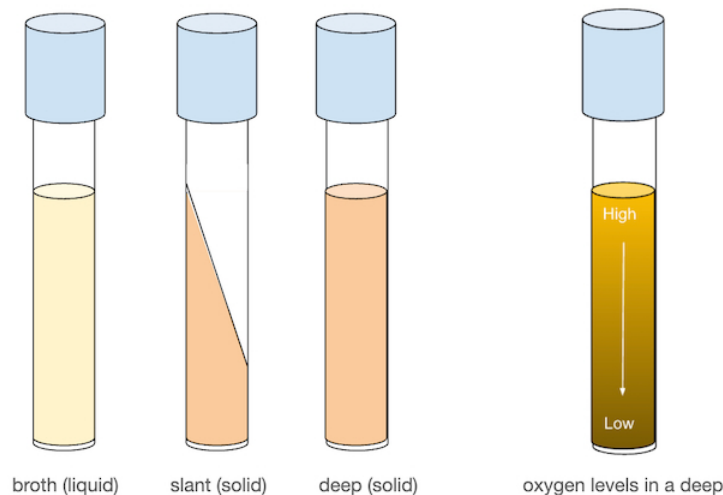


Figure 5.2: Examples of different types of media in culture tubes.

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**”.

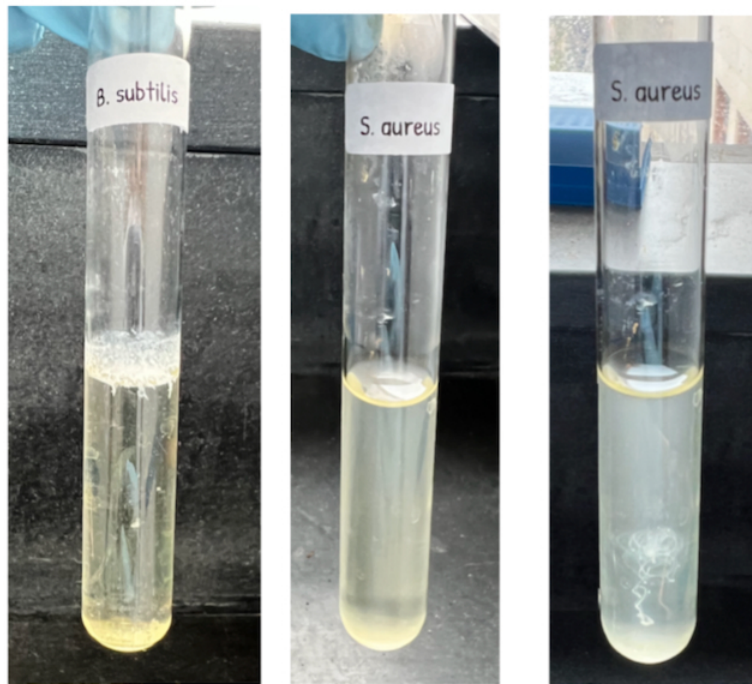


Figure 5.3: Left - a visible pellicle forms at the surface of a *B. subtilis* broth culture. Middle - characteristic turbidity seen in *S. aureus* broths. Right - sediment from the bottom of the *S. aureus* broth can be seen by gently re-suspending the cells at the bottom of the tube.

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).

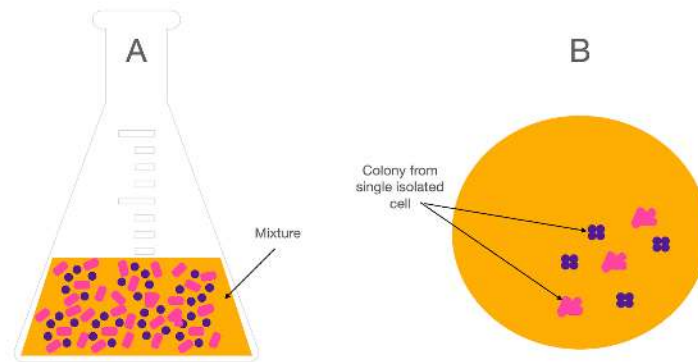


Figure 5.8: Bacteria grown in a mixed culture (A) can be isolated by streaking them out on a solid surface (B).

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)

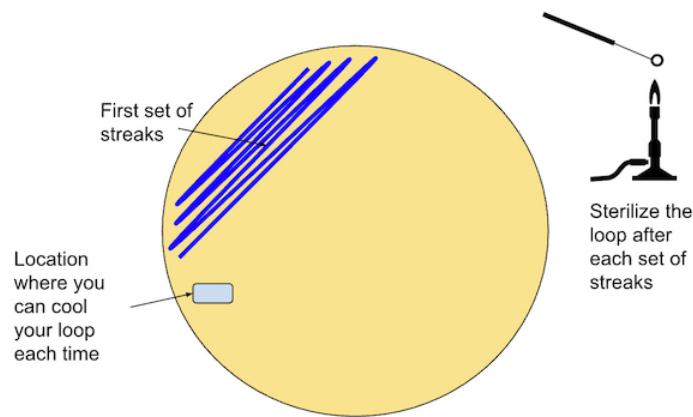


Figure 5.9

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)

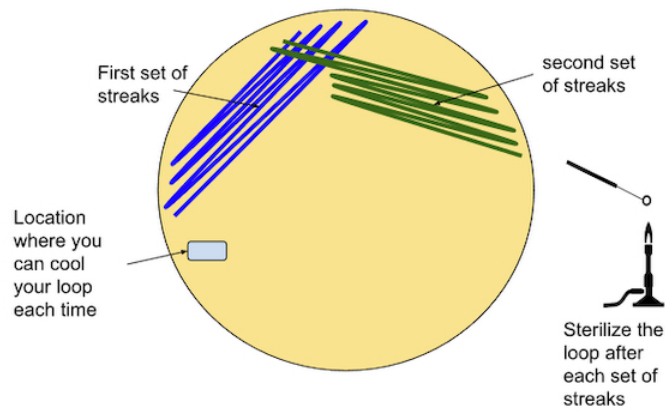


Figure 5.10

1. Sterilize the inoculating loop again.
2. Touch the loop to the agar surface (again) to cool it down.
3. Create the third set of 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)

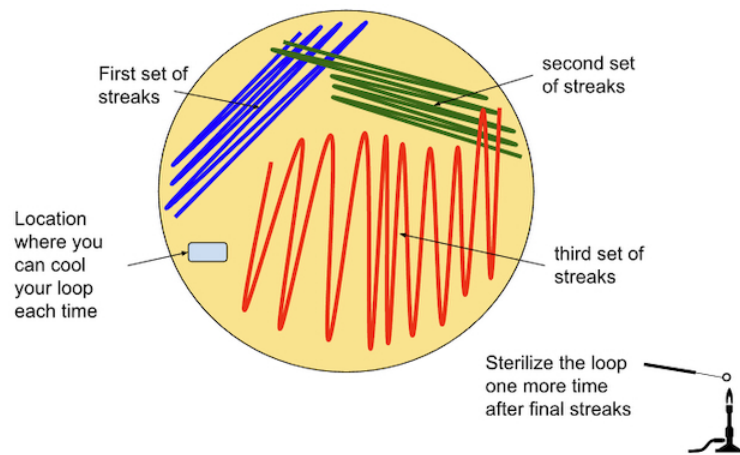


Figure 5.11

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?

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6: 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**”.

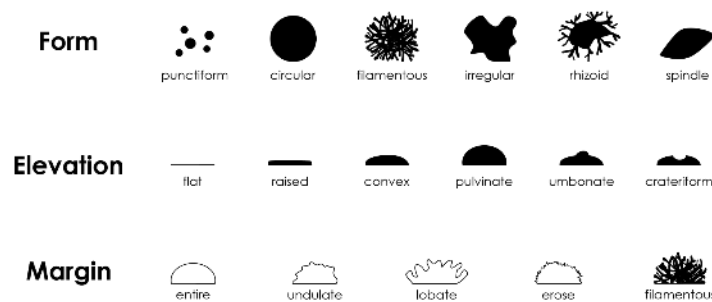


Figure 6.1: Illustration of the various ways that you can describe colony morphology of organisms grown on solid media. [Macedo, CC BY-SA 4.0](#), via Wikimedia Commons

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 6.2)



Figure 6.2: 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!).

