

MICROBIOLOGY LAB SP25



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CHAPTER OVERVIEW

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- 1.1: Required Materials
- 1.2: Laboratory Safety
- 1.3: Microscopy
- 1.4: Prokaryotic Cells
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1.1: Required Materials

Safety/Microscopy

- Immersion Oil
- Clean Slides
- Lens paper
- Lens cleaner
- Prepared Slides
 - “e” slide
 - Helminth
 - Protozoa
 - Paramecium
 - Amoeba
 - Algae
 - Fungus
 - Bacteria
 - Cocci
 - Rods
 - Vibrio
 - Endospore

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1.2: Laboratory Safety

Learning Objectives

- Demonstrate awareness of the safety hazards present in the microbiology laboratory.
- Practice good laboratory safety.

Laboratory Safety Rules for Microbiology Lab Classes

There are many hazards associated with microbiology laboratories. Adherence to the following policies will help you, your classmates, and others who use the laboratory space to stay safe:

1. Do not eat, drink, store food, or smoke in the laboratory.
2. Decontaminate the workbenches at the beginning of laboratory.
3. Bring only necessary equipment to your work area.
4. Store personal belongings so no trip hazards are present in the laboratory.
5. Do not wear shirts with sleeves that hang down (fire hazard).
6. Tie or pull back long hair (fire hazard).
7. Do not carry flasks, test tubes, or bottles from their tops. Always carry from the base.
8. Never taste or smell anything in the laboratory unless your instructor has indicated that it is safe to do so.
9. Avoid touching your mouth and eyes during class unless you thoroughly wash your hands first.
10. Use hot gloves or appropriate glassware holders to handle hot glassware.
11. Report any spills or supply and equipment breakage to your instructor immediately.
12. Report any injuries to your instructor immediately.
13. Make sure all cuts are covered during laboratory.
14. Decontaminate the workbenches at the end of laboratory.
15. Thoroughly wash your hands at the end of class.
16. Follow all instructions for disposing of cultures and chemicals. Do not put any chemicals or cultures down the sink unless your instructor indicated it is safe to do so.
17. Cleanup all glassware, cultures, and supplies at the end of class. The classroom should look the same at the beginning and at the end of laboratory.

Attributions

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1.3: Microscopy

Learning Objectives

- Tell the importance of using microscopes in microbiology.
- Successfully use the metric system for length including unit conversion calculations.
- Define resolution.
- Define magnification.
- Determine/calculate total magnification for each objective lens.
- Identify the structures of a light microscope.
- Identify the functions of the structures of a light microscope.
- Successfully use and care for a light microscope.
- Differentiate between light microscopy and electron microscopy.
- Differentiate between TEM and SEM and the appropriate uses for each.

Early Microscopy

The first microscope was developed in 1590 by Dutch lens grinders Hans and Zacharias Jansen. In 1667, Robert Hooke described the microscopic appearance of cork and used the term cell to describe the compartments he observed. Anton van Leeuwenhoek was the first person to observe living cells under the microscope in 1675—he described many types of cells, including bacteria. Since then more sophisticated and powerful scopes have been developed that allow for higher magnification and clearer images.

Microscopy is used by scientists and health care professionals for many purposes, including diagnosis of infectious diseases, identification of **microorganisms** (microscopic organisms) in environmental samples (including food and water), and determination of the effect of pathogenic (disease-causing) microbes on human cells. This exercise will familiarize you with the microscopes we will be using to look at various types of microorganisms throughout the semester.

Metric Units & Microscopy

Unlike typical length measurement units used in the United States (inches, feet, yards, miles), microscopy uses the **metric system** (nanometers, micrometers, millimeters). Scientific measurements use the metric system. The metric system is also used for measurement in every country in the world except in three countries: Liberia, Myanmar, and The United States. Although these measurements are likely new for you, they are very user-friendly since the metric system uses units that are all related to the meter (the base unit) and are different from each other by powers of 10.

Metric Units for Length

Since microscopes are concerned with the size (length) of structures and the magnification of these structures, we will consider the metric system as it relates to length. The base unit of length in the metric system is the **meter** (abbreviated as **m**). One meter is about 3.3 feet long. For units that are larger or smaller than meters, a prefix is added to the beginning of "meter" to indicate the magnitude of difference from the meter unit (base unit):

Unit of Length	Abbreviation	Size in relationship to a meter (meter is the base unit)
kilometer	km	1,000 or 10^3
hectometer	hm	100 or 10^2
decameter	dam	10 or 10^1
meter (base unit)	m	1 or 10^0
decimeter	dm	0.1 or 10^{-1}
centimeter	cm	0.01 or 10^{-2}
millimeter	mm	0.001 or 10^{-3}
micrometer	μm	0.000001 or 10^{-6}
nanometer	nm	0.000000001 or 10^{-9}
picometer	pm	0.000000000001 or 10^{-12}

Sizes of Cells & Microbes

Cells are typically measured using the micrometer (μm) unit, but some subcellular structures may be reported in nanometers (nm) measurements. To give you a sense of cell size, a typical human red blood cell is about eight millionths of a meter or eight micrometers (abbreviated as $8\ \mu\text{m}$) in diameter; the head of a pin is about two thousandths of a meter (two mm) in diameter. That means about 250 red blood cells could fit on the head of a pin!

Use this interactive tool to get a sense of how big microbes are in comparison to some familiar objects.*

***microbes in this interactive tool include:** amoeba proteus, paramecium, baker's yeast, *E. coli* bacterium, measles virus, hiv, phage, influenza virus, hepatitis virus, and rhinovirus

Converting Metric Length Units

Converting Units to Meters (the Base Unit)

Using the table above that distinguishes units by prefix, to convert unit measurements back to the base unit, **simply multiply the number you are converting with the number in the "size relationship to a meter" column**. For example:

Using your microscope you measure a cell as $20\ \mu\text{m}$ ("twenty micrometers"). To convert $20\ \mu\text{m}$ to meters, multiply $20\ \mu\text{m}$ by 0.000001:

$$20\ \mu\text{m} \times 0.000001 = 0.00002\ \text{m}$$

or

$$20 \mu\text{m} \times 10^{-6} = 0.00002 \text{ m}$$

You measure the distance it takes you to get home from school as 19 km ("nineteen kilometers"). To convert 19 km to meters, multiply 19 km by 1,000:

$$19 \text{ km} \times 1,000 = 19,000 \text{ m}$$

or

$$19 \text{ km} \times 10^3 = 19,000 \text{ m}$$

? Exercise 4.1

Convert 2,500 μm to meters.

[Answer](#)

? Exercise 4.2

Convert 12,000 nm to meters.

[Answer](#)

? Exercise 4.3

Convert 35 mm to meters.

[Answer](#)

Converting Meters (the Base Unit) to Other Units

Using the table above that distinguishes units by prefix, to convert the base unit to another unit, **simply divide the number you are converting with the number in the "size relationship to a meter" column**. For example:

You are told that a certain very large cell is 0.0003 m long. To convert 0.0003 m to micrometers (μm), divide 0.0003 m by 0.000001:

$$0.0003 \text{ m} \div 0.000001 = 300 \mu\text{m}$$

or

$$0.0003 \text{ m} \div 10^{-6} = 300 \mu\text{m}$$

You read that running a marathon means running 42,000 m. To convert 42,000 m to kilometers (km), divide 42,000 m by 1,000:

$$42,000 \text{ m} \div 1,000 = 42 \text{ km}$$

or

$$42,000 \text{ m} \div 10^3 = 42 \text{ km}$$

? Exercise 4.4

Convert 0.004 m to μm .

[Answer](#)

? Exercise 4.5

Convert 0.3 m to mm.

[Answer](#)

? Exercise 4.6

Convert 0.00000085 m to nm.

[Answer](#)

Light Microscopes vs. Electron Microscopes

Most microscopes used in college biology laboratories are classified as **light microscopes** (see the figure, part (a) below) and may also be called **compound microscopes** since they use two lenses whose magnifications compound (multiply). Visible light passes through the specimen and is bent through the lens system to enable the user to see the specimen. Light microscopes are advantageous since they are capable of viewing living organisms. However, since individual cells are generally transparent, their components are not distinguishable unless they are colored with special **stains** (colored chemicals that makes cells appear to have color such as pink, blue, or purple). Stained cells are dead cells, but staining is an important approach to better see cells and cell structures.

Due to the magnification limits of light microscopes, in order to gain a better understanding of cellular structure and function, scientists typically use **electron microscopes** that use electrons (subatomic particles) to carry the microscopic images instead of using light particles (photons). Electron microscopes also typically require staining the sample with heavy metals to be able to visualize the sample.

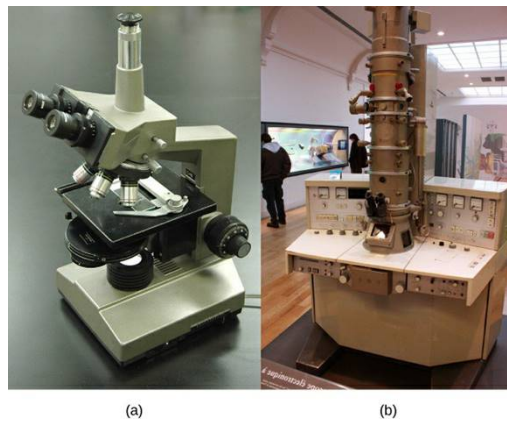


Figure 1: (a) Most light microscopes used in a college biology labs can magnify cells up to approximately 1000 times (1000X) and have a resolution of about 200 nm ("two hundred nanometers"). (b) Electron microscopes provide a much higher magnification, 100,000X, and have a resolution of 50 pm ("fifty picometers").

To see the difference in magnification and detail possible when comparing light microscopes and electron microscopes, compare the images of *Salmonella* (a type of bacteria) in the figure below.

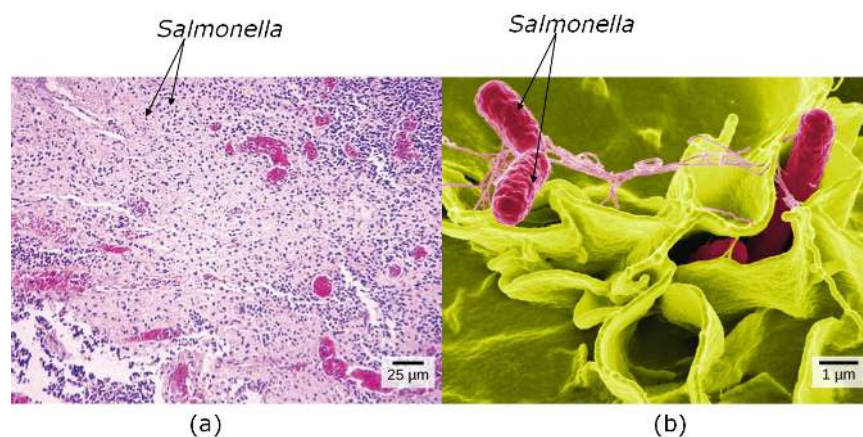


Figure 2: (a) These *Salmonella* bacteria appear as tiny purple dots when viewed with a light microscope (*Salmonella* are stained to appear purple). (b) This scanning electron microscope micrograph shows *Salmonella* bacteria (in red) invading human cells (yellow). Comparing *Salmonella* bacteria when using a light microscope (a), and *Salmonella* bacteria when using an electron microscope (b), observe the comparative increase in magnification with electron microscopy, the higher level of detail in electron microscopy, and the differences in appearance between light and electron microscopy.

The Light Microscope

What does it mean to be microscopic? Objects are said to be microscopic when they are too small to be seen with the unaided eye—they need to be magnified (enlarged) for the human eye to be able to see them. This includes human cells and many other types of cells that you will be studying in this class. The microscope you will be using uses visible light and two sets of lenses to produce a magnified image. The total magnification will depend on which objective lens you are using—the highest magnification possible on these microscopes is typically 1000X—meaning that objects appear 1000X larger than they actually are.

Resolution vs. Magnification

Magnification refers to the process of making an object appear larger than it is; whereas **resolution** is the ability to see objects clearly enough to tell two distinct objects apart. Although it is possible to magnify above 1000X, a higher magnification would result in a blurry image. (Think about magnifying a digital photograph beyond the point where you can see the image clearly). This is due to the limitations of visible light (details that are smaller than the wavelength of light used cannot be resolved).

The limit of resolution of the human eye is about 0.1 mm ("zero point one millimeters"), or 100 μm ("one-hundred micrometers"). Objects that are smaller than this cannot be seen clearly without magnification. Since most cells are much smaller than 100 μm , we need to use microscopes to see them.

The **limit of resolution of a standard brightfield light microscope**, also called the **resolving power**, is $\sim 0.2 \mu\text{m}$, or 200 nm. Biologists typically use microscopes to view all types of cells, including plant cells, animal cells, protozoa, algae, fungi, and bacteria. The nucleus and chloroplasts of eukaryotic cells can also be seen—however smaller organelles and viruses are beyond the limit of resolution of the light microscope.

Resolution is the ability of the lenses to distinguish between two adjacent objects as distinct and separate.

A compound light microscope has a maximum resolution of 0.2 μm , this means it can distinguish between two points $\geq 0.2 \mu\text{m}$, any objects closer than 0.2 μm will be seen as 1 object. Shorter wavelengths of light provide greater resolution. This is why we often have a blue filter over our light source in the microscope, it helps to increase resolution since its wavelength is the shortest in the visible light spectrum. Without resolution, no matter how much the image is magnified, the amount of observable detail is fixed, and regardless of how much you increase the size of the image, no more detail can be seen. At this point, you will have reached the limit of resolution or the resolving power of the lens. This property of the lens is fixed by the design and construction of the lens. To change the resolution, a different lens is often the only answer.

Parts of a Light Microscope

The microscope is one of the microbiologist's most important tools. It allows for the visualization of small particles, including microbes, which are too small to be seen with the human eye. With the help of proper illumination, a microscope can magnify a specimen and optically resolve fine detail. This introduction to microscopy will include an explanation of features and adjustments of a compound **brightfield light microscope**, which magnifies images using a two lens system.

These microscopes combine an **ocular lens** (located in the eyepiece) and an **objective lens** (located above the stage and attached to a revolving nosepiece). Objective lenses can be selected based on the amount of magnification needed. Objective lenses are changed by turning the revolving nosepiece. The ocular lens remains constant and is not changed during normal microscope operation.

To view a sample placed on the microscope stage, the **course focus knob** and the **fine focus knob** are used to move the stage up and down. This will enable focusing on the sample.

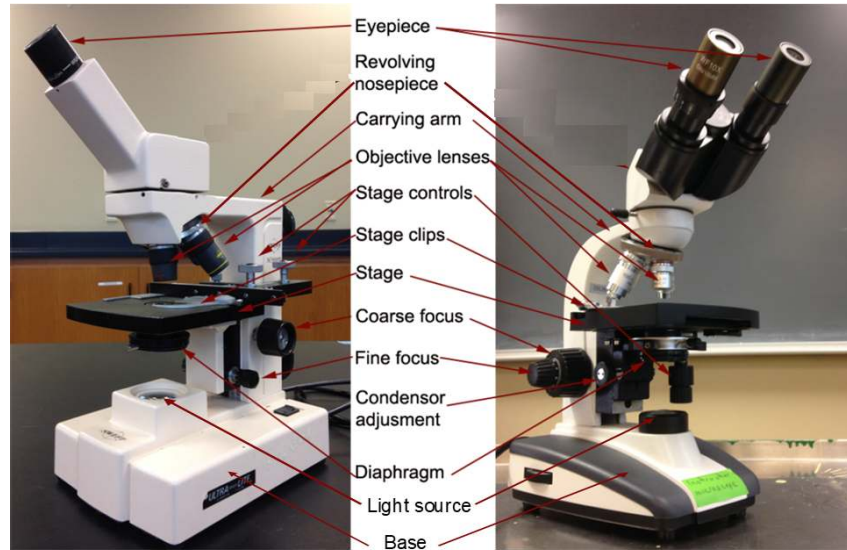


Figure 3: Parts of compound light microscopes. Some models are slightly different. Here, two different models are shown and labeled to indicate the parts of each.

Microscope Part	Description and Function of Microscope Part
eyepiece	location for looking into the microscope; holds ocular lenses (lenses magnify the image; typically have 10X magnification)
revolving nosepiece	contains the objective lenses; the nosepiece turns to change the objective lenses (changes the magnification)
objective lenses	mounted in the revolving nosepiece; typically microscopes have four objective lenses: 4X, 10X, 40X and 100X
stage clips	holds the microscope slide in place; located on the stage
stage	a platform located below the objectives where the slide is placed
stage controls	knobs that move the slide around the stage by moving the stage clips to position the specimen in the desired position
mechanical stage	the mechanism that moves the stage clips around the stage to move the slide; the stage control knobs control the mechanical stage
diaphragm	located beneath the condenser; regulates the amount of light by opening or closing its aperture
light source/ illuminator	a light source; usually a bulb built in the base of the microscope; there may be a dial to regulate the intensity of light depending on the model of microscope
condenser	a system that focuses the light coming up from the illuminator onto the specimen on the slide
arm	a structure of the microscope that connects the base to the head; it may be straight or curved depending on the model of microscope
base	the wide bottom of the microscope; supports the microscope on a bench or table
coarse focus	large knob located on both sides of the microscope below the stage; focuses the image by moving the stage rapidly; should only be used when using the 4X or 10X objective lenses; using the coarse focus with the 40X or 100X objective lenses could damage the lens or crack the slide
fine focus	small knob located on both sides of the microscope below the stage; focuses the image by moving the stage in small increments; used most with the 40X objective lens and 100X objective lens but can be used to fine-tune focus at lower objective lenses (4X and 10X)

Tips for Success Using a Light Microscope

1. Center the sample over the light shining up through the stage.
2. Begin with the lowest magnification objective lens in place (usually the 4X objective lens). Make sure it clicks into place over the sample and the light shining up through the stage.
3. Turn the course focus to bring the stage up as close as it can go to the objective lens. At this point, you should be close to being in focus.
4. Look into the eyepiece and slowly turn the course focus until you can see the sample. Many samples have been stained to have color (usually you can look for color - often pink, purple, or blue - you may be able to see the color on the slide with your naked eye).
5. Increase the objective lens magnification one step at a time. Focus the microscope each step (with course focus at 4X and 10X objective lenses and with fine focus at 40X and 100X objective lenses).
6. If you lose the sample, return back to the lowest magnification objective. This may seem like you are wasting time, but in reality it is the fastest way to find your sample again.

Detailed Instructions for Using a Light Microscope

Learning microscopy is very important in microbiology to examine the microscopic organisms that we are studying (bacteria, protozoa, fungi, etc.). Here is how we work with these microscopes:

Carrying a Compound Light Microscope

To take the microscope out of the cabinet or off of the cart using the following instructions:

1. Use your dominant hand to hold the arm of the microscope, and your non-dominant hand to hold the base of the microscope.
2. Carry the microscope upright. That way the ocular lenses located in the eyepiece do not fall out.
3. Make sure the electrical cord is wrapped or removed from the microscope, avoid tripping on the cord.

To return the microscope to the cabinet or to the cart use the following instructions:

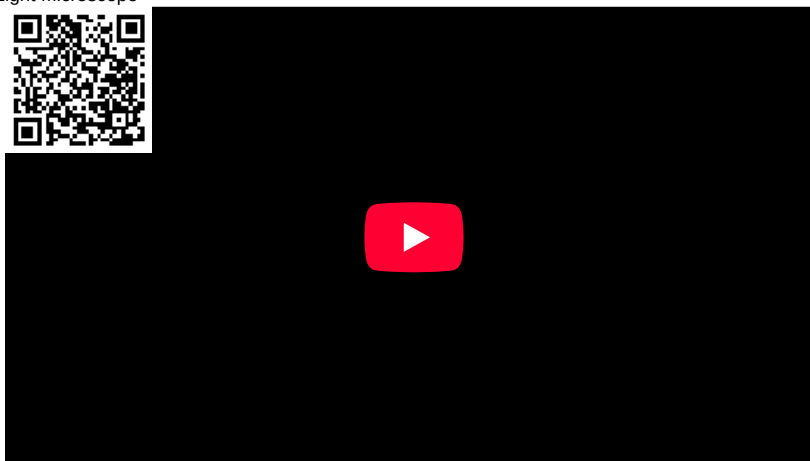
1. Turn off the microscope.
2. Turn the revolving nosepiece to have lowest magnification objective, usually the 4X objective lens.
3. Disconnect from power source.
4. Wrap cord around the microscope.
5. If you used oil immersion, (the 100X objective), **use lens paper only** to remove any remnant of immersion oil on the 100X objective lens.
6. Place the microscope back in the cabinet or on the cart using the transport instructions above.

Focusing a Compound Light Microscope

1. Place the microscope on a table in front of you and plug it in such that the cord does not create a trip hazard for you or others in the laboratory.
2. Turn the revolving nosepiece to have lowest magnification objective in place, usually 4X. The objective should click when properly in place.
3. Place the slide on the stage of the microscope and properly position the stage clips such that the clips "hug" the slide on either side and are **not** on top of the slide since this could crack the slide (NOTE: there are some models of microscopes where stage clips do sit on top of the slide - ask your instructor about proper use of the stage clips for the model of microscope you are using).
4. Turn on the microscope and confirm that the light is on.
5. Move the stage control knobs to position the specimen on the slide directly over the path of the light.
6. **With the 4X objective in place**, turn the coarse focus knob to bring the stage as close to the objective lens as possible.
7. Look through the microscope and use the coarse focus knob to focus on the sample. Make sure the slide is in very sharp focus before moving on. Check with your instructor if you are uncertain.
8. To focus on the specimen using higher magnification than with the 4X objective, use the next steps:
9. With the specimen focused with the 4X objective, re-center the specimen using the stage adjustment knobs. **DO NOT CHANGE THE FOCUS!**
10. Change to the next highest objective lens **without changing any other settings on the microscope**.
11. Use **ONLY** the **fine focus** knob to modify the focus. The specimen should mostly be in focus and only minor adjustments should be necessary. Continue focusing until the image is sharp and clear.
12. Re-center the specimen using the stage adjustment knobs. **DO NOT CHANGE THE FOCUS!**
13. If moving to the next highest objective lens, change to the objective lens to the next highest **without changing any other settings on the microscope**.
14. Use **ONLY** the fine **focus knob** to adjust the focus. Continue focusing until the image is sharp and clear.

****If you get lost on your sample, go back to 40X magnification (objective says '4X'), re-focus, and follow the steps above again.****

Video Walkthrough for Using a Light Microscope



Magnification

The optical system of a compound microscope consists of two lens systems: one found in the objective(s) lens(es) (Fig. 2, part 3); the other in the ocular (eyepiece) (Fig. 2 part 1). The objective lens system is found attached to a rotating nosepiece (Fig. 2, part 2). A microscope usually has three or four objectives that differ in their magnification and resolving power. **Magnification** is the apparent increase in size of an object. **Resolving power** is the term used to indicate the ability to distinguish two objects as separate. The most familiar example of resolving power is that of car headlights at night: at a long distance away, the headlights appear as one light; as the car approaches, the light becomes oblong, then barbell-shaped, and finally it becomes *resolved* into two separate lights. Both resolution and magnification are necessary in microscopy in order to give an apparently larger, finely detailed object to view.

Look at the engravings on the objective lenses and note both the magnification (for example: 10X, 40X, 100X) and the resolution given as N.A. = numerical aperture, from which the limit of resolution can be calculated:

$$\text{microscope resolution limit} = (\text{wavelength})/(\text{numerical aperture} \times 2)$$

At a wavelength of 550 nm (0.55 μm), the 100X objective lens with a N.A. of 1.25 has a resolving power of 0.22 μm . Visible light has of wavelength from about 400-750 nanometers (nm). Since the limit of resolution decreases at the shorter wavelengths, microscopes are usually fitted with a blue filter. The resolving power of the lens separates the details of the

specimen, and the magnification increases the apparent size of these details so that they are visible to the human eye. Without both resolution and magnification, you would either see nothing (good resolution, no magnification) or a big blur (poor resolution, good magnification).

The objective lens system produces an image of the specimen, which is then further magnified by the ocular lens (eyepiece). The magnification of this lens is engraved on the ocular. The total magnification of the microscope is determined by the combination of the magnification of the objective lens and ocular lens that is in use, that is:

$$\text{total magnification} = (\text{objective lens magnification}) \times (\text{ocular lens/eyepiece magnification})$$

For example, with a 10X objective lens and a 10X ocular, the **total magnification** of the microscope is 100X. If the objective lens is changed to a 20X objective, then the total magnification is now 200X, whereas if a 10X objective is used with a 12.5X ocular lens, the total magnification is now 125X. When viewing a sample, **magnifications are reported as the total magnification**. Therefore, if making an illustration or taking a photograph of a magnified sample, report the magnified view as the total magnification (not as the magnification of the objective lens). Likewise, if you are examining a magnified image taken by another person, the magnification they report is the total magnification (not the objective lens). When reading laboratory instructions for viewing a sample with the microscope, total magnification will be given (not the objective lens).

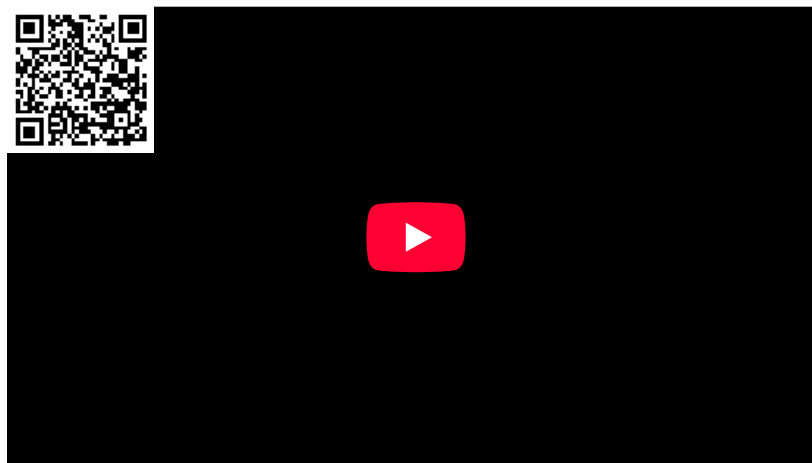
The use of objective and ocular lenses with different magnifications allows greater flexibility when using the compound microscope. Due to the size of most bacteria (ranges widely from ~1 µm to over 100 µm), generally we require the use of the 100X *oil immersion lens* with a 10X ocular lens to view bacteria in a standard brightfield light microscope.

Sample Illumination

The objective lens and ocular lens systems can only perform well under optimal illumination conditions. To achieve these conditions, the light from the light source (bulb) must be centered on the specimen. The parallel light rays from the light source are focused on the specimen by the condenser lens system. The condenser can move up and down to affect this focus. Finally, the amount of light entering the condenser lens system is adjusted using the condenser diaphragm. It is critical that the amount of light be appropriate for the size of the objective lens receiving the light. This is important to give sufficient light, while minimizing glare from stray light, which could otherwise reduce image detail. The higher the magnification and resolving power of the lens, the more light is needed to view the specimen.

Oil Immersion

Objective lenses used for observing very small objects such as bacteria are almost always **oil immersion lenses**. With an oil immersion lens, a drop of oil is placed between the specimen and the objective lens so that the image light passes through the oil. Without the oil, light passing through the glass microscope slide and specimen would be **refracted** (bent) when it entered the air between the slide and the objective lens. This refracted light might still be able to contribute to the image of the specimen if the objective lens is large. However, at the higher magnification, the objective lens is small, so is unable to capture this light. The loss of this light leads to loss of image detail. Therefore, at higher magnifications, the area between the slide and the lens is modified to have the same (or nearly the same) refracting qualities (refractive index) as the glass and specimen by the addition of immersion oil. Watch the video below and [read this article for more information about oil immersion](#).



To use an oil immersion lens, place a drop of oil on top of the dried specimen on the slide and carefully focus the microscope so that the objective lens is immersed in the oil. Any lens, which requires oil, is marked "oil" or "oil immersion." Conversely, any lens not marked "oil" should *not be* used with oil and is generally not sealed against oil seeping into and ruining the objective.

Electron Microscopes (EM)

In contrast to light microscopes, **electron microscopes** use a beam of electrons instead of a beam of light. Not only does this allow for higher magnification and, thus, more detail, it also provides higher resolving power. The method used to prepare the specimen for viewing with an electron microscope kills the specimen. Electrons have short wavelengths (shorter than photons of light) that move best in a vacuum, so living cells cannot be viewed with an electron microscope.

The best type of EM to examine a cell's internal structures such as vesicles, storage granules, ribosomes, mitochondria, and nuclei is **transmission electron microscopy (TEM)** since it is designed to allow electrons to pass through a thin sample. Samples must be prepared for TEM by embedding the sample, such as a cell, in a tiny plastic block, and using either a glass knife or a diamond knife to thin-section the sample using a **microtome** (laboratory equipment that makes thin sections). As a result, thin slices of cells can be observed with TEM, exposing intracellular details. An example of a thin-sectioned skin cell magnified by TEM is shown in the figure, part (a) below.

Another common type of EM is **scanning electron microscopy (SEM)**. SEM works much differently than TEM since SEM radiates electrons onto a sample's surface and these electrons are reflected back by the sample to elucidate the surface details of the sample. As a result, SEM is an excellent technique for examining the three-dimensional surface of an un-sectioned specimen and viewing only the external surfaces of cells and tissues. An example of an SEM image of skin cells with yeast infecting them is shown in the figure, part (b), below.

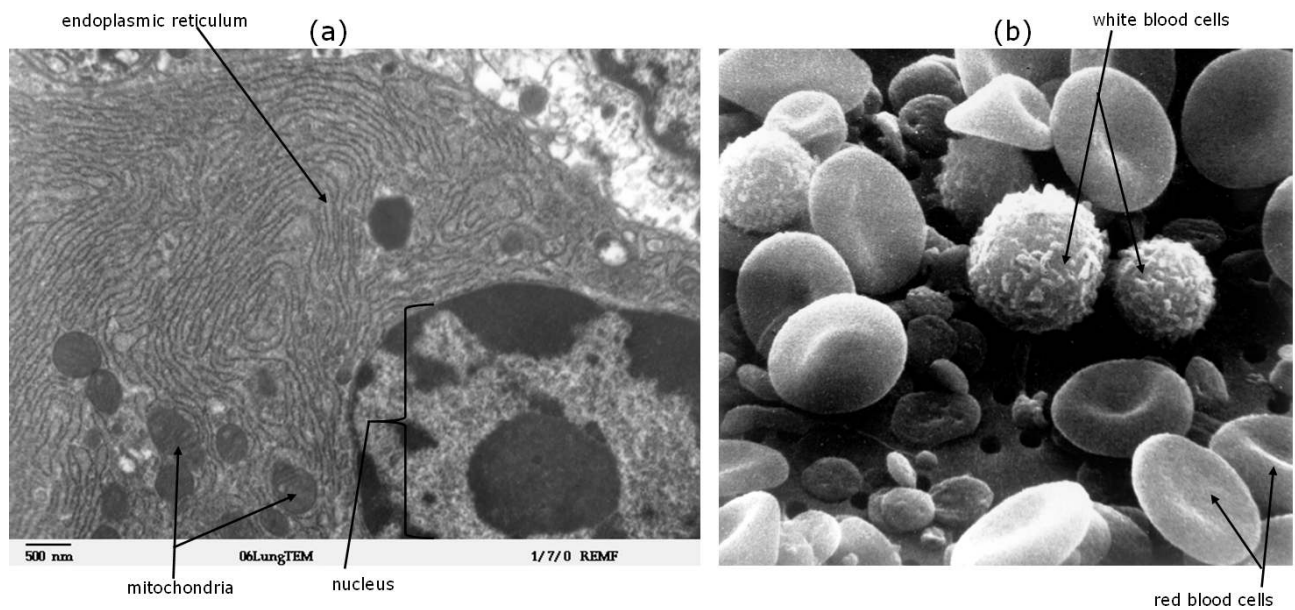


Figure 4: (a) A lung cell that has been thin-sliced and **imaged with TEM**. Thin-slicing a cell with TEM exposes the internal structures such as the nucleus and mitochondria. (b) Exterior of blood cells **imaged with SEM**. SEM images only allow surface visualizations of cells and tissues.

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1.4: Prokaryotic Cells

Learning Objectives

- Distinguish between prokaryotic cells and eukaryotic cells in terms of structure, size, and the types of organisms that have these cell types.
- Identify structures of bacterial cells in models and diagrams, including details of Gram-positive and Gram-negative cell walls and flagella.
- Describe the functions of the structures found in prokaryotic cells.
- Define endospore.
- Identify the structures and explain the functions of endospores.
- Identify and name bacterial cell shapes and arrangements using correct terminologies.
- Name the correct terms of flagellar arrangements from descriptions, illustrations, and images.

Bacteria are Prokaryotic Cells

Cells fall into one of two broad categories: **prokaryotic** and **eukaryotic**. Prokaryotic cells do not have a nucleus (membrane-bound structure that surrounds the cell's DNA). Only eukaryotic cells have a nucleus. Bacteria and archaea are the forms of life that are composed of prokaryotic cells, whereas plants, animals, fungi, and protists (including protozoa) are all composed of eukaryotic cells. In addition to prokaryotic and eukaryotic cells differing from each other based on absence or presence of a nucleus, prokaryotic cells are typically much smaller than eukaryotic cells and also have fewer organelle structures inside of their cells.