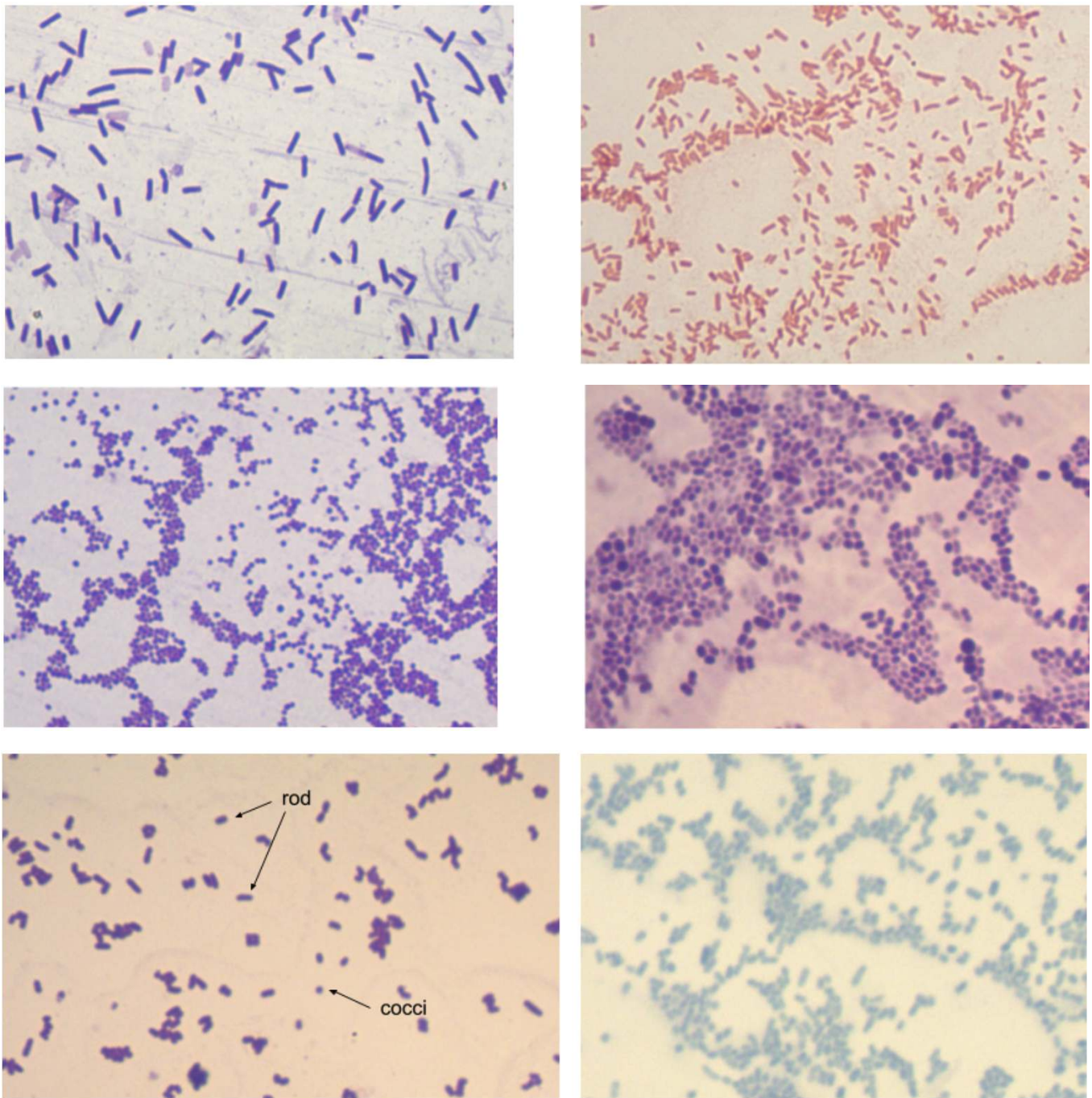


Figure 6.3: Clockwise from top left: Cell morphology of *B. cereus* stained with crystal violet, *E. coli* stained with safranin, *L. lactis* stained with crystal violet and showing coccobacillus morphology, *P. vulgaris* stained with methylene blue, *C. xerosis* stained with crystal violet showing pleomorphic 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 6.4)



Figure 6.4

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 6.5).



Figure 6.5

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 6.6).



Figure 6.6

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 6.7).



Figure 6.7

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 6.4 below.

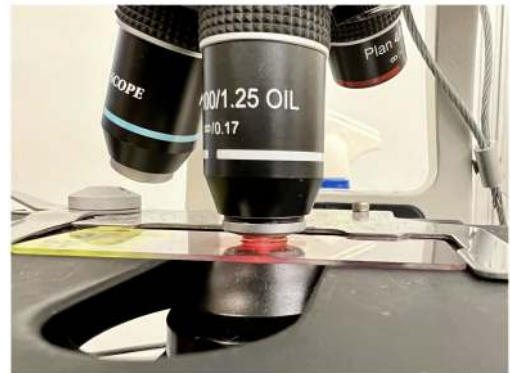
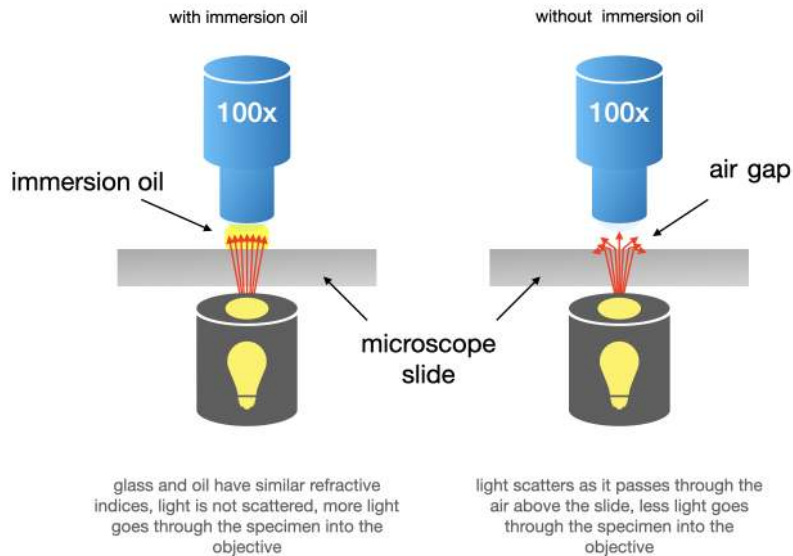


Figure 6.8: Left - Principles of oil immersion microscopy. Right - A microscope slide with immersion oil visible between the slide and the 100x objective.

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.

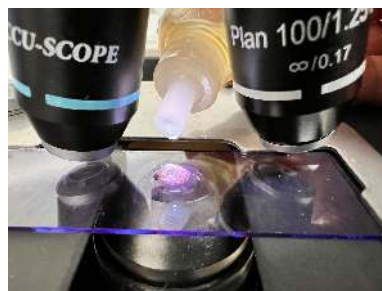


Figure 6.9: A drop of immersion oil is placed right on top of the specimen before advancing to the 100x objective.

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!

- 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

- Use the terms in Figure 6.1 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.
- Follow the procedural diagram in Figure 6.34 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.
- Heat-fix each slide as described in Figure 6.34. 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.
- Place the slide on the metal stain rack over the sink. Cover the smears with the stain using the following times:
- Crystal violet: stain for 30 to 60 seconds
- Safranin: stain for up to 1 minute
- Methylene blue: stain for 30 to 60 seconds
- Grasp the slide with a slide holder and hold it at an angle. Gently rinse the slide with
- 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.
- Gently blot dry in a tablet of bibulous paper. Do not rub.
- Observe the slide using the oil-immersion lens. Record your observations of cell morphology and arrangement in the datasheet.
- 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?

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7: 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		
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 7.1)

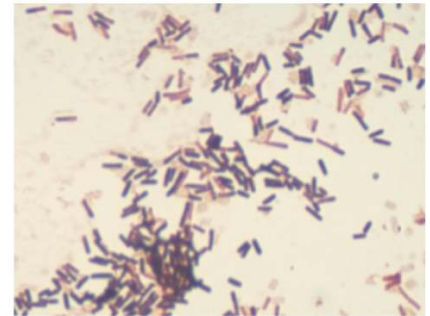
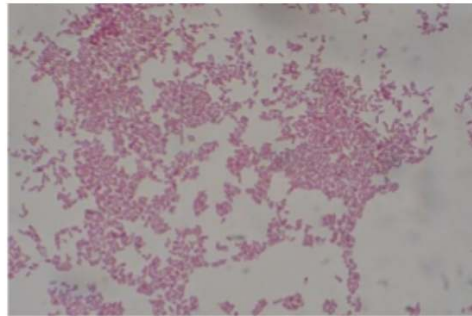
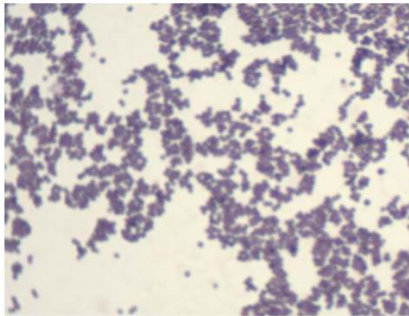


Figure 7.1: Gram Staining. Left - *S. aureus*, a Gram-positive coccus. Middle - *E. coli*, a Gram-negative rod (Dr Graham Beards, CC BY-SA 4.0, via Wikimedia Commons). Right - *B. subtilis*, a Gram-positive rod exhibiting Gram stain variability

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.

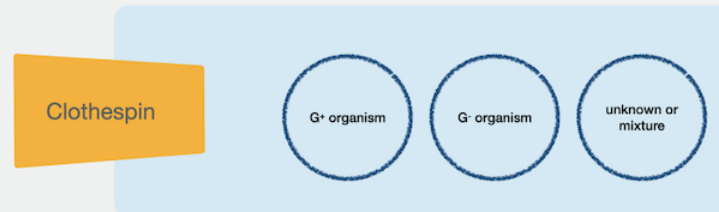


Figure 7.2: Example of how to organize specimens on the microscope slide prior to staining

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. Squirtting 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.

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8: 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		
3.	Counterstain with methylene blue	Methylene blue is needed to stain the now-transparent non-acid-fast cells.		

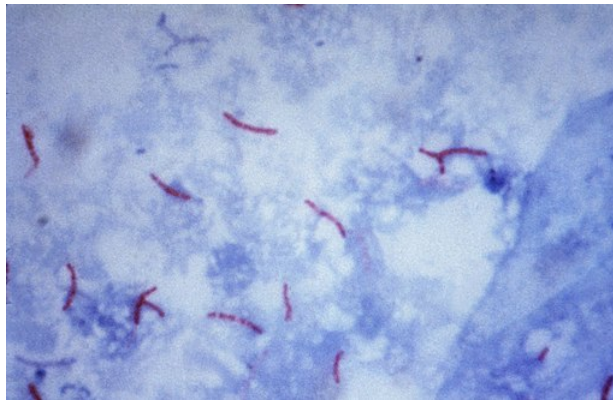


Figure 8.1: Acid Fast Stain of Mycobacterium tuberculosis. CDC/Dr. George P. Kubica, Public domain, via Wikimedia Commons

Endospore Stains

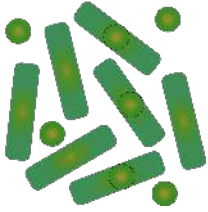
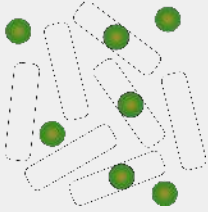
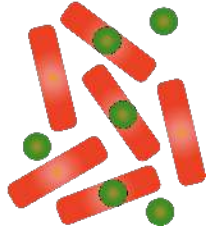
Another differential staining technique, which also requires heat to assist penetration of the primary stain, is the endospore stain. Endospores are structures made by some (not all!) Gram-positive bacteria to withstand long periods of harsh conditions. Endospores consist of bacterial DNA in a tough 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	
3.	Counterstain with safranin	Safranin is needed to stain the now-transparent vegetative cells	

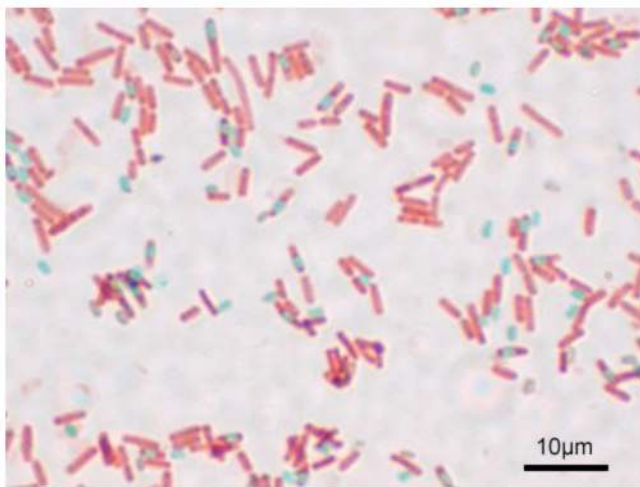


Figure 8.2: Endospore stains. Left - *B. subtilis* showing central endospores. [Y tambe \(original uploader\)](#), CC BY-SA 3.0, via Wikimedia Commons Right - *Clostridium botulinum* showing terminal endospores. [CDC/ Courtesy of Larry Stauffer, Oregon State Public Health Laboratory](#), public domain.