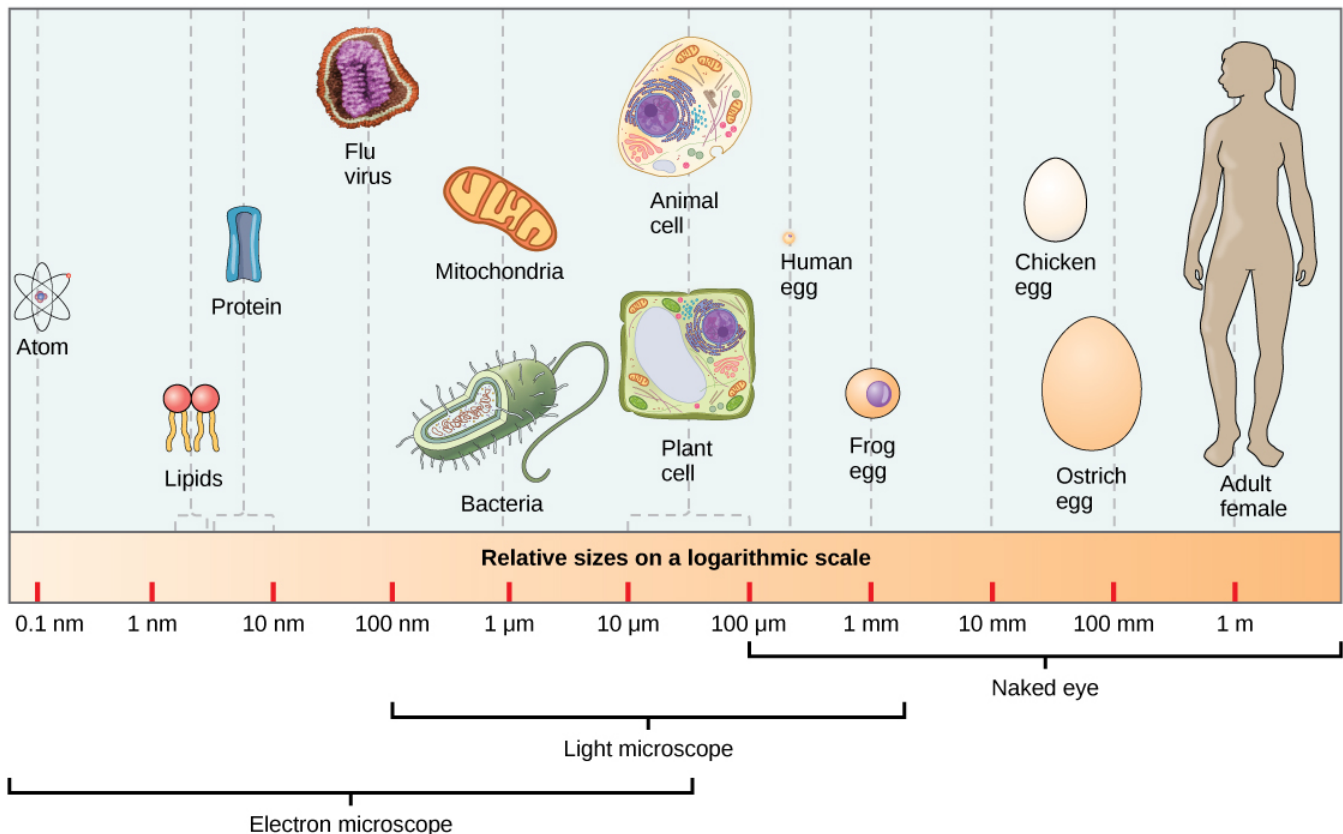


Figure 1: This figure shows relative sizes of microbes on a logarithmic scale (recall that each unit of increase in a logarithmic scale represents a 10-fold increase in the quantity being measured). The smallest cells in the world are prokaryotic cells (including bacteria) that tend to be between 1 μm and 10 μm long. Eukaryotic cells are usually larger than prokaryotic cells (between 10 μm and 100 μm, but sometimes they can be much larger such as a frog egg that is 1 mm and can be viewed with the naked eye)

All cells (both prokaryotic and eukaryotic) share four common components:

1. a **plasma membrane**, an outer covering that separates the cell's interior from its surrounding environment

2. **cytoplasm**, consisting of a jelly-like cytosol within the cell in which other cellular components are found
3. **DNA**, the genetic material of the cell
4. **ribosomes**, which synthesize proteins (prokaryotic ribosomes differ from eukaryotic ribosomes in several ways)

A prokaryote is a simple, mostly single-celled (unicellular) organism that lacks a nucleus, or any other membrane-bound organelle. Prokaryotic DNA is found in a central part of the cell: the **nucleoid**

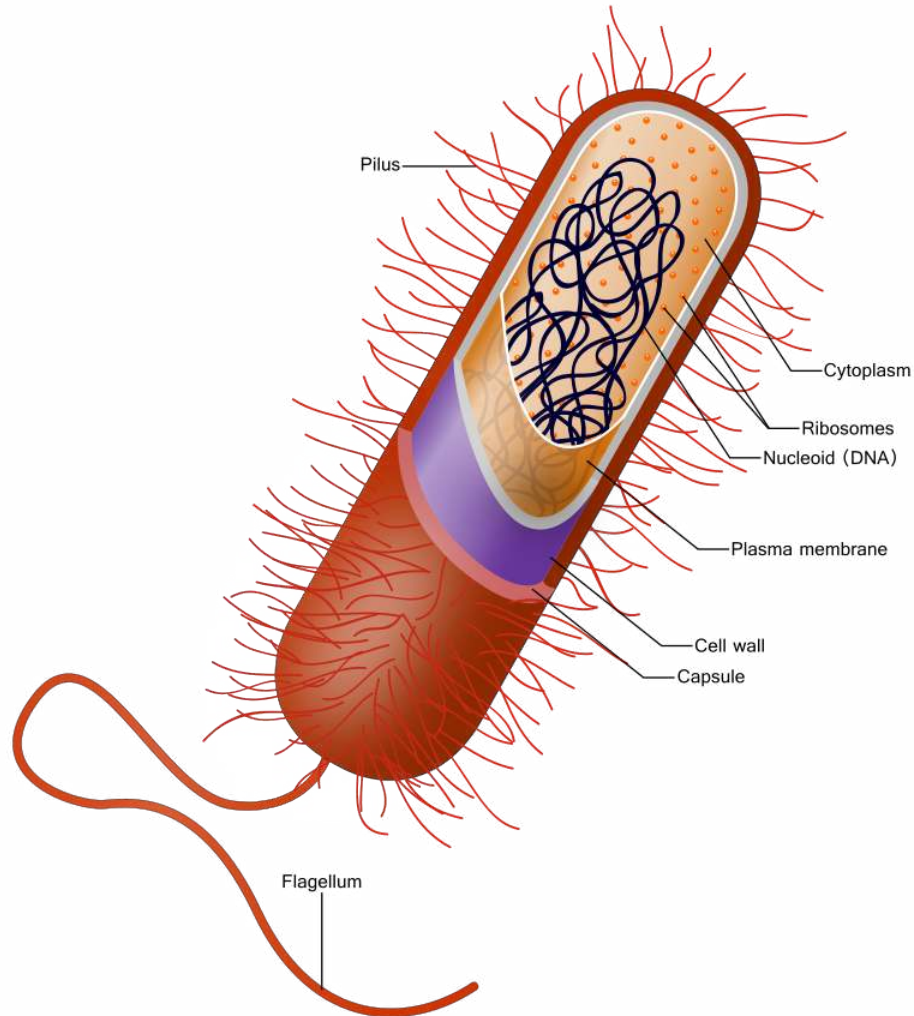


Figure 2: This figure shows the generalized structure of a prokaryotic cell. Prokaryotes have chromosomal DNA localized in a nucleoid, ribosomes, a cell membrane, and a cell wall. The other structures shown are present in some, but not all, bacteria (flagellum, pili, capsule).

prokaryotic cell structure	function of this cell structure
capsule	enables the cell to attach to surfaces in its environment (including attachment to a host in pathogenic [disease-causing] species)
cell wall	acts as an extra layer of protection, helps the cell maintain its shape, and prevents dehydration
cytoplasm	semifluid inside of the cell that holds cell components and is the site of the cell's metabolism (its chemical reactions that keep it alive)
flagellum (singular) / flagella (plural)	a whip-like tail that rotates to move a cell; prokaryotic cells can have no flagella, one flagellum, or multiple flagella depending on the species
nucleoid	a central region within a prokaryotic cell composed of the cell's chromosome (its DNA); the chromosome is a single DNA molecule that is in a circular and comprises the genome of the cell; this chromosome wraps and twist onto itself to form the nucleoid
plasma membrane	a fluid membrane that separates the inside of the cell from the outside of the cell; this structure is semipermeable (some materials can pass through the membrane and others cannot)
plasmid	(not shown in the figure) a small DNA molecule (often circular) found in the cytoplasm; plasmids are much smaller than the DNA chromosome in the nucleoid; plasmids can be exchanged by cells or picked up by cells to acquire new traits; plasmids can carry antibiotic resistance genes and therefore create challenges for treating some infections when they are shared between cells; prokaryotic cells can survive without plasmids and do not always contain a plasmid, while some may contain multiple plasmids
pilus (singular) / pili (plural)	important for attachment to surfaces or other cells, including host cells for pathogenic (disease-causing) cells; some pili, called "sex pili," can be used to exchange genetic material (DNA) between cells during a process called conjugation
ribosome (singular) / ribosomes (plural)	small structures found in the cytoplasm that build proteins; proteins are needed to conduct a cell's metabolism, form structures, and do so many other things within a cell; instructions for building different types of proteins come through chemical messages originating in the DNA

Bacterial Cell Shapes & Arrangements

Bacterial cells can have a variety of cell shapes that differ for different species. Each cell shape has a specific terminology used to describe it:

- **cocci** - round
- **bacilli** - rods
- **vibrios** - curved rods
- **spirochetes** - long, thin, wavy, and helical/corkscrew
- **spirilla** - long, thin wavy, and not helical/corkscrew

Bacteria that are cocci can be found arranged as:

- **solitary** - single cells unattached to other cells (may still be seen attached to another cell during division process)

- **diplococci** - cells attached as pairs
- **tetrads** - cells are grouped as four attached cells
- **sarcina** - cells are grouped as eight attached cells
- **streptococci** - cells are arranged in long chains resembling a string of pearls
- **staphylococci** - cells are arranged in large bunches

Bacteria that are bacilli can be found arranged as:

- **solitary** - single cells unattached to other cells (may still be seen attached to another cell during division process)
- **diplobacilli** - cells attached as pairs
- **streptobacilli** - cells are arranged in long chains end-to-end
- **palisades** - cells arranged lined up next to each other long-ways and resembles pickets in a picket fence

To see illustrations and images of cell shapes and cell arrangements as well as more details see [Bacterial Cell Morphology & Arrangement](#) in the Simple Stain chapter.

Bacterial Cell Wall Structures

Most bacteria are divided into two major groups: Gram-positive bacteria and Gram-negative bacteria based on the cell wall composition (can be differentiated by using the Gram stain procedure on the bacteria). Knowing the Gram reaction of a clinical isolate (isolated bacterial species from a patient) can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.

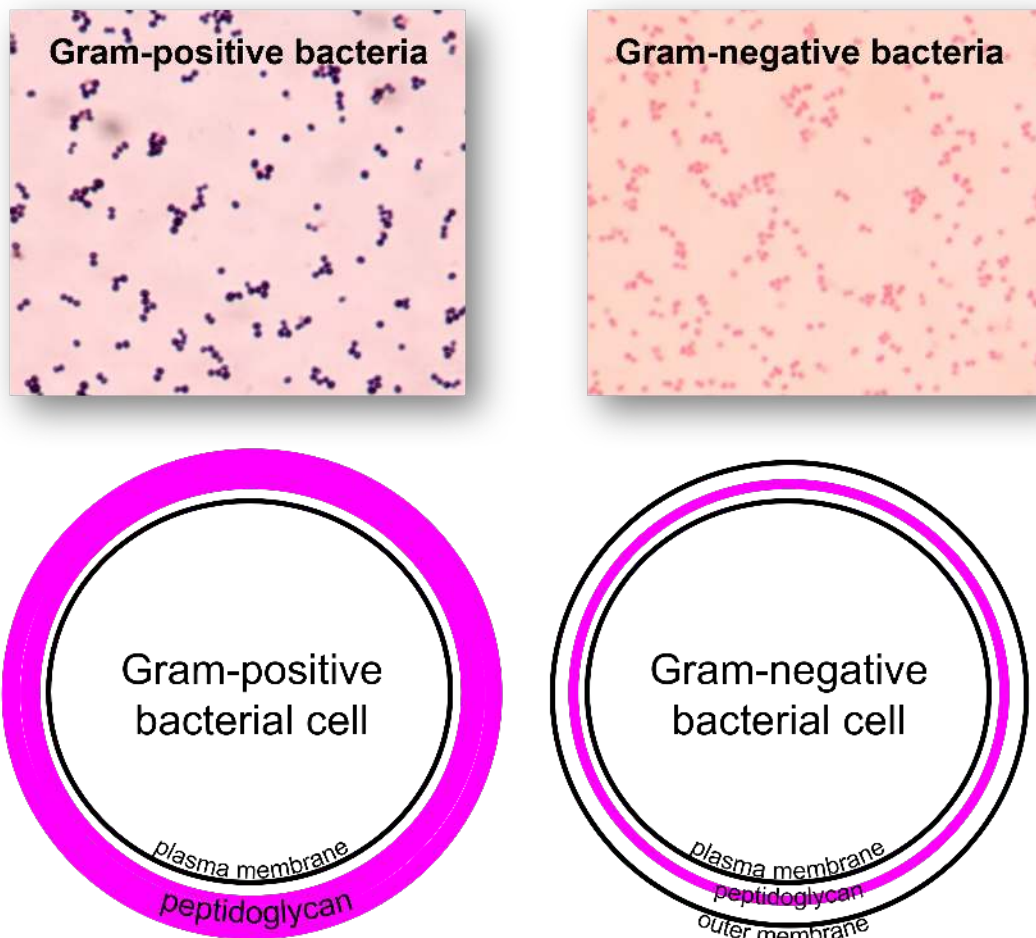


Figure 3: (Top) Microscopic images of bacteria stained with the Gram stain. (Top right) Gram-positive bacterial species stain purple with the Gram stain. (Top left) Gram-negative bacterial species stain pink with the Gram stain. (Bottom) Diagrams illustrating major structural components of Gram-positive bacterial cell walls and Gram-negative bacterial cell walls. (Bottom right) Gram-positive bacterial species have a thick layer of peptidoglycan outside of the plasma membrane and a layer of periplasm. (Bottom left) Gram-negative bacterial species have a thin layer of peptidoglycan outside of the plasma membrane surrounded by periplasm with an outer membrane outside of the peptidoglycan layer. Embedded in the outer membrane of Gram-negative cell walls are lipopolysaccharides (LPS) that are not shown in this diagram.

The results of the Gram stain reflect differences in cell wall composition. **Gram-positive** cells have thick layers of peptidoglycan (a substance composed of carbohydrates and protein subunits) in their cell walls. **Gram-negative** bacteria have very little peptidoglycan. Gram-positive bacteria also have teichoic acids, whereas Gram-negative bacteria do not. Gram-negative cells have an outer membrane that resembles the phospholipid bilayer of the plasma membrane. The outer membrane contains **lipopolysaccharides (LPS)**, which are released as **endotoxins** when Gram-negative cells die. This can be of concern to a person with an infection caused by a Gram-negative organism.

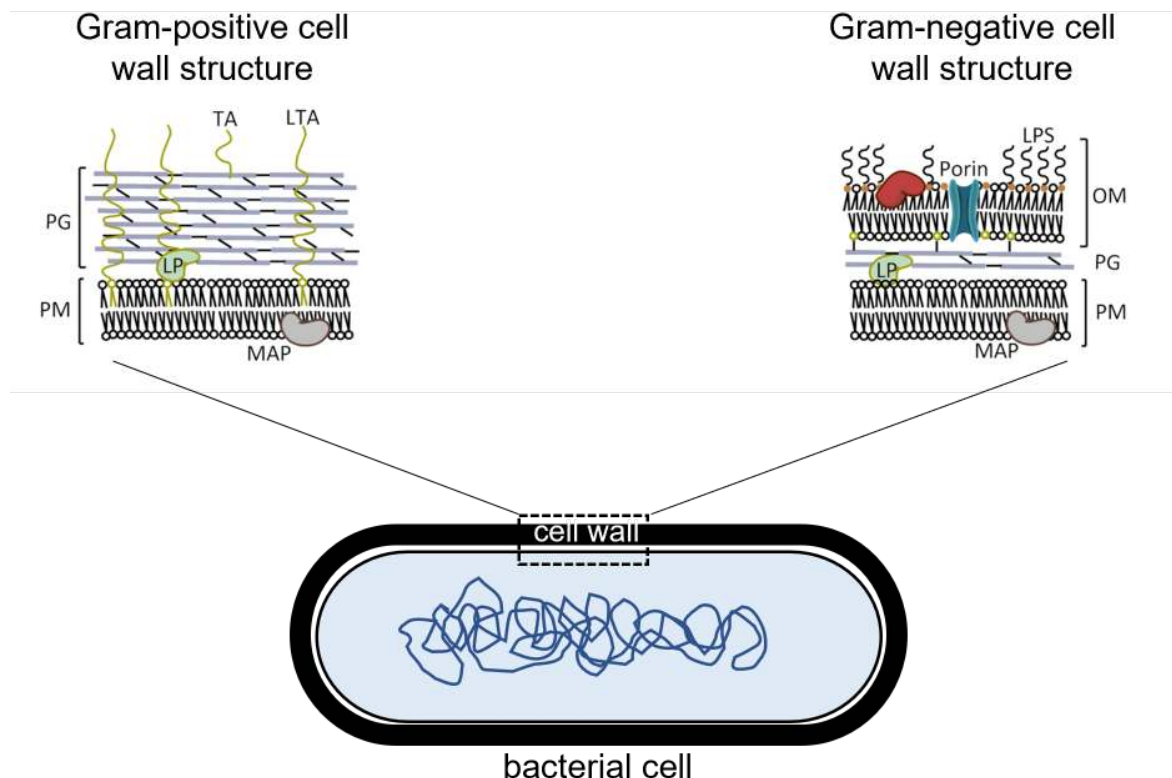


Figure 4: Detailed view of the structure of bacterial cell walls for Gram-positive species and for Gram-negative species. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across the peptidoglycan layer. (Right) Gram-negative cell walls consist of a thin layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM). Gram-negative cell walls have an outer membrane (OM) beyond the peptidoglycan layer containing lipopolysaccharide (LPS) as well as porin channels that enable some materials to pass across the outer membrane. In Gram-positive and Gram-negative cell wall types lipoprotein (LP) is also found.

Some species are known as **Gram-variable**, and so both Gram-positive and Gram-negative reactions may occur when a Gram-variable species is stained using the Gram stain. The vast majority of bacteria are either Gram-positive or Gram-negative. However, not all bacteria can be stained with the Gram stain (for example, *Mycoplasma* sp., which have no cell wall, stains poorly with the Gram stain).