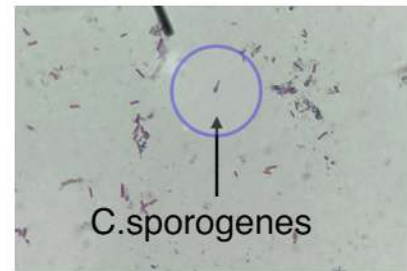
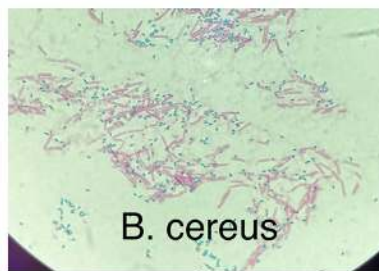
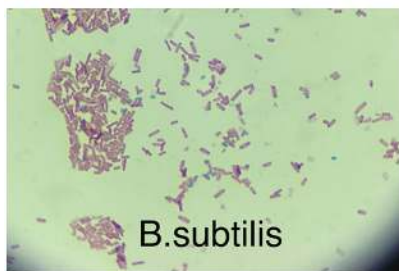


Figure 8.3: Endospore stains from the CCSF Microbiology Lab. Left - *B. subtilis* showing vegetative cells and free spores. Middle - *B. cereus* showing vegetative cells and free spores. Right- *C. sporogenes* showing terminal endospores. Photo credits: Doris Inson, used with permission

Materials

Per Student Group

- Carbofuchsin 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

- 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.
- 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*.
- 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.
- 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.
- 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.

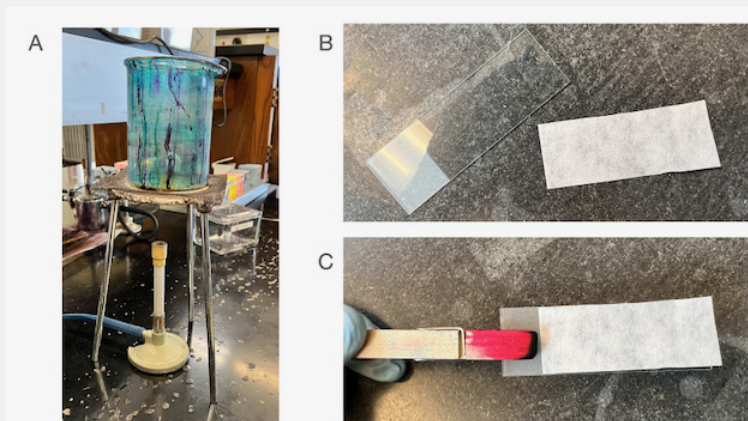


Figure 8.3: Setting up the acid-fast and endospore stains. A. Steam-bath apparatus. B. Cut bibulous paper or paper towel to fit the microscope slide. C. Place the bibulous paper over the dried/fixed smear to help keep the smear moist with stain during primary staining.

For the acid-fast stain:

- 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).
- 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.
- 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.
- 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.
- Still holding the slide on an angle, gently rinse with distilled water into the sink.
- Place the slide on the staining rack and counterstain with methylene blue for one minute.
- Gently rinse the slide with distilled water into the sink.
- Carefully blot the slide dry with fresh bibulous paper. Do not rub.

For the endospore stain:

- 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.

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9: 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*

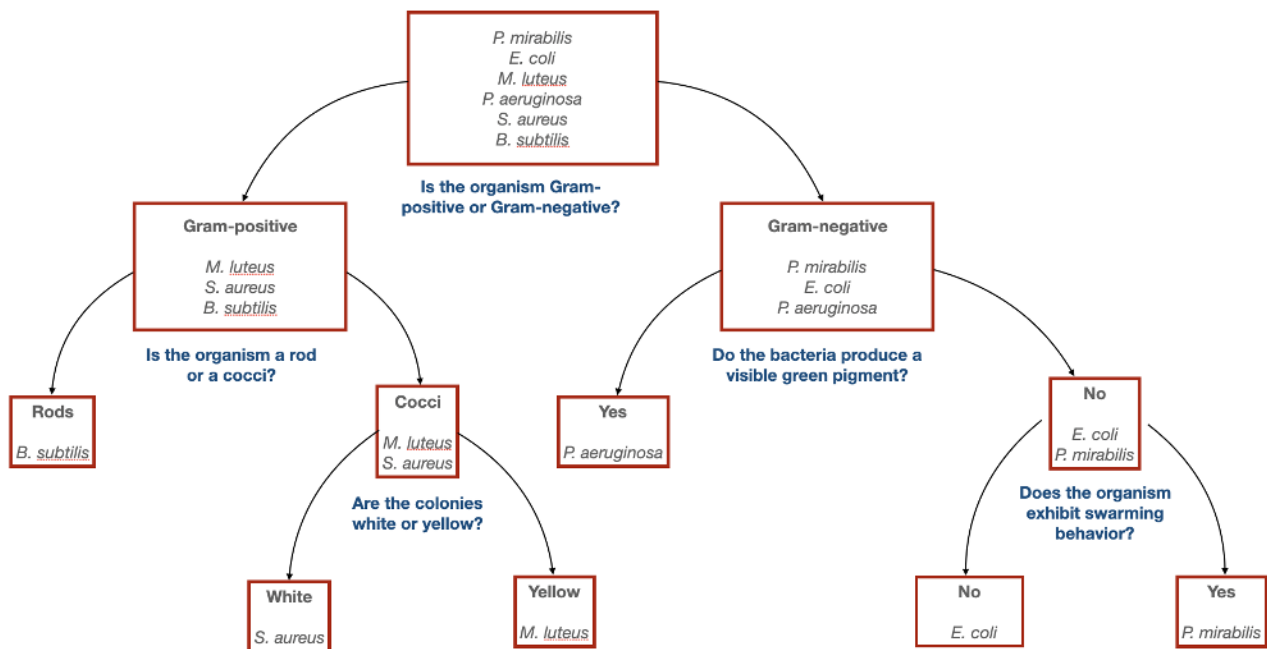


Figure 9.1: Dichotomous key for use in identifying the unknowns in today's lab.

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

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10: 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>E. 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>
<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 10.1).

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.

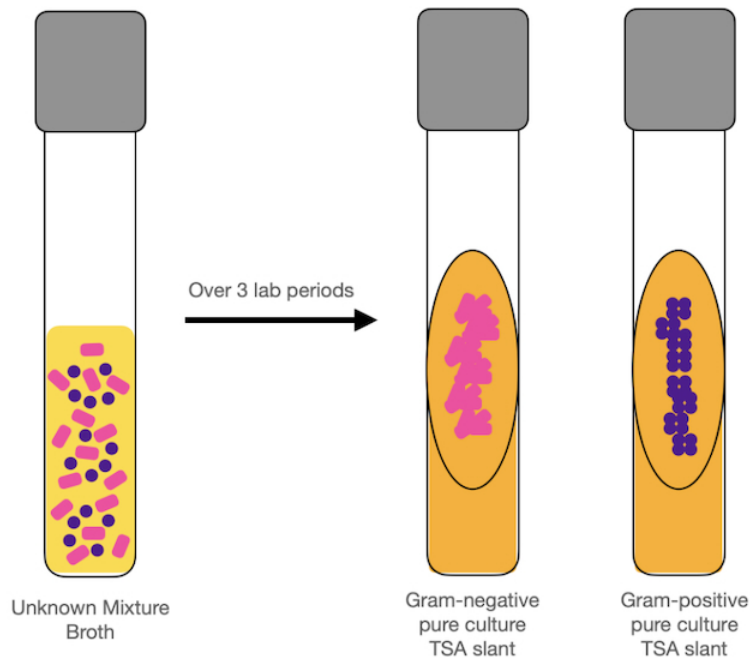


Figure 10.1: In the course of this lab we will isolate your Gram-negative and Gram-positive organisms and subculture them on TSA slants.

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 10.2).



Figure 10.2: 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 10.3).
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 10.3: 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		
	• What does a + result look like? • What does a - result look like?	Gram - Test Result, description, and pictures	Gram + Test Result, description, and pictures
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>
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>

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11: 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.

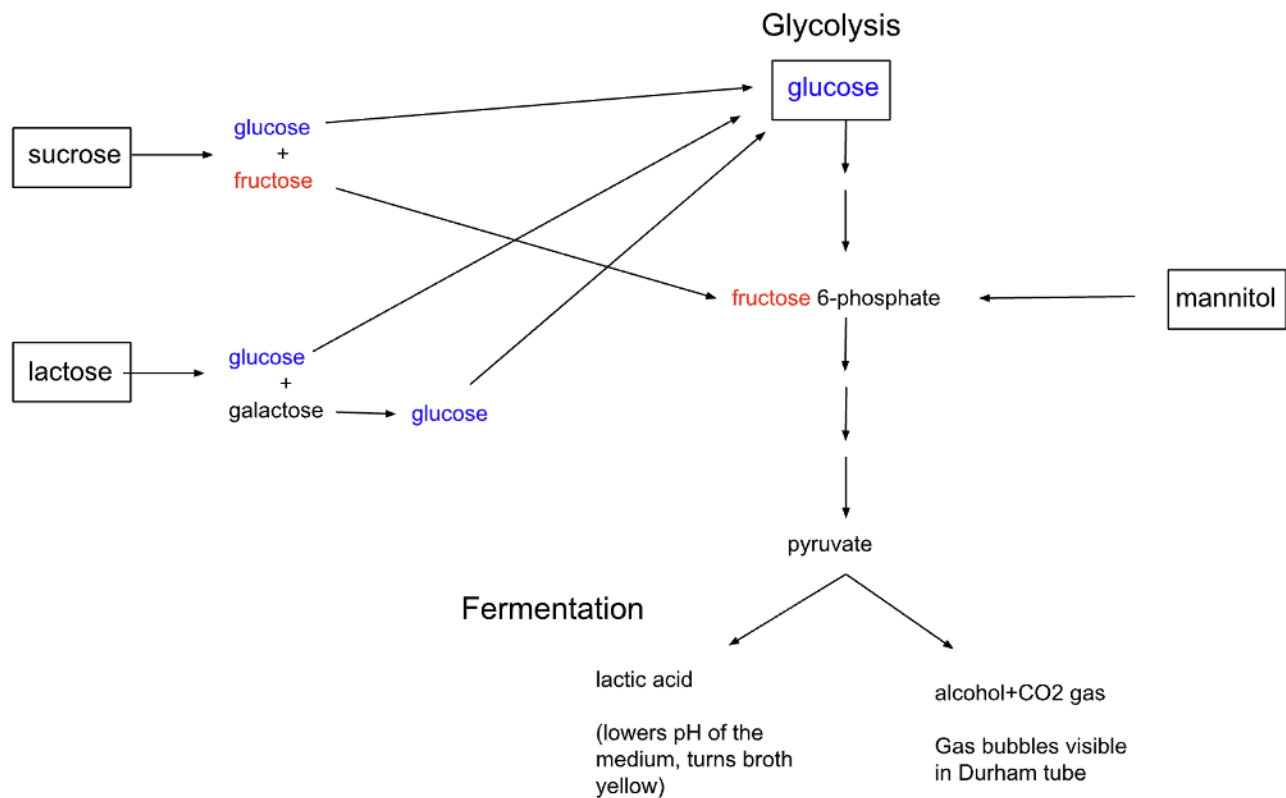


Figure 11.1: Glucose, mannitol, sucrose and lactose can be fermented using glycolysis and fermentation pathways with just one or two additional steps (enzymes).