Bacterial Endospores

An **endospore** is a form of a bacterial cell. Only some species of bacteria can produce endospores. Endospores are made so the cell can survive poor growth or poor survival conditions. An endospore is a mainly inactive version of a cell that is analogous to a survival bunker. When environmental conditions are poor, or even deadly, bacteria that are able to will produce endospores to survive. When environmental conditions improve, endospores can produce the active cells again (active cells are called **vegetative cells**.) Bacterial endospores are the most resistant structures of all living organisms, and they can live in this dormant dehydrated state for hundreds of years (even some documented at thousands of years). The stimulus that triggers sporulation (formation of spores) can vary and may include nutrient depletion, desiccation, chemicals, heat, etc.

Note

Endospores are not for reproduction. One spore forms inside of one vegetative cell (vegetative = metabolically active cell). When environmental conditions improve, one spore germinates to produce one vegetative cell.

Endospore production is a very important characteristic of some bacteria, allowing them to resist adverse environmental conditions such as desiccation, chemical exposure, extreme heat, etc. Endospores were first identified in the 1800s (John Tyndall developed a

process for destroying them with an intermittent heating procedure), although the stain procedures to identify them did not develop until the early twentieth century.

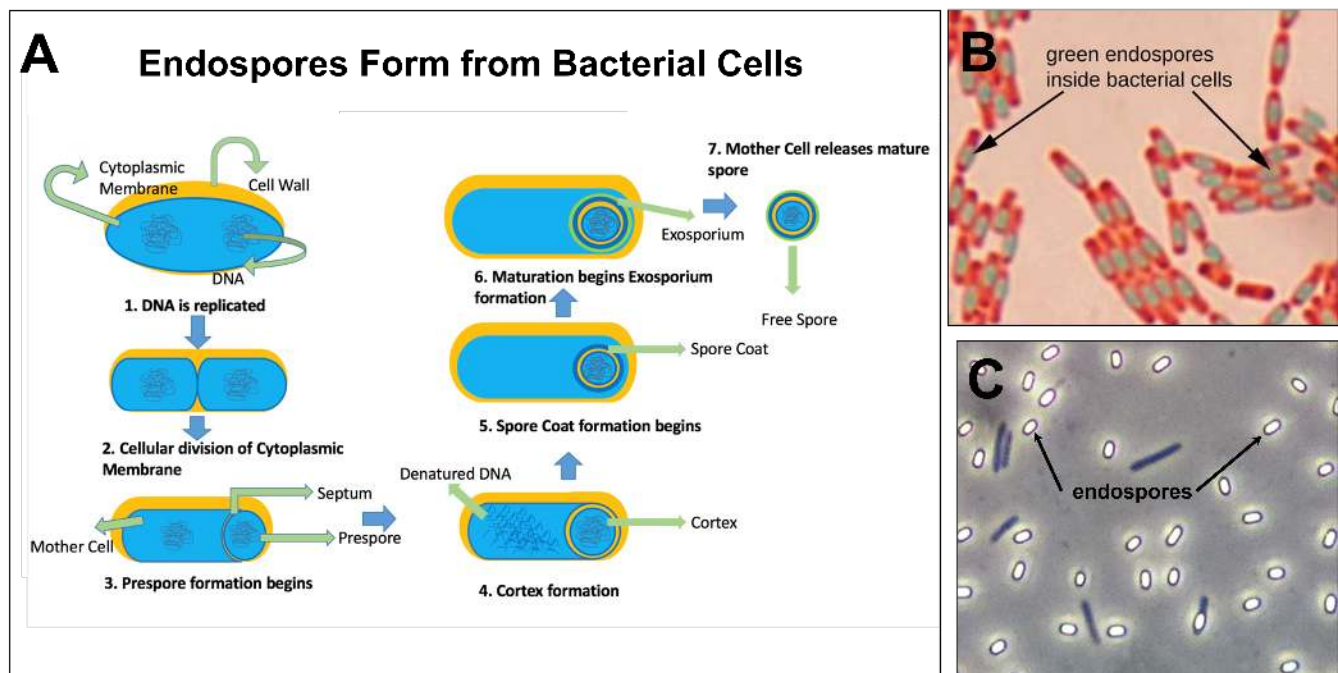


Figure 5: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore 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.

The identification of endospores is very important for the clinical microbiologist who is analyzing a patient's body fluid or tissue since there are not that many spore-forming **genera**. Knowing if the species of bacteria causing an infection forms spores or not helps to narrow down the possible bacterial species causing the infection. In fact, there are two major **pathogenic** (disease-causing) spore-forming genera: *Bacillus* and *Clostridium*. *Bacillus* and *Clostridium* species cause a number of dangerous and lethal diseases such as botulism, gangrene, tetanus, c-diff, and anthrax, to name a few.

Some bacteria have to be put into unfavorable situations (high cell density and starvation are two key triggers) to go into sporulation. Other species will make spores easily without much provocation (e.g. *Bacillus subtilis*). Vegetative cells that have not yet made spores may be in the process of making the spore or will not make them at all. The vegetative cell is metabolically active, whereas the spore is not. The location where an endospore is within a vegetative cell is also useful for distinguishing bacterial species. Endospores may be located in a **terminal** (end of the cell), **subterminal** (near the end of the cell), or **central** (middle of the cell) position. A particular species of the genus will form spores in a specific area, producing another useful taxonomic identification tool and therefore useful in identifying the species of bacteria.

Bacterial Flagella

Flagella are structures used by cells to move in aqueous environments. Bacterial flagella act like propellers. They are stiff spiral filaments composed of flagellin protein subunits that extend outward from the cell and spin in solution. The **basal body** is the motor for the flagellum and is embedded in the plasma membrane. A hook region connects the basal body to the filament. Gram-positive and gram-negative bacteria have different basal body configurations due to differences in cell wall structure.

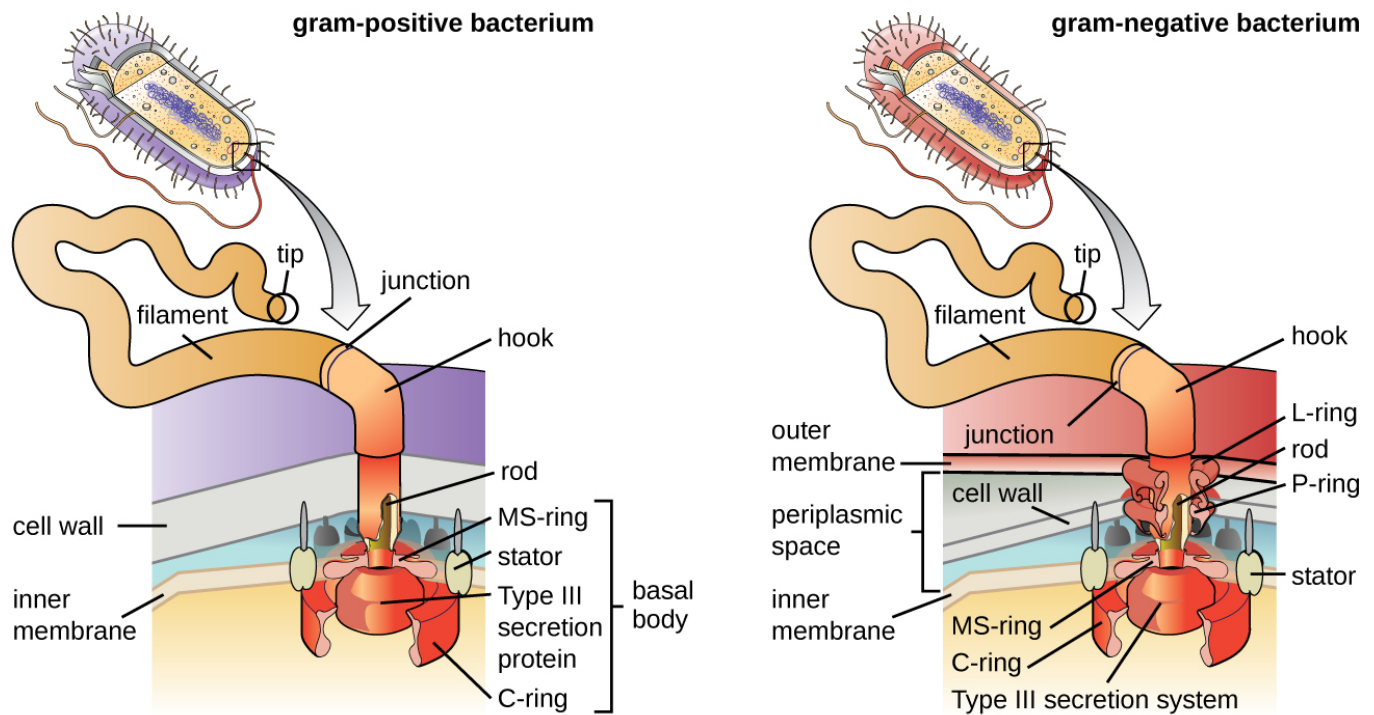


Figure 6: The basic structure of a bacterial flagellum consists of a basal body, hook, and filament. The basal body composition and arrangement differ between gram-positive and gram-negative bacteria. (credit: modification of work by "LadyofHats"/Mariana Ruiz Villareal)

Different types of motile bacteria exhibit different arrangements of flagella. A bacterium with a singular flagellum, typically located at one end of the cell (polar), is said to have a **monotrichous** flagellum. An example of a monotrichously flagellated bacterial pathogen is *Vibrio cholerae*, the gram-negative bacterium that causes cholera. Cells with **amphitrichous** flagella have a flagellum or tufts of flagella at each end. An example is *Spirillum minor*, the cause of spirillary (Asian) rat-bite fever or sodoku. Cells with **lophotrichous** flagella have a tuft at one end of the cell or both ends of the cell. The gram-negative bacillus *Pseudomonas aeruginosa*, an opportunistic pathogen known for causing many infections, including "swimmer's ear" and burn wound infections, has lophotrichous flagella. Flagella that cover the entire surface of a bacterial cell are called **peritrichous** flagella. The gram-negative bacterium *E. coli* shows a peritrichous arrangement of flagella.

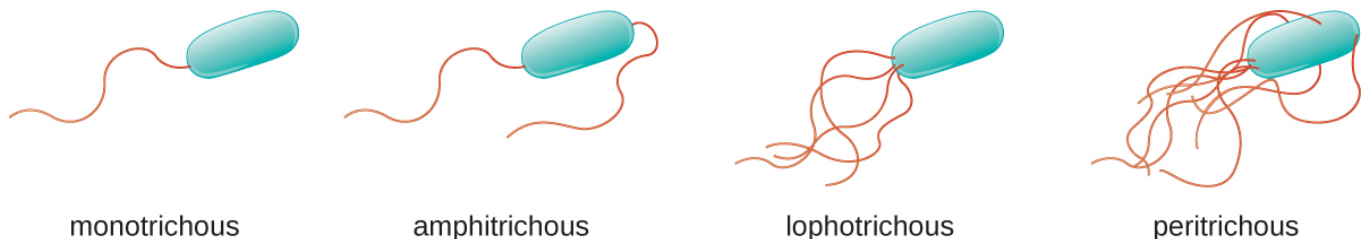
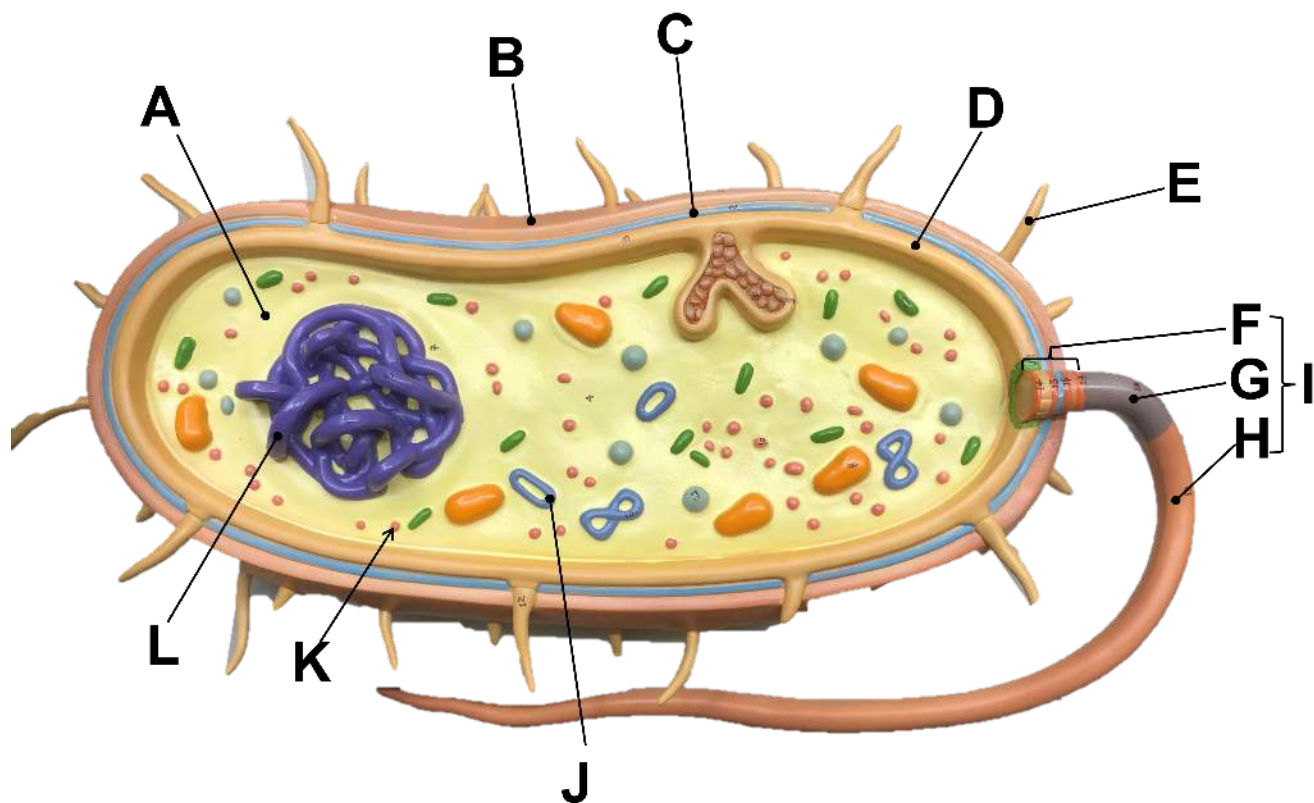
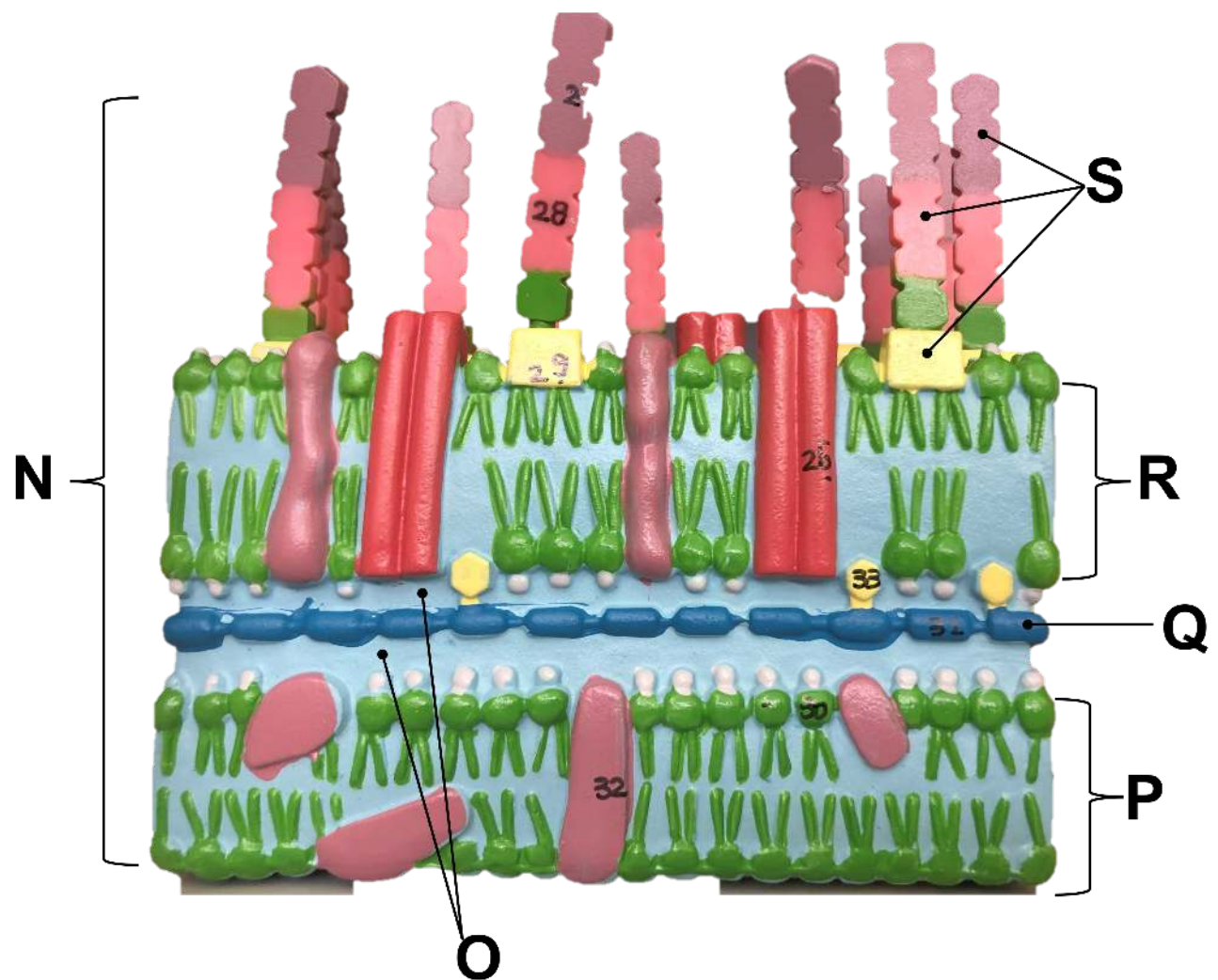