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.

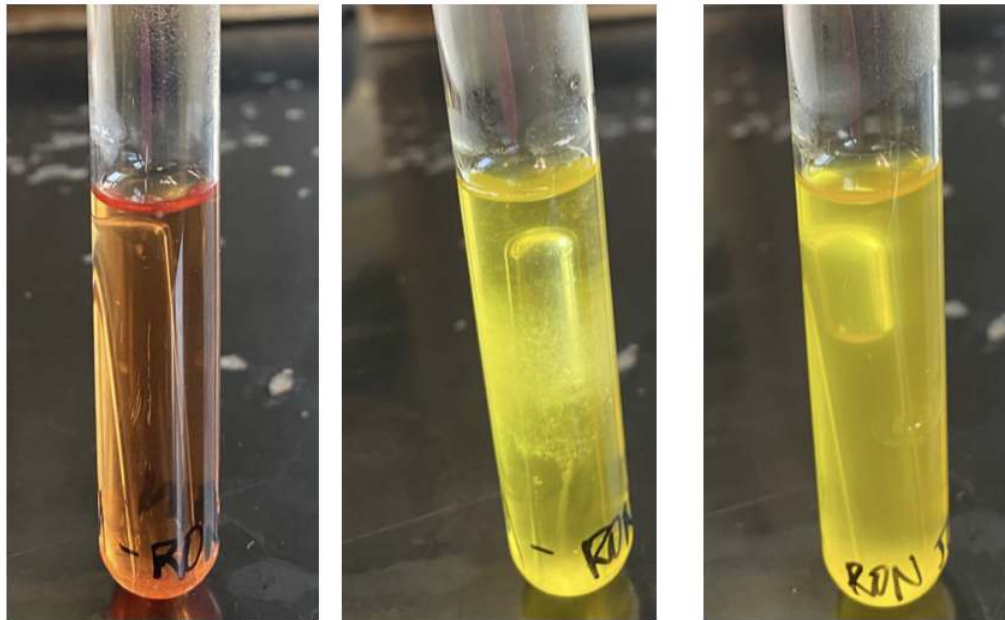


Figure 11.2: Phenol Red Glucose Fermentation Broth Results. Left - no acid produced. Middle - fermentation occurred producing acid. Right - fermentation occurred producing acid and gas. Photo credits - Ron John Vargas, used with permission.

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.

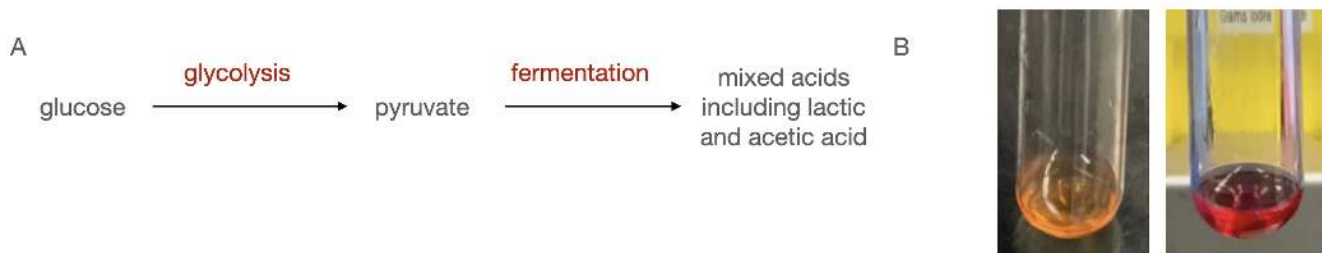


Figure 11.3:A. The pathway that produces mixed acids. B. A methyl-red positive result is shown on the right. A MR negative result is shown on the left. Photo credits - I.H., used with permission

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.

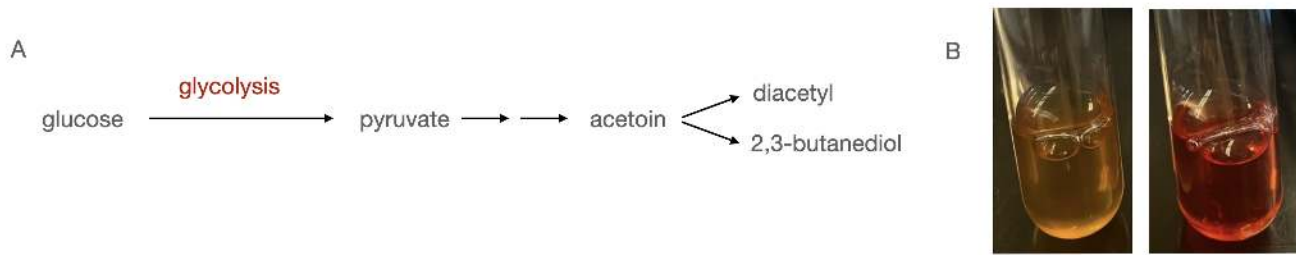


Figure 11.4:A. Acetoin is an intermediate in the breakdown of glucose to 2,3-butanediol. B. A VP positive result is shown on the right. A VP negative result is shown on the left. Photo credits - Maithy Nguyen, used with permission

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.

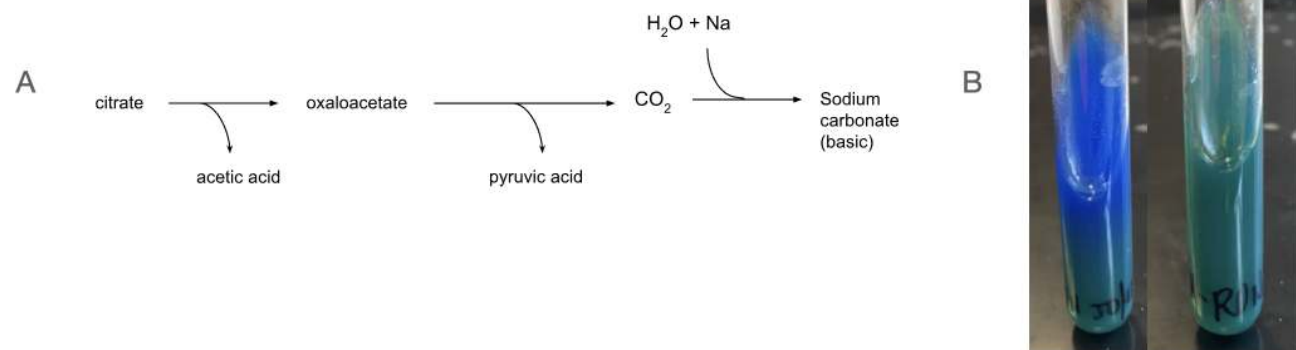


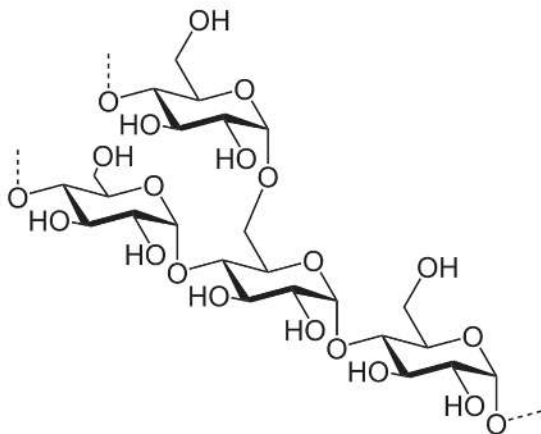
Figure 11.5: A. When citrate is used as a source of carbon, basic products such as sodium carbonate are produced, raising the pH. B. A citrate-positive result is shown on the left. A citrate-negative result is shown on the right. Photo credits - Ron John Vargas, used with permission.

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.

A



B

Before addition of iodine

After addition of iodine

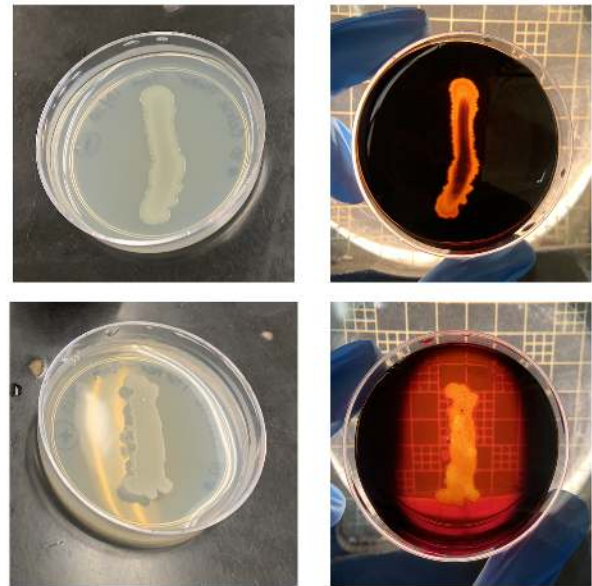


Figure 11.6: A - A small section of a starch molecule showing alpha 1,4 and alpha 1,6 glycosidic linkages. Citation: Starch. (2023, June 4). In *Wikipedia*. [Starch molecule](#) [en.Wikipedia.org] B. Starch hydrolysis detection on starch plates. The organism on the top does not hydrolyze starch. The 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 11.7: 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.

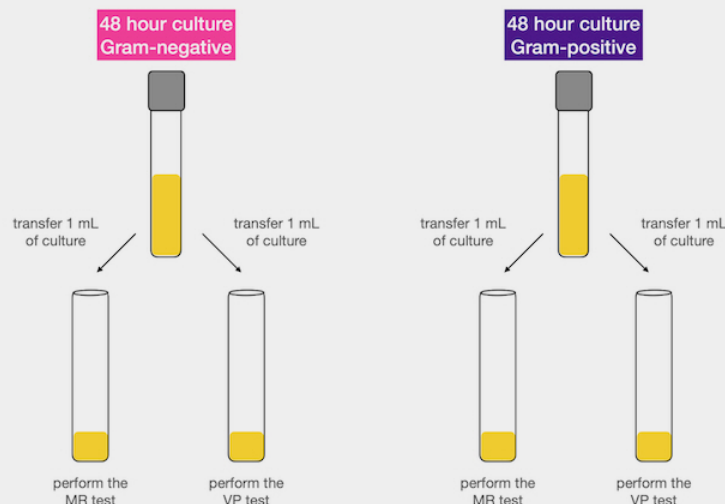


Figure 11.8: Schematic for performing the MR-VP tests.

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

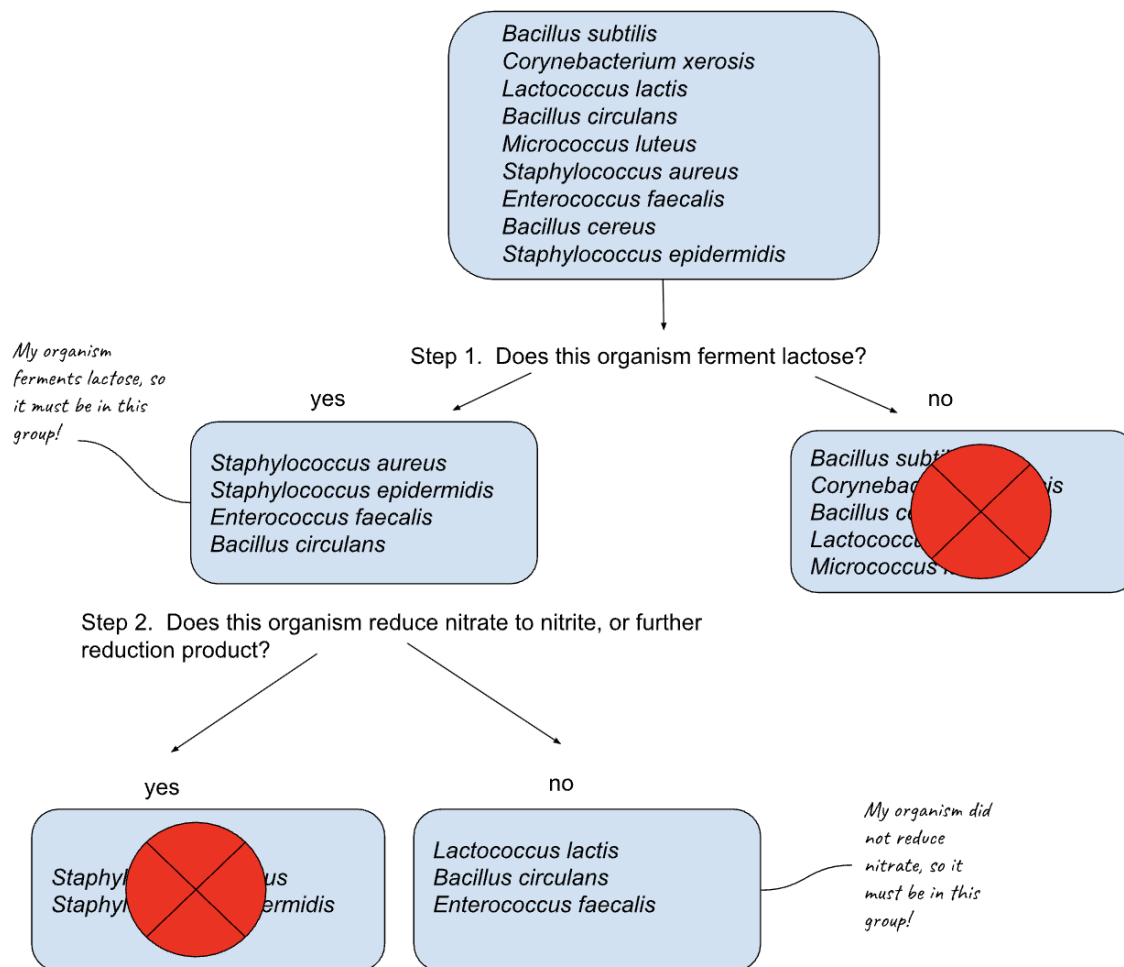


Figure 11.9: Example dichotomous key for the unknown project.

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12: 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.

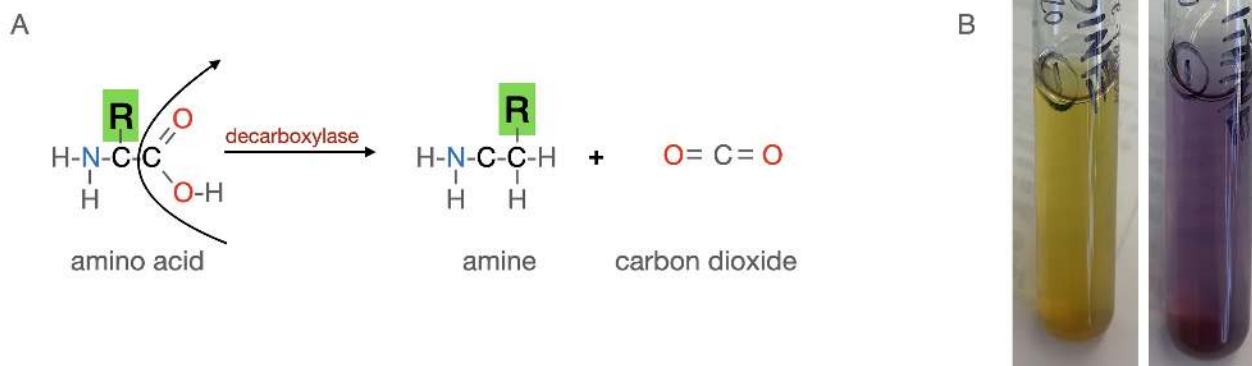


Figure 12.1: The decarboxylase test. A. The decarboxylation reaction showing how the carboxylic acid is removed, forming carbon dioxide. B. Left - a negative decarboxylase result. Right - a positive decarboxylase result. Photo Credit - Celeste Towner, used with permission.

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.

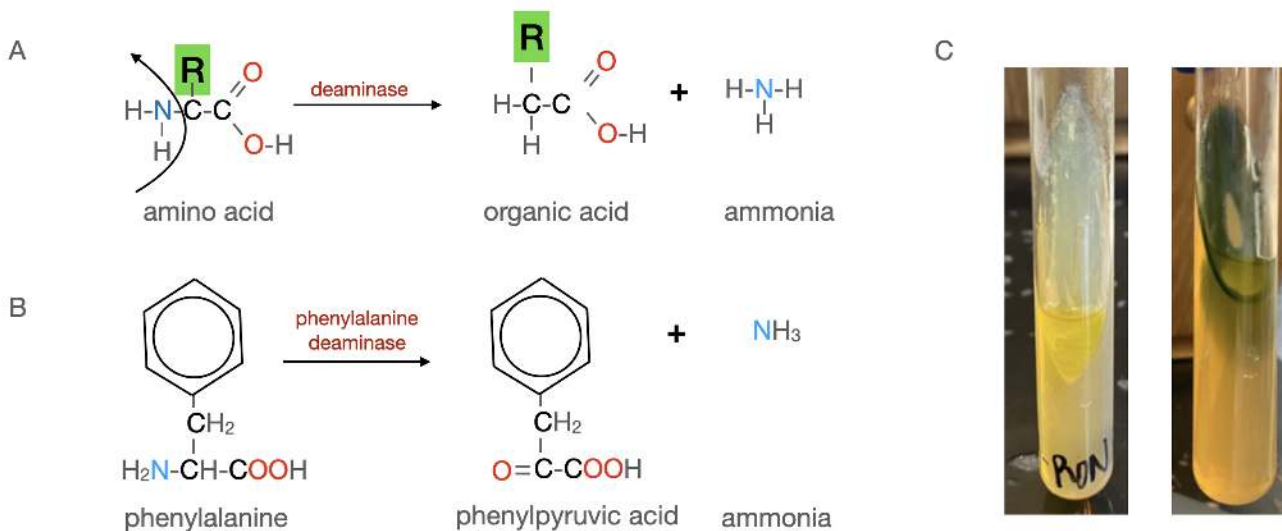


Figure 12.2: Phenylalanine deaminase test. A. General amino acid deamination B. phenylalanine deaminase produces phenylpyruvic acid C. Right - positive deamination reaction. Left - negative result. Photo credits: Ron John Vargas, (negative result), I.H. (positive result), used with permission

SIM Tests

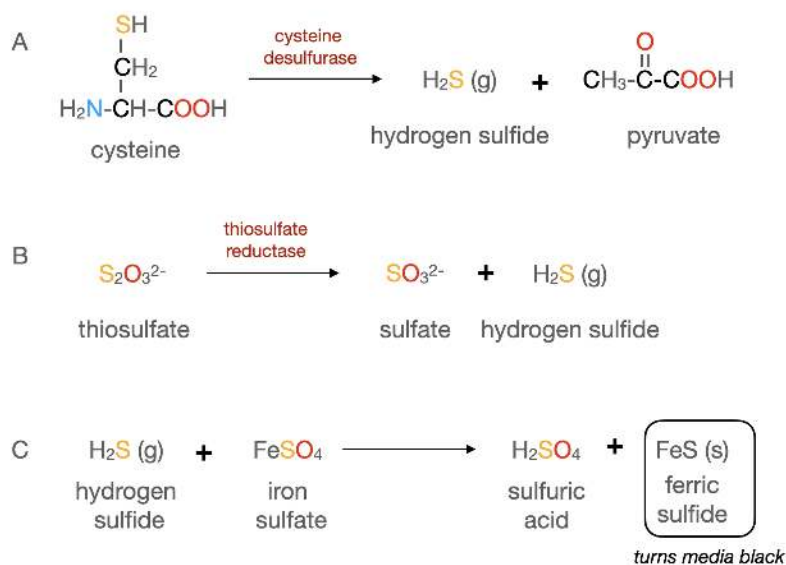
The SIM tests, again only performed on our Gram-negative bacteria, are actually three tests in one tube. In this tube, we will test for sulfur reduction, the production of indole, and bacterial motility. Both sulfur and indole production are the result of amino acid metabolism. The observation of motility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



D



Figure 12.3: 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.