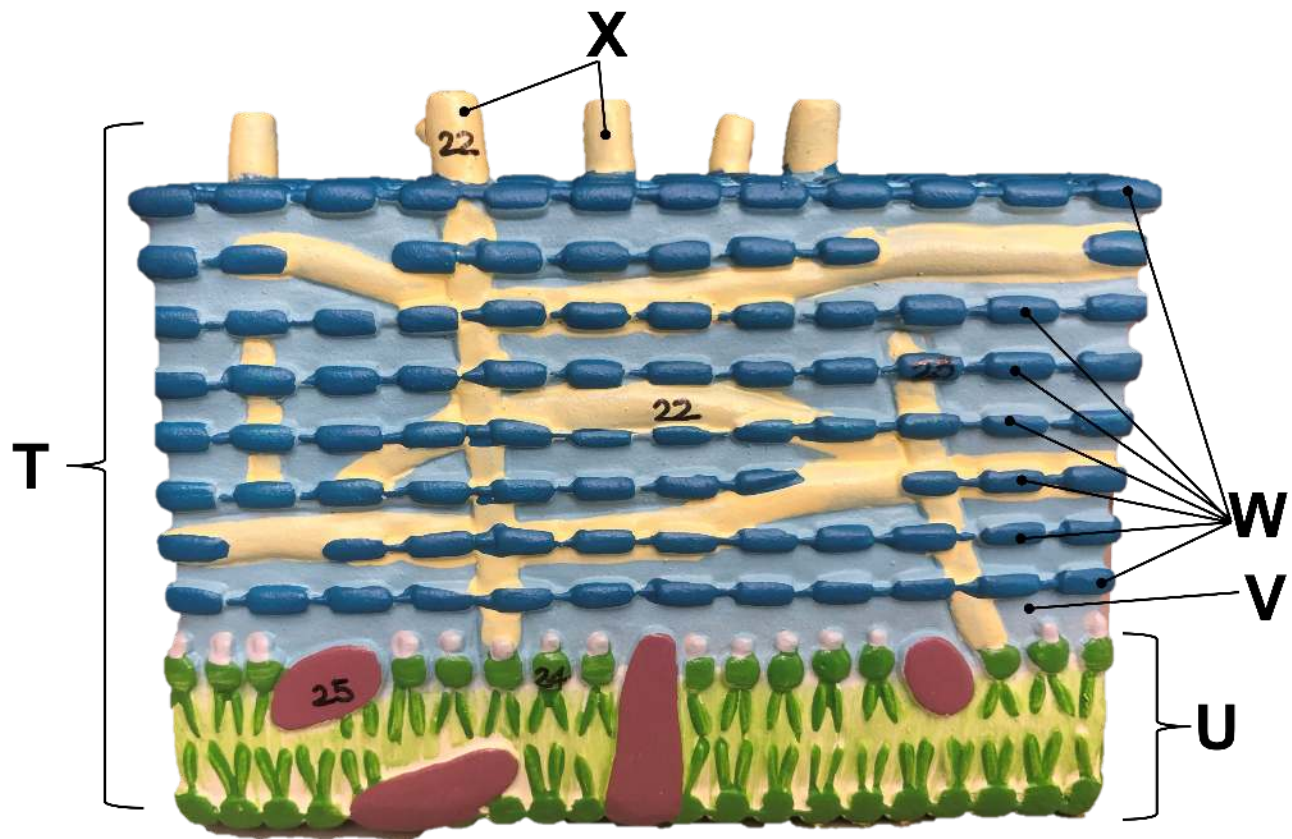
Figure 7: Flagellated bacteria may exhibit multiple arrangements of their flagella. Common arrangements include monotrichous, amphitrichous, lophotrichous, or peritrichous.

Laboratory Instructions

Identify Prokaryotic Cell Model Structures







1. Carefully examine cell models of bacterial cells.
2. Identify the following structures on the cell models:

Prokaryotic structure	Diagram letter
basal body (of flagellum)	
capsule	
cell wall	
cytoplasm	
filament (of flagellum)	
flagellum	
Gram-negative cell wall	
Gram-positive cell wall	
hook (of flagellum)	
lipopolysaccharide	
nucleoid	
outer membrane	
peptidoglycan (thick layer)	
peptidoglycan (thin layer)	
periplasm (2 places)	
pilus	
plasma membrane (3 places)	
plasmid	
ribosome	
teichoic acid	

CARMEN QUESTIONS

1. Use a microscope to examine the different bacterial types. Identify the specimen, total magnification, and bacterial shape/arrangement for each specimen.

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1.5: Eukaryotic Cells

Learning Objectives

- Differentiate between eukaryotic cell and prokaryotic cell structures.
- Identify the structures and functions of components of eukaryotic cells.
- Name the categories of microorganisms that are eukaryotic.
- Provide a description that differentiates each type of eukaryotic microorganism from the other types of eukaryotic microorganisms.
- Examine specimens of different types of eukaryotic microorganisms and identify structures of these microorganisms, especially the nucleus.

Eukaryotic Cells

The cells of eukaryotic organisms have several distinguishing characteristics. Above all, eukaryotic cells are defined by the presence of a nucleus surrounded by a complex nuclear membrane. Also, eukaryotic cells are characterized by the presence of membrane-bound organelles in the cytoplasm. Organelles such as mitochondria, the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, and peroxisomes are held in place by the **cytoskeleton**, an internal network that supports transport of intracellular components and helps maintain cell shape. The genome of eukaryotic cells is packaged in multiple, rod-shaped chromosomes as opposed to the single, circular-shaped chromosome that characterizes most prokaryotic cells.

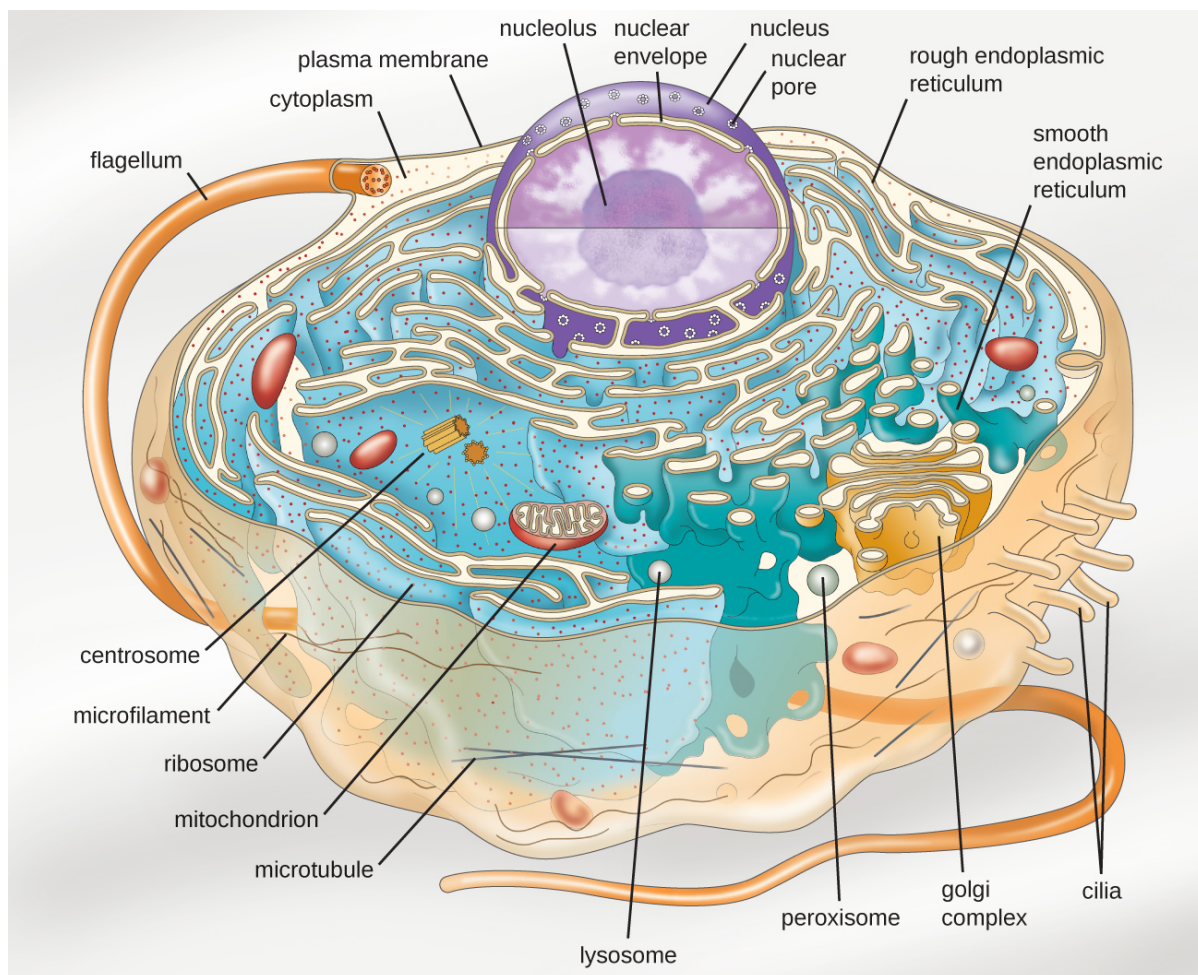


Figure 1: An illustration of a generalized, single-celled eukaryotic organism. Note that cells of eukaryotic organisms vary greatly in terms of structure and function, and a particular cell may not have all of the structures shown here.

Table 1: A comparison of prokaryotic cells and eukaryotic cells.

Summary of Cell Structures			
	Prokaryotes		Eukaryotes
	Bacteria	Archaea	
size	~0.5–1 μm	~0.5–1 μm	~5–20 μm
surface area-to-volume ratio	High	High	Low
nucleus	No	No	Yes
genome characteristics	- Single chromosome - Circular - Haploid - Lacks histones	- Single chromosome - Circular - Haploid - Contains histones	- Multiple chromosomes - Linear - Haploid or diploid - Contains histones
cell division	Binary fission	Binary fission	Mitosis, meiosis
membrane lipid composition	- Ester-linked - Straight-chain fatty acids - Bilayer	- Ether-linked - Branched isoprenoids - Bilayer or monolayer	- Ester-linked - Straight-chain fatty acids - Sterols - Bilayer
cell wall composition	- Peptidoglycan, or - None	- Pseudopeptidoglycan, or - Glycopeptide, or - Polysaccharide, or - Protein (S-layer), or - None	- Cellulose (plants, some algae) - Chitin (fungi) - Silica (some algae) - Most others lack cell walls
motility structures	Rigid spiral flagella composed of flagellin	Rigid spiral flagella composed of archaeal flagellins	Flexible flagella and cilia composed of microtubules
membrane-bound organelles	No	No	Yes
endomembrane system	No	No	Yes (ER, Golgi, lysosomes)
ribosomes	70S	70S	80S in cytoplasm and rough ER 70S in mitochondria, chloroplasts

Table 2: Functions of Eukaryotic Cell Structures.

Cell Structure	Function
cell wall	structure outside of the plasma membrane in some types of species; maintains cell shape
centrioles / centrosome	organizes microtubules (particularly important during cell division to move the chromosomes and separate them correctly into the two daughter cells)
chloroplast	conducts photosynthesis
chromatin	DNA with its associated proteins
cilia	external-facing protein fibers that wave to move a cell
cytoplasm	semi-fluid surrounding cellular structures with dissolved molecules; location of many cellular metabolic reactions
cytoskeleton	microtubules, intermediate filaments, and microfilaments (provides a cell shape, structure, can be used for cell movement or moving materials around the cell)
flagellum / flagella	a whip-like tail that moves a cell
Golgi complex / apparatus / body	modifies proteins and packages them into vesicles for transport to their destinations
lysosome	digests food and waste materials
mitochondria / mitochondrion	makes ATP (an energy-rich molecule) using nutrients
nuclear membrane / nuclear envelope	membrane enclosing the nucleus; protein-lined pores allow materials to move in and out of the nucleus
nuclear pore	protein-lined pores allow materials to move in and out of the nucleus
nucleolus	a condensed region within the cell nucleus where ribosomes are formed
nucleus	contains chromatin, a nuclear envelope, and a nucleolus
peroxisomes	metabolizes oxygen-containing waste
ribosomes	makes proteins
rough endoplasmic reticulum	membranes associated with ribosomes where the ribosomes make membrane proteins and proteins for secretion out of the cell
smooth endoplasmic reticulum	membranes without ribosomes; make lipids; detoxification

Microbes with Eukaryotic Cells

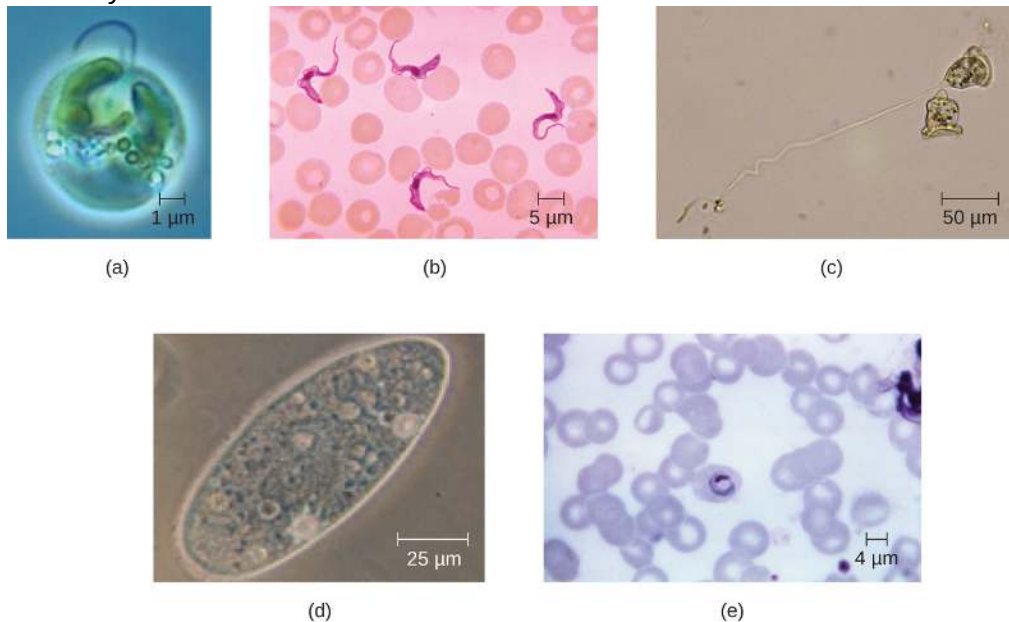


Figure 2: Eukaryotic cells come in a variety of cell shapes. (a) Spheroid *Chromulina* (a species of algae). (b) Fusiform shaped *Trypanosoma* (a parasitic protozoan shown with red blood cells of its host). (c) Bell-shaped *Vorticella* (a free-living protozoan). (d) Ovoid *Paramecium* (a free-living protozoan). (e) Ring-shaped *Plasmodium ovale* (a parasitic protozoan living inside a red blood cell of its host). (credit a: modification of work by NOAA; credit b, e: modification of work by Centers for Disease Control and Prevention)

Eukaryotic organisms include protozoans, algae, fungi, plants, and animals. Some eukaryotic cells are independent, single-celled microorganisms, whereas others are part of multicellular organisms.

Eukaryotic microbes are an extraordinarily diverse group, including species with a wide range of life cycles, morphological specializations, and nutritional needs. Although more diseases are caused by viruses and bacteria than by microscopic eukaryotes, these eukaryotes are responsible for some diseases of great public health importance. For example, the protozoal disease malaria was responsible for 584,000 deaths worldwide (primarily children in Africa) in 2013, according to the World Health Organization (WHO). The protozoan parasite *Giardia* causes a diarrheal illness (giardiasis) that is easily transmitted through contaminated water supplies. In the United States, *Giardia* is the most common human intestinal parasite. Although it may seem surprising, parasitic worms are included within the study of microbiology because identification depends on observation of microscopic adult worms or eggs. Even in developed countries, these worms are important parasites of humans and of domestic animals. There are fewer fungal pathogens, but these are important causes of illness, as well. On the other hand, fungi have been important in producing antimicrobial substances such as penicillin.

Protozoa

Protozoa are nonphotosynthetic, motile eukaryotic organisms that are always unicellular. Historically, these microorganisms have been classified as protozoa because they were "animal like" unicellular eukaryotic organisms that did not fit with other eukaryotic taxonomic groupings (plants, fungi, or animals).

Protozoans inhabit a wide variety of habitats, both aquatic and terrestrial. Many are free-living, while others are parasitic, carrying out a life cycle within a host or hosts and potentially causing illness. There are also beneficial symbionts that provide metabolic services to their hosts. During the feeding and growth part of their life cycle, they are called **trophozoites**; these feed on small particulate food sources such as bacteria. While some types of protozoa exist exclusively in the trophozoite form, others can develop from trophozoite to an encapsulated cyst stage when environmental conditions are too harsh for the trophozoite. A **cyst** is a cell with a protective wall, and the process by which a trophozoite becomes a cyst is called **encystment**. When conditions become more favorable, these cysts are triggered by environmental cues to become active again through **excystment**.