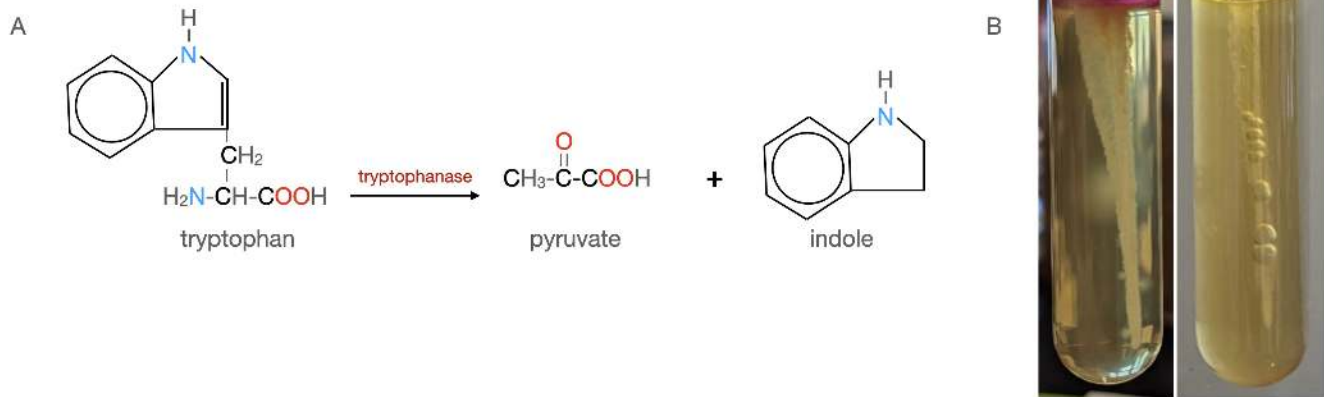


Figure 12.4: A. Tryptophanase catalyzes the breakdown of tryptophan into pyruvate and indole. B. Indole can be detected by adding Kovac's reagent to the SIMs deep, resulting in a cherry red layer on the top. Left - indole positive tube. Right - negative result. Maithy Nguyen (negative result), used with 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 H₂S 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.

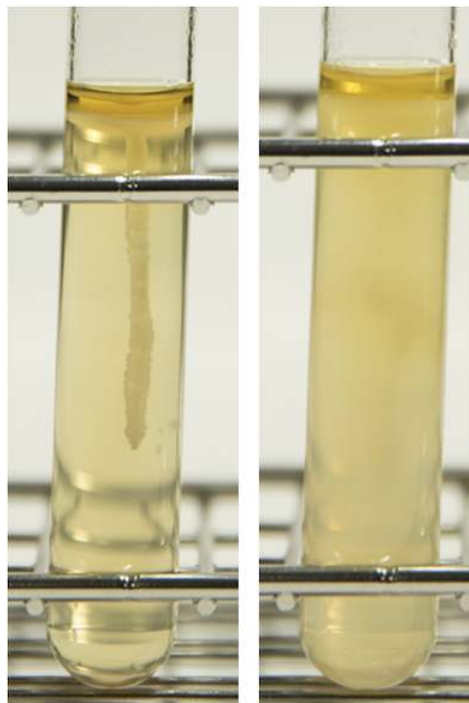


Figure 12.5: Motility in SIMs media. The tube on the right illustrates the cloudy radiating growth typical of a motile organism. The tube on the left is typical of a non-motile organism. [A doubt](#), CC BY-SA 4.0, and [A doubt](#), CC BY-SA 4.0, both via Wikimedia Commons. Both images cropped at top, 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 (N_2).

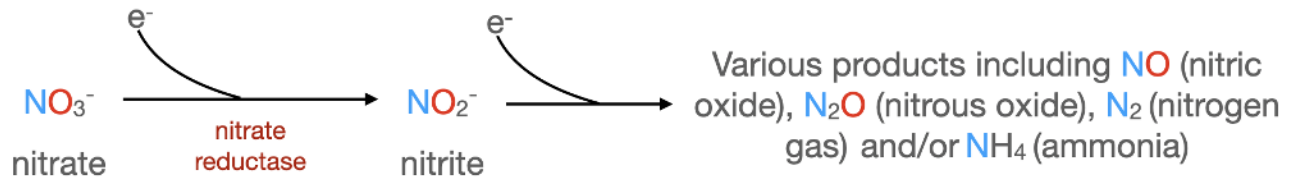


Figure 12.6: Possible results of nitrate reduction. Many organisms “stop” at the production of nitrite, however ammonia, and nitrogen gas are also made by different organisms. Ammonia can be used by bacteria for amino acid synthesis. Denitrifiers, which are important ecologically can reduce nitrate all 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 12.6. 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.

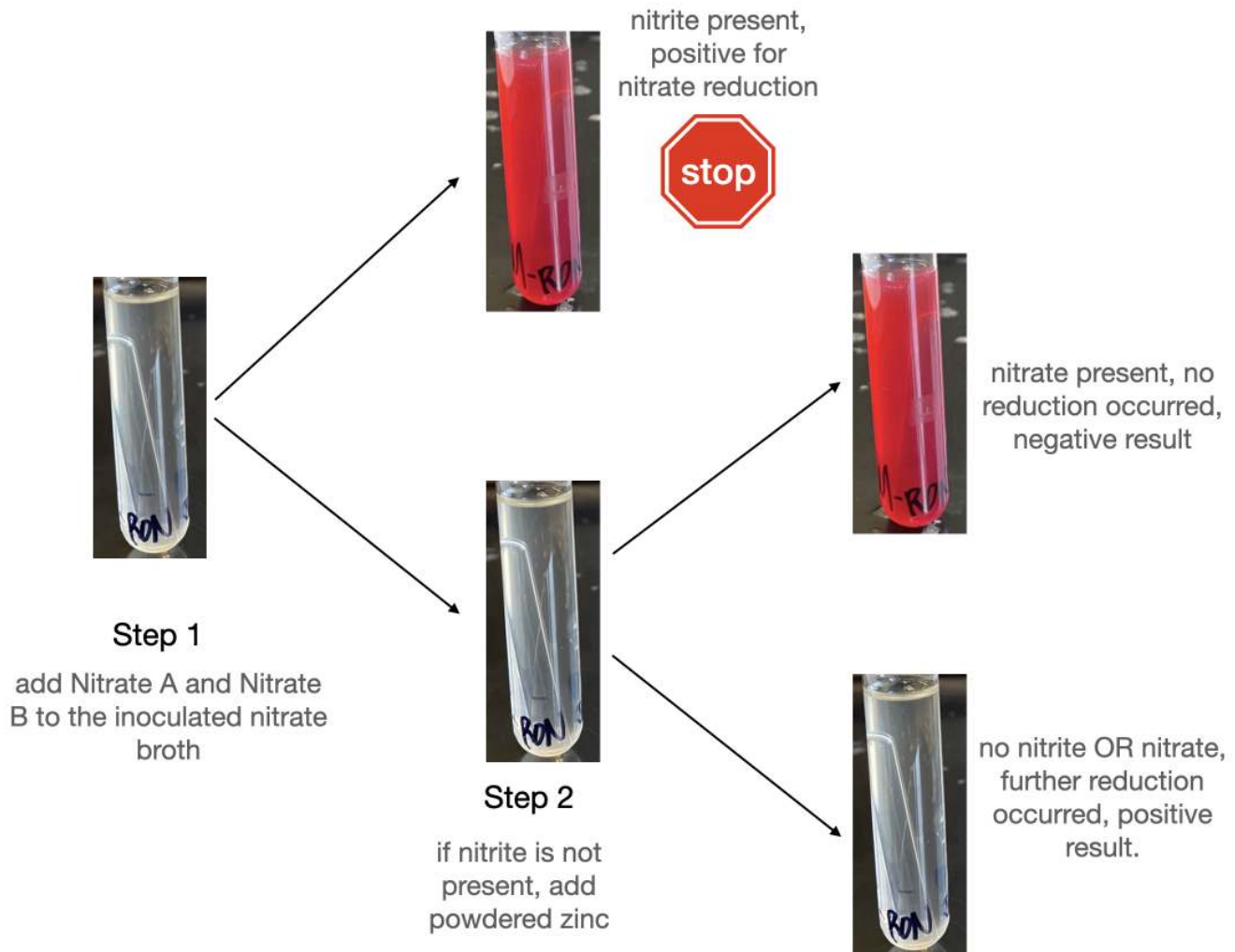


Figure 12.7: Schematic diagram showing testing for nitrate reduction and possible outcomes. If after the addition of Nitrate Reagent A and Reagent B, the tube turns red, this is a positive result. Zinc can be used to differentiate between tubes where no reduction has occurred and tubes where nitrate 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 liquefy 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.

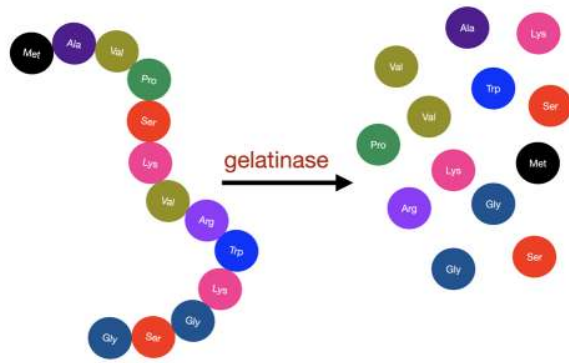


Figure 12.8: Left - Gelatinases break the bonds between individual amino acids in the gelatin protein. Right - The presence of gelatinase will result in liquefaction of the gelatin. Top tube - a negative result. Bottom tube - a positive result.

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.

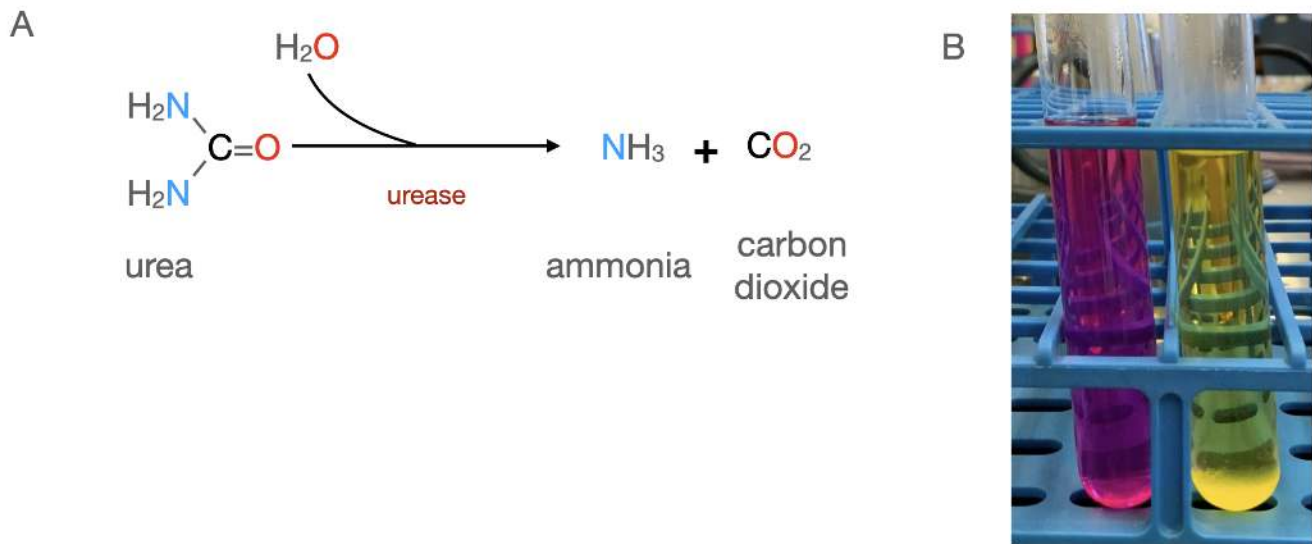


Figure 12.9: A. The hydrolysis of urea results in the production of ammonia, which makes the broth more basic (raises the pH). B. A positive result is shown on the left. A negative result is shown on the right. Photo credit - I.H., used with permission

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.