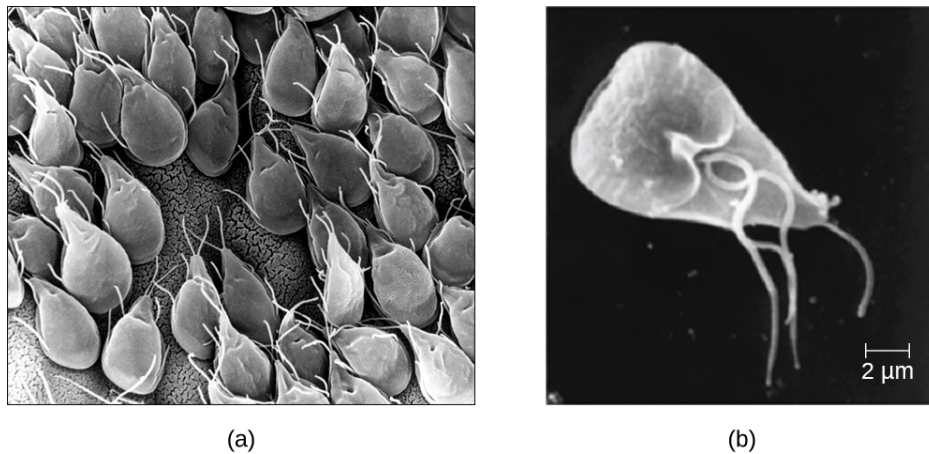


Figure 3: (a) A scanning electron micrograph shows many *Giardia* parasites in the trophozoite, or feeding stage, in a gerbil intestine. (b) An individual trophozoite of *G. lamblia*, visualized here in a scanning electron micrograph. This waterborne protozoan causes severe diarrhea when ingested. (credit a, b: modification of work by Centers for Disease Control and Prevention)

Protozoans have a variety of reproductive mechanisms. Some protozoans reproduce asexually and others reproduce sexually; still others are capable of both sexual and asexual reproduction. In protozoans, asexual reproduction occurs by binary fission, budding, or schizogony. In **schizogony**, the nucleus of a cell divides multiple times before the cell divides into many smaller cells. The products of schizogony are called merozoites and they are stored in structures known as schizonts. Protozoans may also reproduce sexually, which increases genetic diversity and can lead to complex life cycles. Protozoans can produce haploid gametes that fuse through **syngamy**. However, they can also exchange genetic material by joining to exchange DNA in a process called conjugation. This is a different process than the conjugation that occurs in bacteria. The term protist conjugation refers to a true form of eukaryotic sexual reproduction between two cells of different mating types. It is found in **ciliates**, a group of protozoans, and is described later in this subsection.

All protozoans have a plasma membrane, or **plasmalemma**, and some have bands of protein just inside the membrane that add rigidity, forming a structure called the **pellicle**. Some protists, including protozoans, have distinct layers of cytoplasm under the membrane. In these protists, the outer gel layer (with microfilaments of actin) is called the **ectoplasm**. Inside this layer is a sol (fluid) region of cytoplasm called the **endoplasm**. These structures contribute to complex cell shapes in some protozoans, whereas others (such as amoebas) have more flexible shapes.

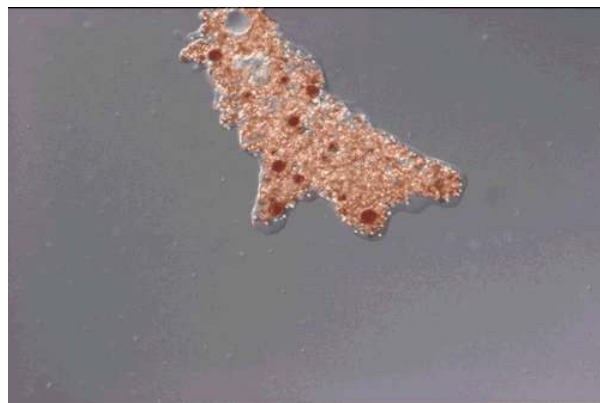


Figure 4: Amoeba motion involves formation of pseudopodia ("false feet"). These extensions enable the cell to creep along in a liquid environment and also means that the shape of the cell is constantly changing.

Different groups of protozoans have specialized feeding structures. They may have a specialized structure for taking in food through phagocytosis, called a **cytostome**, and a specialized structure for the exocytosis of wastes called a **cytoproct**. Oral grooves leading to cytostomes are lined with hair-like cilia to sweep in food particles. Protozoans are heterotrophic. Protozoans that are **holozoic** ingest whole food particles through phagocytosis. Forms that are **saprozoic** ingest small, soluble food molecules.

Many protists have whip-like flagella or hair-like cilia made of microtubules that can be used for locomotion. Other protists use cytoplasmic extensions known as pseudopodia (“false feet”) to attach the cell to a surface; they then allow cytoplasm to flow into the extension, thus moving themselves forward.

Protozoans have a variety of unique organelles and sometimes lack organelles found in other cells. Some have **contractile vacuoles**, organelles that can be used to move water out of the cell for osmotic regulation (salt and water balance). Mitochondria may be absent in parasites or altered to kinetoplasts (modified mitochondria) or hydrogenosomes.

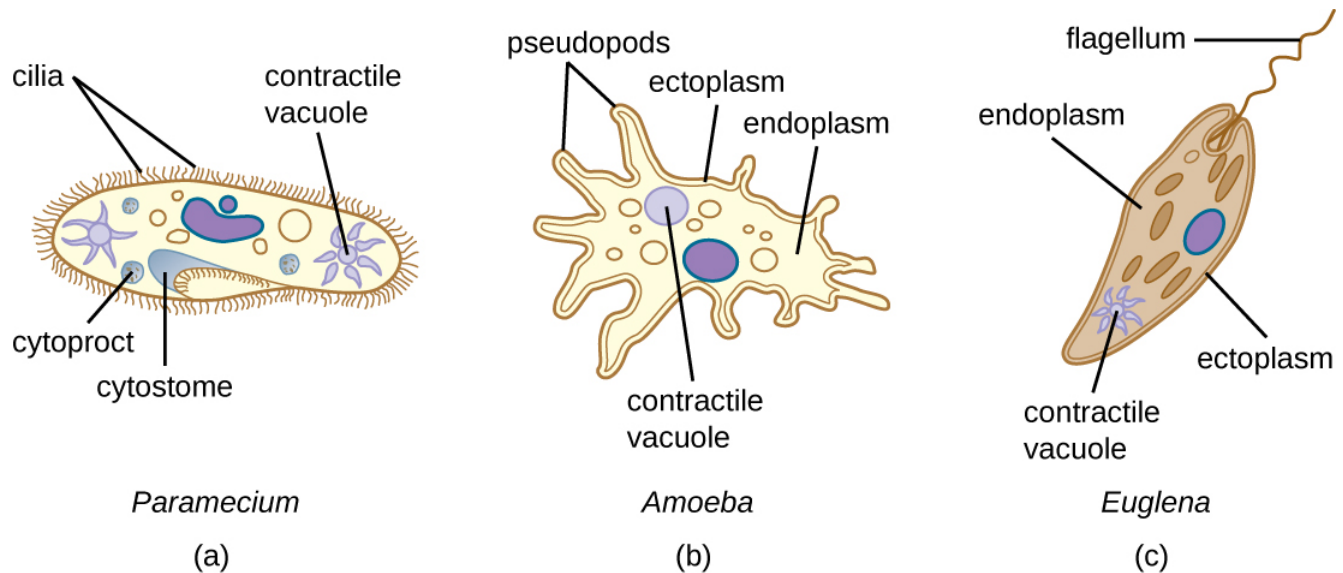


Figure 5: (a) *Paramecium* spp. have hair-like appendages called cilia for locomotion. (b) *Amoeba* spp. use lobe-like pseudopodia to anchor the cell to a solid surface and pull forward. (c) *Euglena* spp. use a whip-like structure called a flagellum to propel the cell.

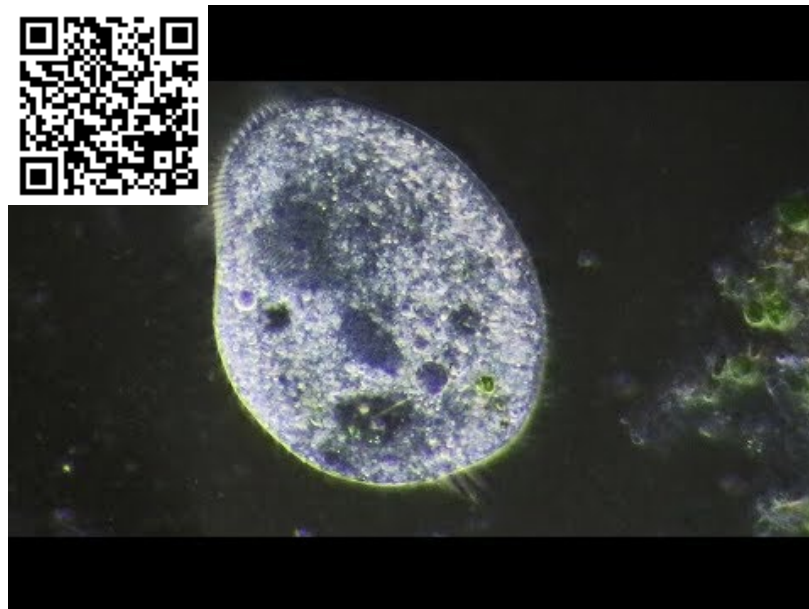


Figure 6: A free-living ciliated cell with contractile vacuole pulsating to regulate water and salt balance in the cell.

Algae

The **algae** are autotrophic eukaryotes that can be unicellular or multicellular and are distinct from other eukaryotic groupings (plants, fungi, and animals). These organisms are found in the supergroups Chromalveolata (dinoflagellates, diatoms, golden algae, and brown algae) and Archaeplastida (red algae and green algae). They are important ecologically and environmentally because they are responsible for the production of approximately 70% of the oxygen and organic matter in aquatic environments. Some types of algae, even those that are microscopic, are regularly eaten by humans and other animals. Additionally, algae are the source

for **agar**, agarose, and **carrageenan**, solidifying agents used in laboratories and in food production. Although algae are typically not pathogenic, some produce toxins. Harmful **algal blooms**, which occur when algae grow quickly and produce dense populations, can produce high concentrations of toxins that impair liver and nervous-system function in aquatic animals and humans.

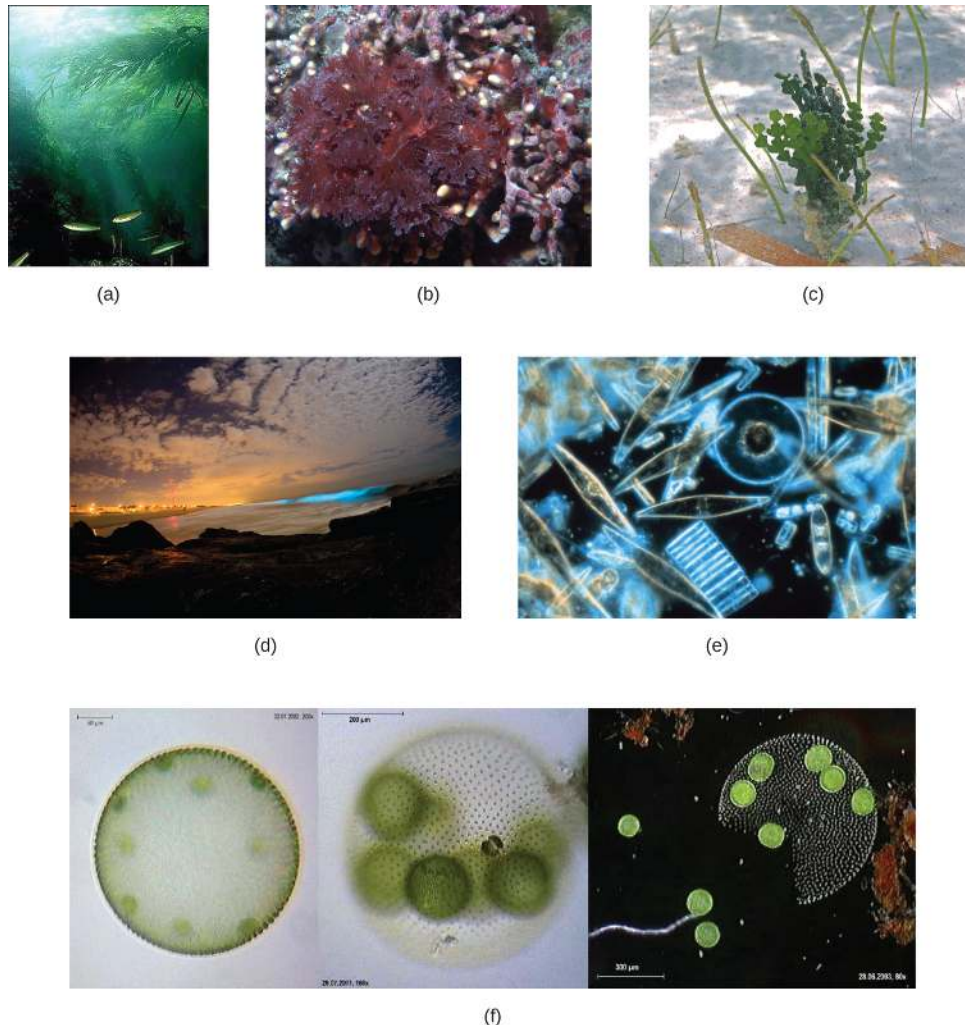


Figure 7: Some of the diversity of algae. (a) These large multicellular kelps are members of the brown algae. Note the “leaves” and “stems” that make them appear similar to green plants. (b) This is a species of red algae that is also multicellular. (c) The green alga *Halimeda incrassata*, shown here growing on the sea floor in shallow water, appears to have plant-like structures, but is not a true plant. (d) Bioluminescence, visible in the cresting wave in this picture, is a phenomenon of certain dinoflagellates. (e) Diatoms (pictured in this micrograph) produce siliceous tests (skeletons) that form diatomaceous earths. (f) Colonial green algae, like volvox in these three micrographs, exhibit simple cooperative associations of cells. (credit a, e: modification of work by NOAA; credit b: modification of work by Ed Bierman; credit c: modification of work by James St. John; credit d: modification of work by “catalano82”/Flickr; credit f: modification of work by Dr. Ralf Wagner)

Like protozoans, algae often have complex cell structures. For instance, algal cells can have one or more chloroplasts that contain structures called **pyrenoids** to synthesize and store starch. The chloroplasts themselves differ in their number of membranes, indicative of secondary or rare tertiary endosymbiotic events. Primary chloroplasts have two membranes—one from the original cyanobacteria that the ancestral eukaryotic cell engulfed, and one from the plasma membrane of the engulfing cell. Chloroplasts in some lineages appear to have resulted from secondary endosymbiosis, in which another cell engulfed a green or red algal cell that already had a primary chloroplast within it. The engulfing cell destroyed everything except the chloroplast and possibly the cell membrane of its original cell, leaving three or four membranes around the chloroplast. Different algal groups have different pigments, which are reflected in common names such as red algae, brown algae, and green algae.

Some algae, the seaweeds, are macroscopic and may be confused with plants. Seaweeds can be red, brown, or green, depending on their photosynthetic pigments. Green algae, in particular, share some important similarities with land plants; however, there are also

important distinctions. For example, seaweeds do not have true tissues or organs like plants do. Additionally, seaweeds do not have a waxy cuticle to prevent desiccation. Algae can also be confused with cyanobacteria, photosynthetic bacteria that bear a resemblance to algae; however, cyanobacteria are prokaryotes.

Algae have a variety of life cycles. Reproduction may be asexual by mitosis or sexual using gametes.

Fungi

The fungi comprise a diverse group of organisms that are heterotrophic and typically saprozoic (consume dead organic matter). In addition to the well-known macroscopic fungi (such as mushrooms and molds), many unicellular yeasts and spores of macroscopic fungi are microscopic. For this reason, fungi are included within the field of microbiology.

Fungi are important to humans in a variety of ways. Both microscopic and macroscopic fungi have medical relevance, with some pathogenic species that can cause **mycoses** (illnesses caused by fungi). Some pathogenic fungi are opportunistic, meaning that they mainly cause infections when the host's immune defenses are compromised and do not normally cause illness in healthy individuals. Fungi are important in other ways. They act as decomposers in the environment, and they are critical for the production of certain foods such as cheeses. Fungi are also major sources of antibiotics, such as penicillin from the fungus *Penicillium*.

Fungi have well-defined characteristics that set them apart from other organisms. Most multicellular fungal bodies, commonly called molds, are made up of filaments called **hyphae**. Hyphae can form a tangled network called a **mycelium** and form the **thallus** (body) of fleshy fungi. Hyphae that have walls between the cells are called **septate hyphae**; hyphae that lack walls and cell membranes between the cells are called nonseptate or **coenocytic hyphae**.