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13: 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).

- β -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.
- α -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 13.1: 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.

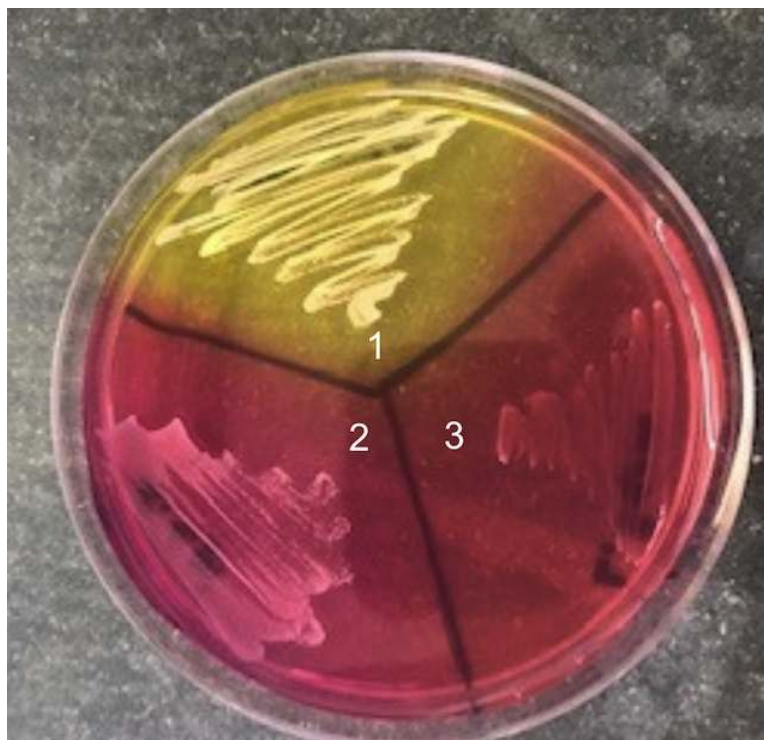


Figure 13.2: Mannitol Salt Agar (MSA) results. 1 - *S. aureus* grows on MSA and ferments mannitol. 2, 3 - *S. epidermidis* and *L. fermentum* both grow on MSA, but neither ferments mannitol. Photo Credit, Ying Liu, used with permission.

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*.

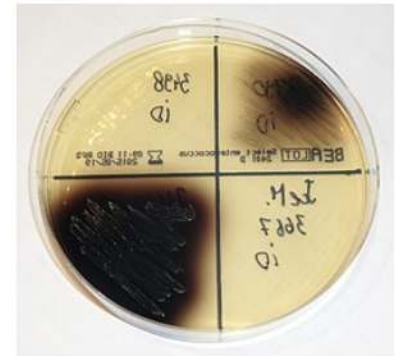
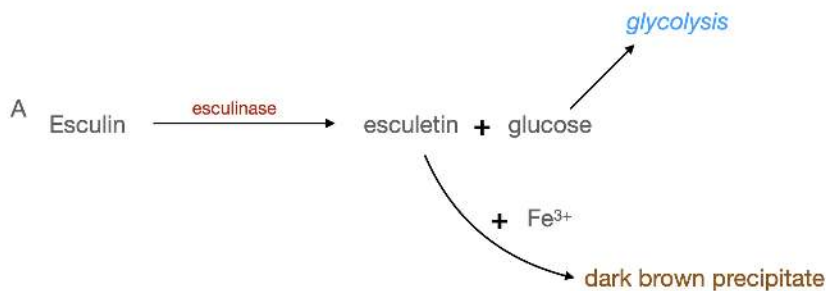


Figure 13.3: A. Esculin can be used by bacteria that make the enzyme esculinase to generate glucose for energy. A bile esculin plate showing the brown precipitate formed when *Enterococcus* is grown on the medium. [Stefan Walkowski, CC BY-SA 4.0](#), via Wikimedia Commons

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](#).

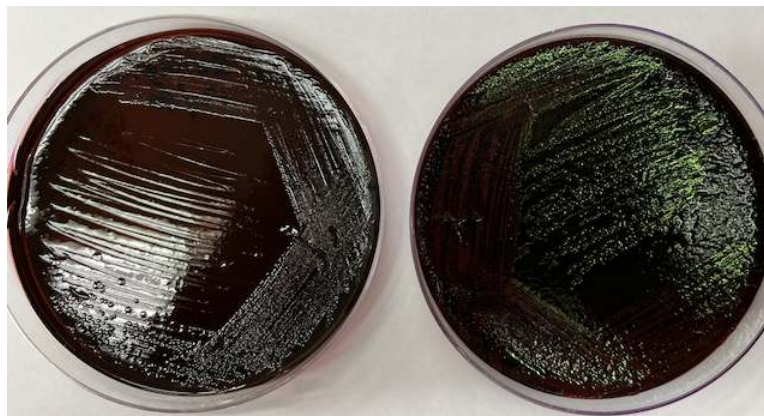


Figure 13.4: Typical growth on EMB. Left - a non-lactose fermenter. Right - a vigorous lactose fermenter such as *E. coli* produces a characteristic green sheen.

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 (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*.

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



Figure 13.5: 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 H_2S 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 H_2S 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.

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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).

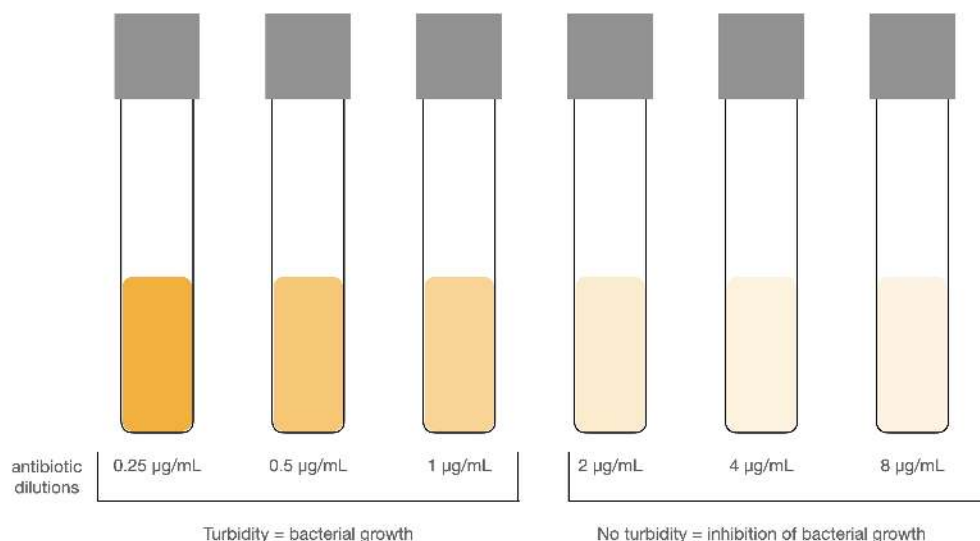


Figure 14.1: Diagram of an antibiotic dilution assay. As the concentration of antibiotic increases, bacterial growth is inhibited.

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).

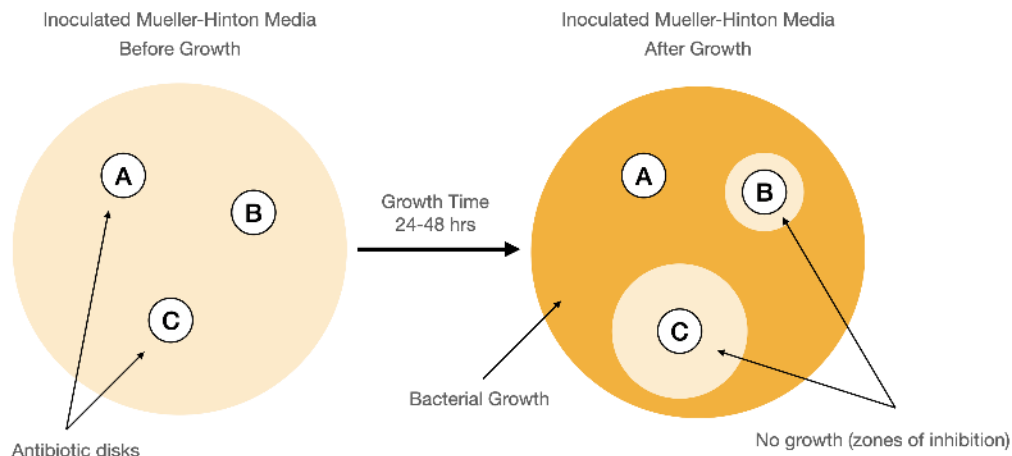


Figure 14.2: Principles of the Disk Diffusion Assay

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).

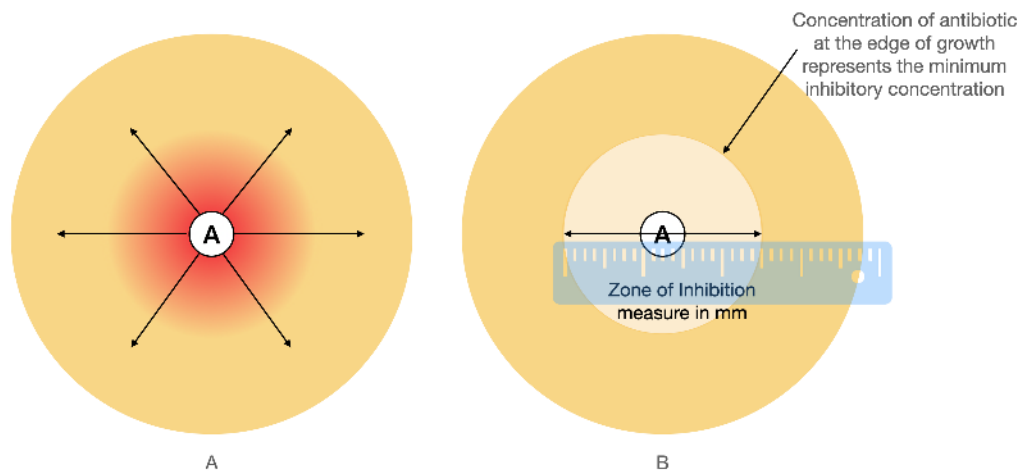


Figure 14.3: A. Antibiotic, shown in red, diffuses outward from the disk. The further from the disk, the lower the concentration. B. The zone of inhibition (ZOI) is measured in millimeters (mm). The minimum inhibitory concentration is the concentration of antibiotic at the very perimeter of growth.

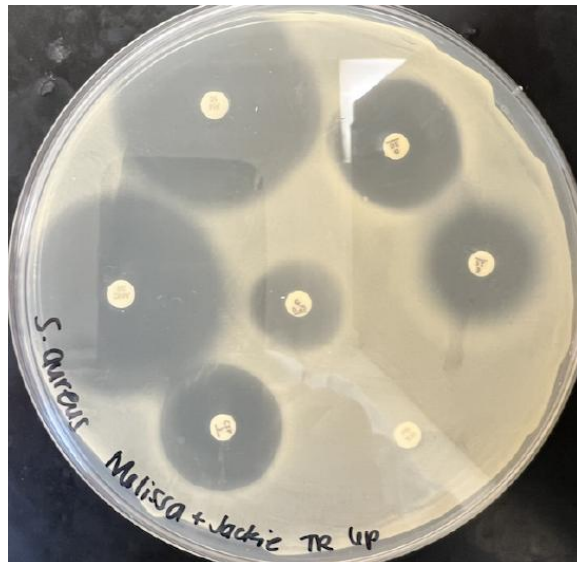


Figure 14.4: Disk diffusion assay performed on *S. aureus*. Note that Polymyxin B (PB 300 disk) does not inhibit *S. aureus* growth.

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	<i>S. aureus</i>		<i>E. coli</i>		<i>P. aeruginosa</i>	
		Zone diameter	S/I/R	Zone diameter	S/I/R	Zone diameter	S/I/R

Questions

1. Overall, which organism was most resistant to antibiotics? Consider the environment where this organism is found. Does this result make sense?
2. Which antibiotic was the most broad-spectrum in this experiment? What mechanism of action does it represent?

3. Which antibiotic was the most narrow spectrum in this experiment? What mechanism of action does it represent?
 4. Define/describe
 1. Mueller Hinton agar
 2. Zones of inhibition
 3. Minimum inhibitory concentration
 5. 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?
-

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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.

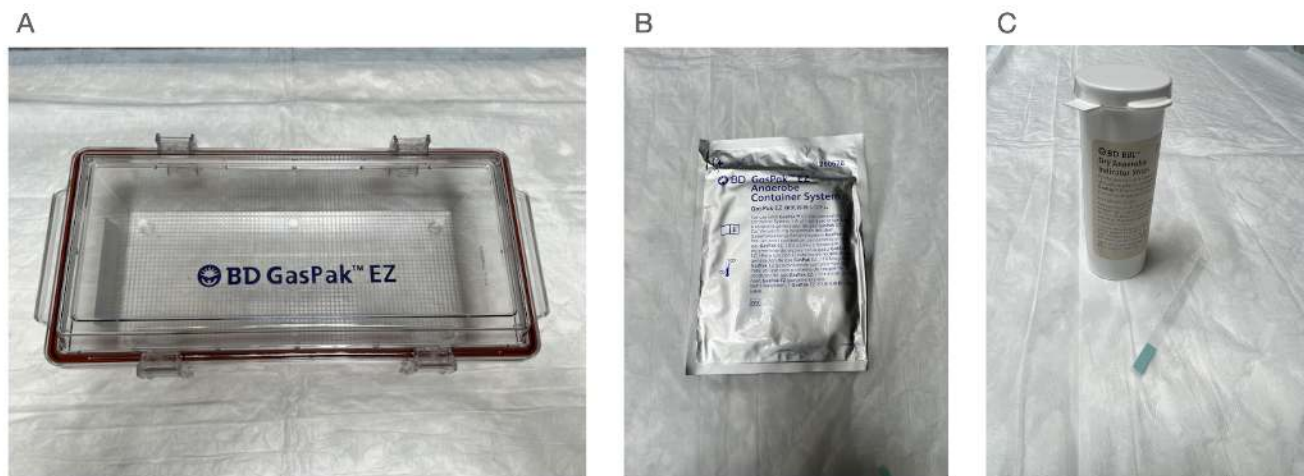


Figure 15.1: A. Anaerobic Chamber for incubating Petri plates in the absence of oxygen. B. Sealed "Gas Pak" for removing oxygen from the chamber. C. Test strips are to be placed in an anaerobic chamber to detect the presence or absence of oxygen. A blue

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.

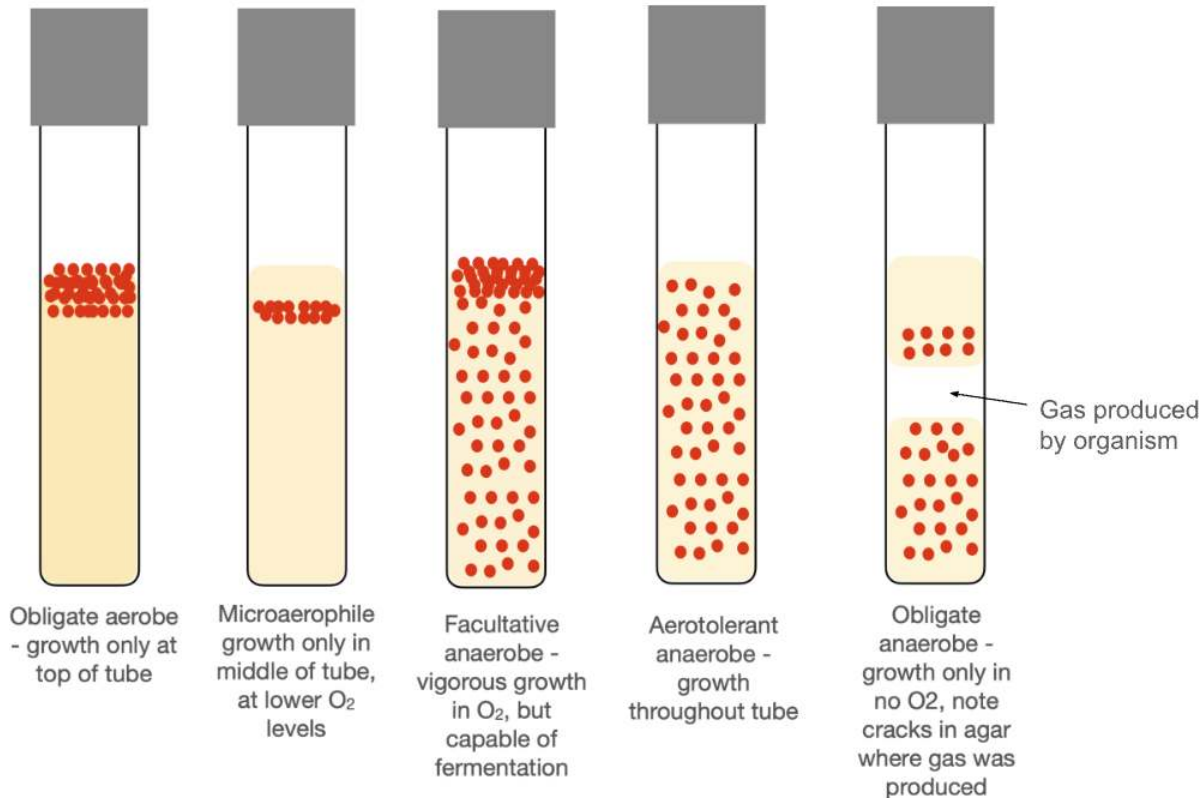


Figure 15.2: Diagram of growth patterns in BHI Shake Tubes of organisms with different oxygen requirements

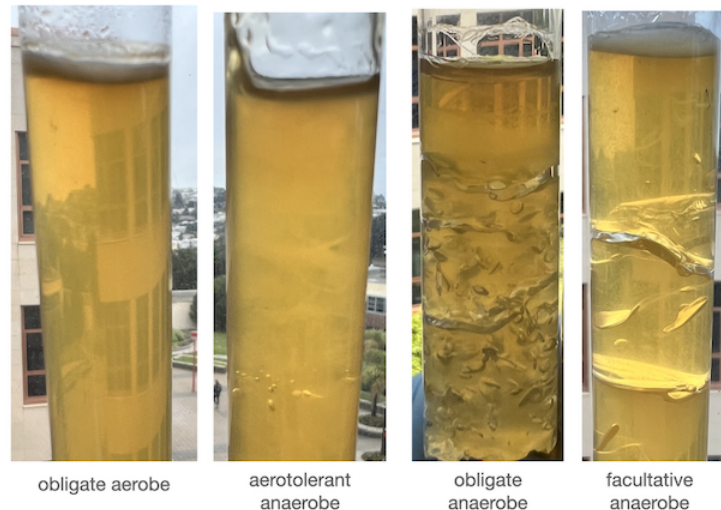


Figure 15.3: Photograph of BHI shake tubes. Note how growth is only observed at the top of the tube inoculated with an obligate aerobe. The aerotolerant organisms demonstrates uniform growth from top to bottom. For the obligate anaerobe, note the absence of growth at the very top of the tube where oxygen 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)

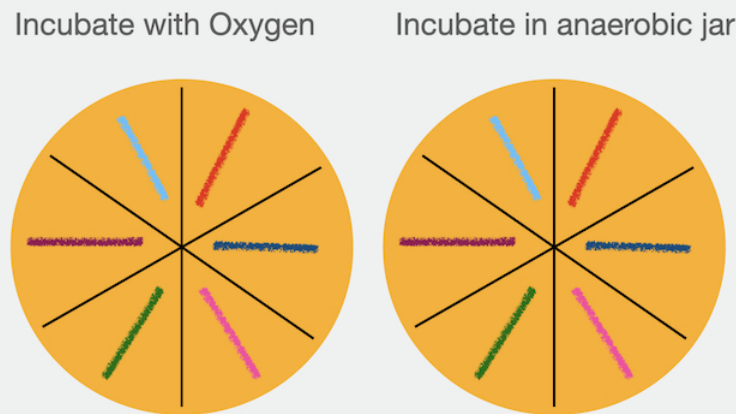


Figure 15.4

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?

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