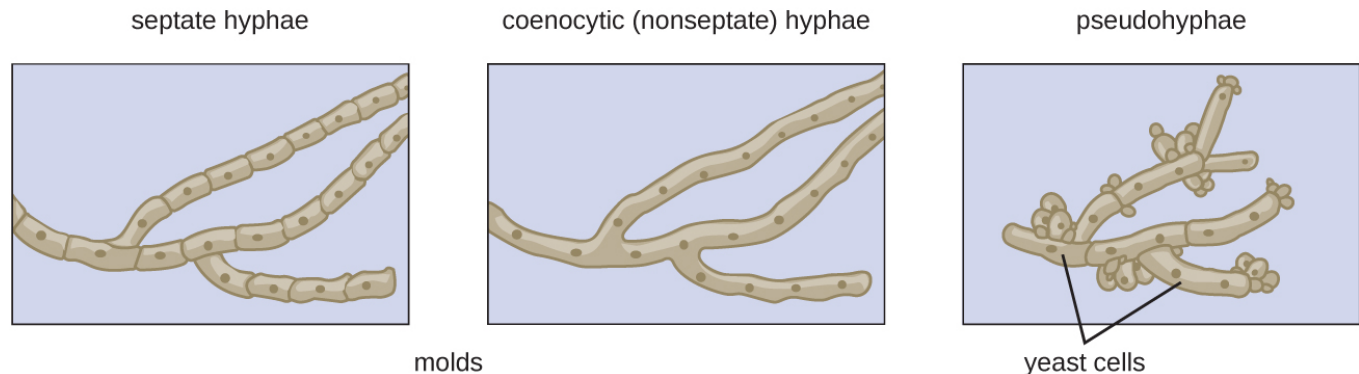


Figure 8: Multicellular fungi (molds) form hyphae, which may be septate or nonseptate. Unicellular fungi (yeasts) cells form pseudohyphae from individual yeast cells.

In contrast to molds, yeasts are unicellular fungi. The **budding yeasts** reproduce asexually by budding off a smaller daughter cell; the resulting cells may sometimes stick together as a short chain or **pseudohypha**.

Some fungi are dimorphic, having more than one appearance during their life cycle. These **dimorphic fungi** may be able to appear as yeasts or molds, which can be important for infectivity. They are capable of changing their appearance in response to environmental changes such as nutrient availability or fluctuations in temperature, growing as a mold, for example, at 25 °C (77 °F), and as yeast cells at 37 °C (98.6 °F). This ability helps dimorphic fungi to survive in diverse environments. Two examples of dimorphic yeasts are the human pathogens *Histoplasma capsulatum* and *Candida albicans*. *H. capsulatum* causes the lung disease histoplasmosis, and *C. albicans* is associated with vaginal yeast infections, oral thrush, and candidiasis of the skin.

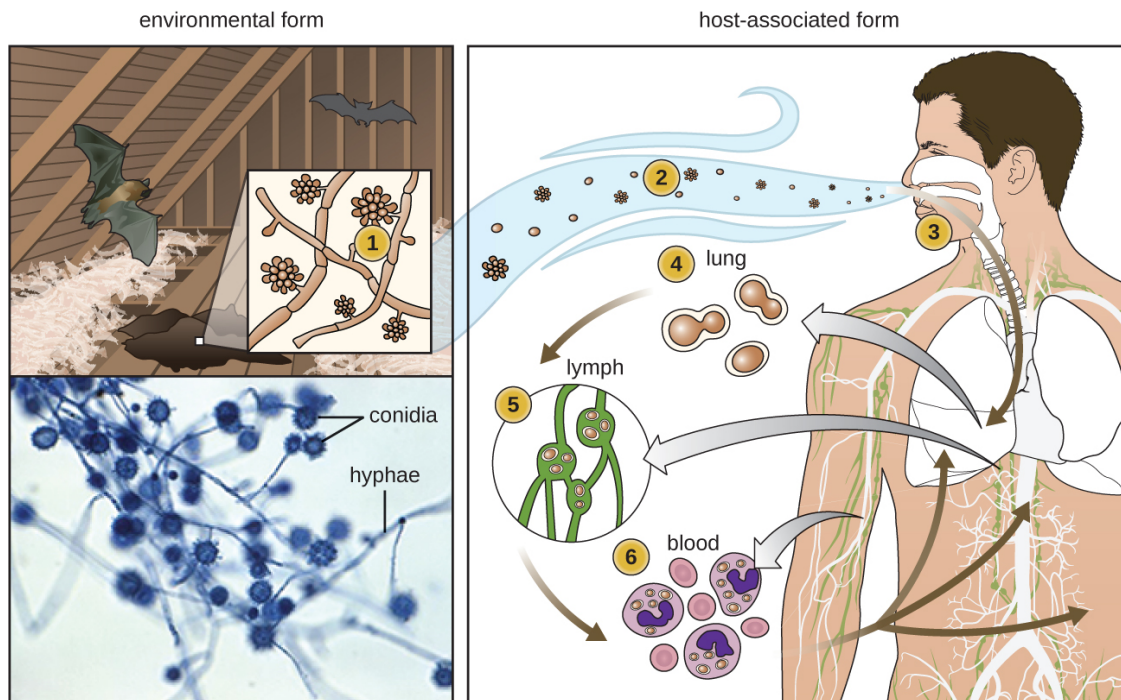


Figure 9: *Histoplasma capsulatum* is a dimorphic fungus that grows in soil exposed to bird feces or bat feces (guano) (top left). It can change forms to survive at different temperatures. In the outdoors, it typically grows as a mycelium (as shown in the micrograph, bottom left), but when the spores are inhaled (right), it responds to the high internal temperature of the body (37 °C [98.6 °F]) by turning into a yeast that can multiply in the lungs, causing the chronic lung disease histoplasmosis. (credit: modification of work by Centers for Disease Control and Prevention)

There are notable unique features in fungal cell walls and membranes. Fungal cell walls contain **chitin**, as opposed to the cellulose found in the cell walls of plants and many protists. Additionally, whereas animals have cholesterol in their cell membranes, fungal cell membranes have different sterols called ergosterols. Ergosterols are often exploited as targets for antifungal drugs.

Fungal life cycles are unique and complex. Fungi reproduce sexually either through cross- or self-fertilization. Haploid fungi form hyphae that have gametes at the tips. Two different mating types (represented as “+ type” and “– type”) are involved. The cytoplasm of the + and – type gametes fuse (in an event called plasmogamy), producing a cell with two distinct nuclei (a **dikaryotic** cell). Later, the nuclei fuse (in an event called karyogamy) to create a diploid zygote. The zygote undergoes meiosis to form **spores** that germinate to start the haploid stage, which eventually creates more haploid mycelia. Depending on the taxonomic group, these sexually produced spores are known as zygospores (in Zygomycota), ascospores (in Ascomycota), or basidiospores (in Basidiomycota).

Fungi may also exhibit asexual reproduction by mitosis, mitosis with budding, fragmentation of hyphae, and formation of asexual spores by mitosis. These spores are specialized cells that, depending on the organism, may have unique characteristics for survival, reproduction, and dispersal. Fungi exhibit several types of asexual spores and these can be important in classification.

Helminths

Parasitic helminths are animals that are often included within the study of microbiology because many species of these worms are identified by their microscopic eggs and larvae. There are two major groups of parasitic helminths: the roundworms (Nematoda) and flatworms (Platyhelminthes). Of the many species that exist in these groups, about half are parasitic and some are important human pathogens. As animals, they are multicellular and have organ systems. However, the parasitic species often have limited digestive tracts, nervous systems, and locomotor abilities. Parasitic forms may have complex reproductive cycles with several different life stages and more than one type of host. Some are **monoecious**, having both male and female reproductive organs in a single individual, while others are **dioecious**, each having either male or female reproductive organs.

Nematoda (Roundworms)

Phylum **Nematoda** (the roundworms) is a diverse group containing more than 15,000 species, of which several are important human parasites. These unsegmented worms have a full digestive system even when parasitic. Some are common intestinal parasites, and their eggs can sometimes be identified in feces or around the anus of infected individuals. *Ascaris lumbricoides* is the largest nematode intestinal parasite found in humans; females may reach lengths greater than 1 meter. *A. lumbricoides* is also very widespread, even in developed nations, although it is now a relatively uncommon problem in the United States. It may cause symptoms ranging from relatively mild (such as a cough and mild abdominal pain) to severe (such as intestinal blockage and impaired growth).

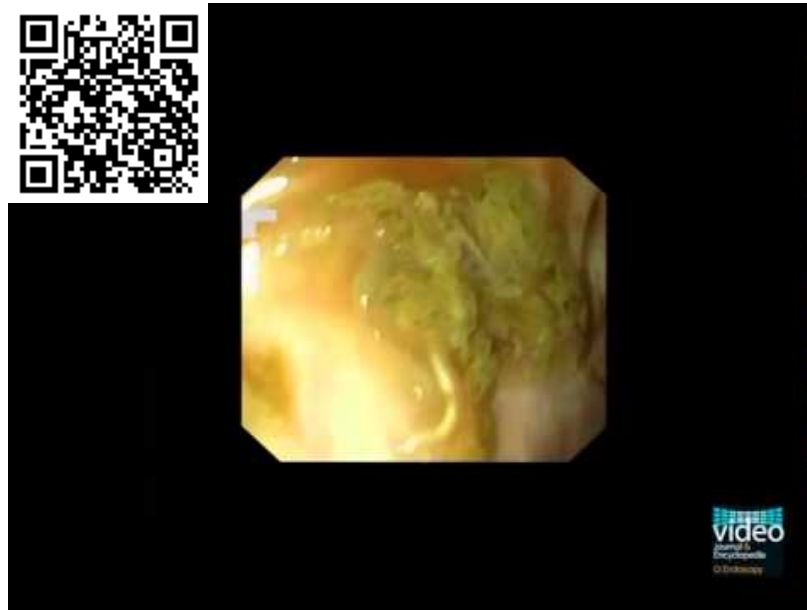


Figure 10: *Enterobius vermicularis* is a type of parasitic roundworm.

Of all nematode infections in the United States, pinworm (caused by *Enterobius vermicularis*) is the most common. Pinworm causes sleeplessness and itching around the anus, where the female worms lay their eggs during the night. *Toxocara canis* and *T. cati* are nematodes found in dogs and cats, respectively, that can be transmitted to humans, causing toxocariasis. Antibodies to these parasites have been found in approximately 13.9% of the U.S. population, suggesting that exposure is common.⁷ Infection can cause larval migrans, which can result in vision loss and eye inflammation, or fever, fatigue, coughing, and abdominal pain, depending on whether the organism infects the eye or the viscera. Another common nematode infection is hookworm, which is caused by *Necator americanus* (the New World or North American hookworm) and *Ancylostoma duodenale* (the Old World hookworm). Symptoms of hookworm infection can include abdominal pain, diarrhea, loss of appetite, weight loss, fatigue, and anemia.

Trichinellosis, also called trichinosis, caused by *Trichinella spiralis*, is contracted by consuming undercooked meat, which releases the larvae and allows them to encyst in muscles. Infection can cause fever, muscle pains, and digestive system problems; severe infections can lead to lack of coordination, breathing and heart problems, and even death. Finally, heartworm in dogs and other animals is caused by the nematode *Dirofilaria immitis*, which is transmitted by mosquitoes. Symptoms include fatigue and cough; when left untreated, death may result.

Platyhelminths (Flatworms)

Phylum **Platyhelminthes** (the platyhelminths) are flatworms. This group includes the flukes, tapeworms, and the turbellarians, which include planarians. The flukes and tapeworms are medically important parasites.

The **flukes** (trematodes) are nonsegmented flatworms that have an oral sucker (and sometimes a second ventral sucker) and attach to the inner walls of intestines, lungs, large blood vessels, or the liver. Trematodes have complex life cycles, often with multiple hosts. Several important examples are the liver flukes (*Clonorchis* and *Opisthorchis*), the intestinal fluke (*Fasciolopsis buski*), and the oriental lung fluke (*Paragonimus westermani*). Schistosomiasis is a serious parasitic disease, considered second in the scale of

its impact on human populations only to malaria. The parasites *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*, which are found in freshwater snails, are responsible for schistosomiasis. Immature forms burrow through the skin into the blood. They migrate to the lungs, then to the liver and, later, other organs. Symptoms include anemia, malnutrition, fever, abdominal pain, fluid buildup, and sometimes death.

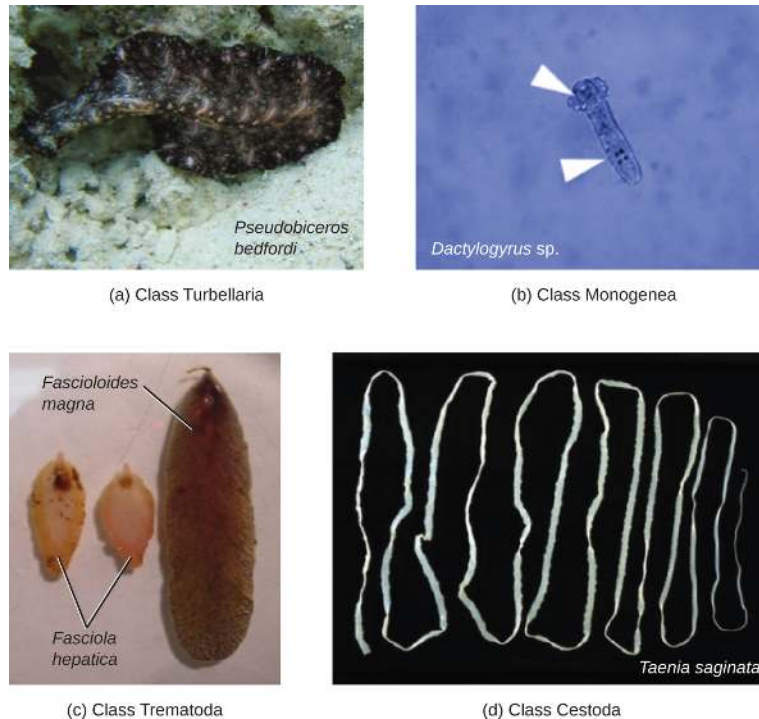


Figure 11: Phylum Platyhelminthes is divided into four classes. (a) Class Turbellaria includes the Bedford's flatworm (*Pseudobiceros bedfordi*), which is about 8–10 cm long. (b) The parasitic class Monogenea includes *Dactylogyrus* spp. Worms in this genus are commonly called gill flukes. The specimen pictured here is about 0.2 mm long and has two anchors, indicated by arrows, that it uses to latch onto the gills of host fish. (c) The Trematoda class includes the common liver fluke *Fasciola hepatica* and the giant liver fluke *Fascioloides magna* (right). The *F. magna* specimen shown here is about 7 cm long. (d) Class Cestoda includes tapeworms such as *Taenia saginata*, which infects both cattle and humans and can reach lengths of 4–10 meters; the specimen shown here is about 4 meters long. (credit c: modification of work by "Flukeman"/Wikimedia Commons)

The other medically important group of platyhelminths are commonly known as **tapeworms** (cestodes) and are segmented flatworms that may have suckers or hooks at the **scolex** (head region). Tapeworms use these suckers or hooks to attach to the wall of the small intestine. The body of the worm is made up of segments called **proglottids** that contain reproductive structures; these detach when the gametes are fertilized, releasing gravid proglottids with eggs. Tapeworms often have an intermediate host that consumes the eggs, which then hatch into a larval form called an oncosphere. The oncosphere migrates to a particular tissue or organ in the intermediate host, where it forms cysticerci. After being eaten by the definitive host, the cysticerci develop into adult tapeworms in the host's digestive system. *Taenia saginata* (the beef tapeworm) and *T. solium* (the pork tapeworm) enter humans through ingestion of undercooked, contaminated meat. The adult worms develop and reside in the intestine, but the larval stage may migrate and be found in other body locations such as skeletal and smooth muscle. The beef tapeworm is relatively benign, although it can cause digestive problems and, occasionally, allergic reactions. The pork tapeworm can cause more serious problems when the larvae leave the intestine and colonize other tissues, including those of the central nervous system. *Diphyllobothrium latum* is the largest human tapeworm and can be ingested in undercooked fish. It can grow to a length of 15 meters. *Echinococcus granulosus*, the dog tapeworm, can parasitize humans and uses dogs as an important host.

CARMEN QUESTIONS

1. Examine each of the microbial eukaryotic cells with the microscope. For each specimen, record the specimen name and identify any cell features that you can observe (e.g., nucleus, plasmids, flagella, etc.).

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1.6: Fungal Parasites

Learning Objectives

- Define and differentiate between yeast and mold.
- Define, identify, and apply the following terms: budding, hyphae, mycelium, septate, coenocytic, sporangium, sporangiophore, sporangiospore, conidia, conidiophore, vesicle
- Describe how *Aspergillus* can cause infection in humans, the risk factors for aspergillosis, and the symptoms of infection.
- Describe how *Coccidioides* can cause infection in humans, the risk factors for infection, and the symptoms of infection.
- Give both the medical and common names for *Coccidioides* infections.
- Tell that antibiotics cannot be used to treat fungal infections and that they must be treated with antifungal medications.

Introduction to Fungi

Mycology is the study of fungi. Fungi are eukaryotic organisms which grow as either yeast or mold. However, there are some fungi that are dimorphic, meaning they can grow as yeast under certain environmental conditions (such as the warm moist lungs in the body) and mold under other conditions (such as in soil in the environment). For example, *Coccidioides immitis*, the fungus responsible for San Joaquin Valley Fever, is an example of a dimorphic fungus.

Fungi grow slower than bacteria and at a lower temperature and lower pH than most bacteria prefer. Sabouraud's agar is selective media for fungi because it incorporates simple nutrients (glucose and peptone) at a pH of 4.5-5.6 which inhibits bacterial growth. Although some yeasts can grow at 36°C, we incubate all fungal cultures at 25 to 30°C for at least one week. Some fungi take three weeks or more to grow.

Yeast

The colonial appearance of most yeast is moist, creamy and white in color and similar in appearance to *Staphylococcus* colonies. Microscopically, yeast cells are unicellular and round to oval, whereas bacteria cells vary in shape (cocci, rods, spirals). Yeast cells are usually five to ten times larger than bacteria and can be visualized at 400X total magnification. Yeast reproduce asexually by **budding** and the newly produced cell, called a bud or **blastospore**, protrudes from the periphery of the parent cell. The blastospore may break off from the parent cell or stay attached. Successive blastospores remaining attached to the original cell result in the formation of **pseudohyphae**.



Figure 1: (Left) Yeast growing on a petri plate. (Right) Microscopic image of yeast cells.

Most yeast have similar macroscopic and microscopic appearances. Biochemical tests, such as carbohydrate assimilation tests, must be performed to identify yeast species. Examples of yeast include *Candida albicans* which is an opportunistic pathogen (thrush, diaper rash, vaginal yeast infections) and *Saccharomyces cerevisiae* which is used to make bread, beer, and wine.

Mold

Fungi that grow as mold produce multicellular filaments called **hyphae** (plural) or **hypha** (singular). Most species of mold hyphal filaments are separated by a cross wall (**septum**) and are called **septate hyphae**. In mold species where the hyphal filaments not separated by cross walls are called **aseptate** or **coenocytic hyphae**. Hyphal filaments intertwined into a mass, known as **mycelia** (plural) or **mycelium** (singular), can be seen macroscopically as fuzzy or hairy, colorful colonies. Some of the hyphae, called **vegetative hyphae**, grow on or down into the agar surface to extract nutrients from the medium. Other hyphae, called **aerial hyphae**, grow above the agar surface and produce asexual reproductive **spores**.



Figure 2: (Left top) Illustration of the structure of septate hyphae. These hyphae appear as long strands that are only one cell wide where cells occur end-to-end with septa that separate the cells. (Left bottom) Illustration of the structure of coenocytic hyphae or nonseptate hyphae. These hyphae appear the same as septate hyphae, except they lack septa, and therefore they are long strands without separations having multiple nuclei. (Middle) Microscopic image of hyphae. (Right) Image of a petri plate with the mold *Penicillium* growing on it. This species is responsible for producing penicillin, the first antibiotic discovered. Mold does not grow on petri plates as single colonies, but instead as a mass of growth that has a fuzzy appearance.

The two types of asexual spores produced by molds are called **sporangiospores** and **conidiospores**. Sporangiospores are produced at the end of aerial hyphae called **sporangiophores** in a saclike structure called a **sporangium**. The sporangia of specific molds have characteristic shapes which can be used to identify the mold species. An example of a mold that produces sporangiospores is the bread mold, *Rhizopus*. Conidiospores are formed on aerial hyphae called **conidiophores**. **Conidia** may be one-celled (**microconidia**) or multicelled (**macroconidia**). Examples of fungi that produce conidiospores are *Penicillium* and *Aspergillus*.

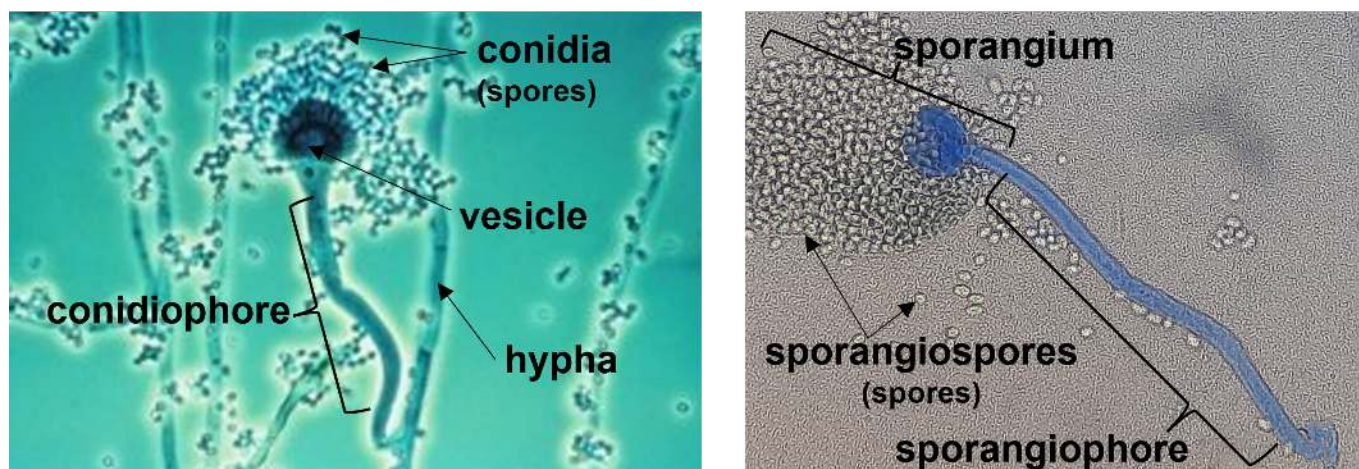


Figure 3: (Left) *Aspergillus* (a species of mold) with its asexual conidiophores leading to a vesicle supporting other cells that bear conidia (spores). (Right) *Mucor* (a species of mold) with its asexual sporangiophore leading to the sac of sporangiospores called the sporangium.

Molds are usually identified in the laboratory by their characteristic macroscopic appearance, hyphal structure (septate or nonseptate) and type of asexual sporulation. Since spore formation is an important identification criterion, slide cultures are performed to observe sporulation without disturbing the hyphal structures.

Aspergillus

Introduction to *Aspergillus* (Cause of Aspergillosis)

Aspergillosis is an infection caused by *Aspergillus*, a common mold (a type of fungus) that lives indoors and outdoors. Most people breathe in *Aspergillus* spores every day without getting sick. However, people with weakened immune systems or lung diseases are at a higher risk of developing health problems due to *Aspergillus*. The types of health problems caused by *Aspergillus* include allergic reactions, lung infections, and infections in other organs.

There are approximately 180 species of *Aspergillus*, but fewer than 40 of them are known to cause infections in humans. *Aspergillus fumigatus* is the most common cause of human *Aspergillus* infections. Other common species include *A. flavus*, *A. terreus*, and *A. niger*.

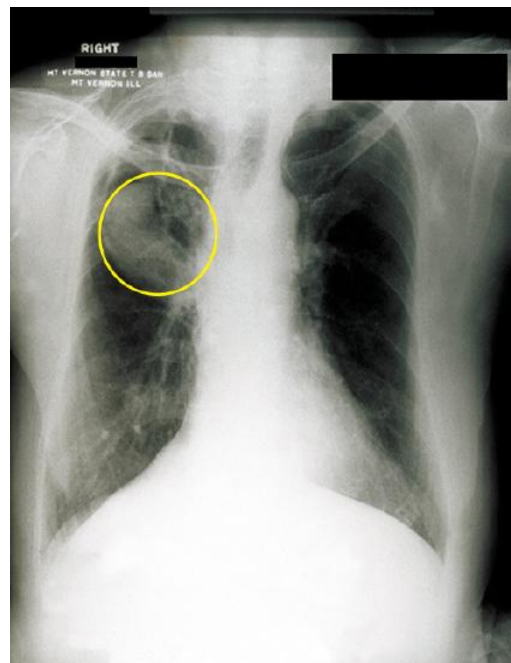


Figure 4: A fungal ball can be observed in the upper lobe of the right lung in this chest radiograph of a patient with aspergilloma. (credit: modification of work by Centers for Disease Control and Prevention)

Healthcare providers consider your medical history, risk factors, symptoms, physical examinations, and lab tests when diagnosing aspergillosis. Imaging tests such as a chest x-ray or a CT scan a patient's lungs or other parts of the body depending on the location of the suspected infection. If a healthcare provider suspects an *Aspergillus* infection in the lungs, they might collect a sample of fluid from your respiratory tract to send to a laboratory. Healthcare providers may also perform a tissue biopsy, in which a small sample of affected tissue is analyzed in a laboratory for evidence of *Aspergillus* under a microscope or in a fungal culture. A blood test can help diagnose invasive aspergillosis early in people who have severely weakened immune systems.

Types of Aspergillosis

- **Allergic bronchopulmonary aspergillosis (ABPA):** Occurs when *Aspergillus* causes inflammation in the lungs and allergy symptoms such as coughing and wheezing, but doesn't cause an infection.²
- **Allergic *Aspergillus* sinusitis:** Occurs when *Aspergillus* causes inflammation in the sinuses and symptoms of a sinus infection (drainage, stuffiness, headache) but doesn't cause an infection.³
- **Azole-Resistant *Aspergillus fumigatus*:** Occurs when one species of *Aspergillus*, *A. fumigatus*, becomes resistant to certain medicines used to treat it. Patients with resistant infections might not get better with treatment.

- **Aspergilloma:** Occurs when a ball of *Aspergillus* grows in the lungs or sinuses, but usually does not spread to other parts of the body.⁴ Aspergilloma is also called a “fungus ball.”
- **Chronic pulmonary aspergillosis:** Occurs when *Aspergillus* infection causes cavities in the lungs, and can be a long-term (3 months or more) condition. One or more fungal balls (aspergillomas) may also be present in the lungs.⁵
- **Invasive aspergillosis:** Occurs when *Aspergillus* causes a serious infection, and usually affects people who have weakened immune systems, such as people who have had an organ transplant or a stem cell transplant. Invasive aspergillosis most commonly affects the lungs, but it can also spread to other parts of the body.
- **Cutaneous (skin) aspergillosis:** Occurs when *Aspergillus* enters the body through a break in the skin (for example, after surgery or a burn wound) and causes infection, usually in people who have weakened immune systems. Cutaneous aspergillosis can also occur if invasive aspergillosis spreads to the skin from somewhere else in the body, such as the lungs.⁶

Clinical Presentation: *Aspergillus*

The different types of aspergillosis can cause different symptoms.¹

The symptoms of **allergic bronchopulmonary aspergillosis (ABPA)** are similar to asthma symptoms, including:

- Wheezing
- Shortness of breath
- Cough
- Fever (in rare cases)

Symptoms of **allergic *Aspergillus* sinusitis**² include:

- Stuffiness
- Runny nose
- Headache
- Reduced ability to smell

Symptoms of an **aspergilloma** (“fungus ball”)³ include:

- Cough
- Coughing up blood
- Shortness of breath

Symptoms of **chronic pulmonary aspergillosis**^{4,5} include:

- Weight loss
- Cough
- Coughing up blood
- Fatigue
- Shortness of breath

Invasive aspergillosis¹ usually occurs in people who are already sick from other medical conditions, so it can be difficult to know which symptoms are related to an *Aspergillus* infection. However, the symptoms of invasive aspergillosis in the lungs include:

- Fever
- Chest pain
- Cough
- Coughing up blood
- Shortness of breath
- Other symptoms can develop if the infection spreads from the lungs to other parts of the body.

Laboratory Instructions: *Aspergillus*

1. Examine *Aspergillus* using a microscope.
2. Identify the following features:
 - "hyphae" or "hypha"

- "conidiophore"
- "vesicle"
- "conidia (spores)" or "conidiospores"

CARMEN QUESTIONS: *Aspergillus*

1. What areas of the human body can become infected with *Aspergillus*?
2. Where does *Aspergillus* live and how are humans exposed to *Aspergillus*?
3. Who populations are most at risk of developing aspergillosis?
4. What is the difference between a mold and yeast?
5. Is *Aspergillus* a mold or a yeast?
6. Name the *Aspergillus* structures that branch from the hyphae to produce asexual spores?
7. What are the *Aspergillus* asexual spores called?
8. Can aspergillosis be treated with antibiotics? Explain your answer.

Coccidioides (Causes Coccidioidomycosis aka Valley Fever)

Introduction to *Coccidioides* (Cause of Coccidioidomycosis aka Valley Fever)

Valley fever is an infection caused by a fungus that lives in the soil. About 20,000 cases are reported in the United States each year, mostly from Arizona and California, and the number of cases is increasing. Valley fever can be misdiagnosed because its symptoms are similar to those of other respiratory illnesses. Here are some important things to know about Valley fever, also called coccidioidomycosis.

The fungus that causes Valley fever, *Coccidioides*, is found in soil in the southwestern United States, parts of Mexico and Central America, and parts of South America. It has also been found in south-central Washington State. The fungus might also live in similar areas with hot, dry climates. People can get Valley fever by breathing in the microscopic fungus from the air in these areas. Valley fever does not spread from person to person.

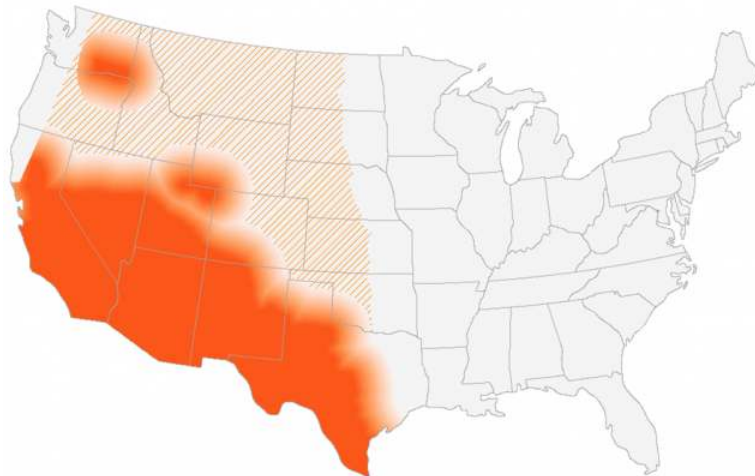


Figure 5: This map shows CDC's current estimate of where the fungus that causes Valley fever lives in the environment in the United States. The fungus is not distributed evenly in the shaded areas, might not be present everywhere in the shaded areas, and can also be outside the shaded areas. Darker shading shows areas where the fungus is more likely to live. Diagonal lines show the potential range of the fungus.

In areas where Valley fever is common, it's difficult to completely avoid exposure to the fungus because it is in the environment. There is no vaccine to prevent infection. That's why knowing about Valley fever is one of the most important ways to avoid delays in diagnosis and treatment. People who have symptoms of Valley fever and live in, work in, or have visited an area where the fungus is common should ask their doctor to test them for Valley fever. Healthcare providers should be aware that Valley fever symptoms are similar to those of other respiratory illnesses and should consider testing for Valley fever in patients with pneumonia symptoms who live in or have traveled to an area where *Coccidioides* lives.



Figure 6: The Valley fever fungus (*Coccidioides*) grows in the dirt and soil, but is too small to see. Fungus spores get in the air when dirt and dust are stirred up by the wind or from digging. People and animals breath in the fungus spores from dust in the air. The fungus usually infects the lungs, but it can be spread to other organs.



The Financial Cost of Valley Fever

Valley fever is a serious, costly illness:

- **Nearly 75%** of people with Valley fever miss work or school
- **As many as 40%** of people who get Valley fever are hospitalized
- The average cost of a hospital stay for a person with Valley fever is **almost \$50,000**
- **About 60–80%** of patients with Valley fever are given one or more rounds of antibiotics before receiving a correct diagnosis and appropriate treatment (antifungal medication is required for treatment)

Clinical Presentation of *Coccidioides* (Cause of Coccidioidomycosis aka Valley Fever)

Many people who are exposed to the fungus never have symptoms. Other people may have symptoms that include:

- Fatigue (tiredness)
- Cough
- Fever

- Shortness of breath
- Headache
- Night sweats
- Muscle aches or joint pain
- Rash on upper body or legs

The symptoms of valley fever can be similar to those of other respiratory illnesses, which may cause delays in diagnosis and treatment. For many people, symptoms go away within weeks or months without any treatment. But healthcare providers may prescribe antifungal medicine for some people to reduce symptoms or prevent the infection from getting worse. People who have severe lung infections or infections that have spread to other parts of the body always need antifungal treatment and may need to stay in the hospital.

Anyone can get Valley fever if they live in, work in, or travel to an area where the fungus lives in the environment. Valley fever can affect people of any age, but it's most common in adults aged 60 and older. Also, certain groups of people may be at higher risk for developing the severe forms of valley fever, such as:

- People who have weakened immune systems, which may include people who:
 - Have HIV
 - Have had an organ transplant
 - Are taking medications such as corticosteroids or tumor necrosis factor (TNF) inhibitors
- Pregnant people
- People who have diabetes
- People who are Black or Filipino

Laboratory Instructions: *Coccidioides* (Cause of Coccidioidomycosis aka Valley Fever)



FIGURE 11.—Sputum culture of *C. immitis* on Sabouraud's medium, showing white, cottony fungus growth.

1. Carefully examine the microscopic image of *Coccidioides* in the image above.

CARMEN QUESTIONS: *Coccidioides* (Cause of Coccidioidomycosis aka Valley Fever)



FIGURE 11.—Sputum culture of *C. immitis* on Sabouraud's medium, showing white, cottony fungus growth.

1. Based in careful examination of the microscopic image of *Coccidioides*, is this fungus growing as yeast or mold?
2. Label the image above to show the location of "hyphae."
3. Are the hyphae of *Coccidioides* septate or coenocytic? How can you tell?
4. How do people become exposed to *Coccidioides*?
5. Give the common name and the medical term for infection with *Coccidioides*.
6. What populations are at greater risk of infection by *Coccidioides*?
7. What are common symptoms of infection by *Coccidioides*?
8. Where geographically can patients become develop coccidioidomycosis?
9. What percentage of people with coccidioidomycosis become hospitalized?
10. Do patients with a *Coccidioides* infection struggle to get a correct diagnosis?
11. When should a healthcare professional consider coccidioidomycosis as a possible diagnosis?
12. Can *Coccidioides* infection be treated with antibiotics? Explain your answer.

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CHAPTER OVERVIEW

2: Lab 2

- 2.1: Required Materials
- 2.2: Aseptic Technique
- 2.3: Plating on Petri Plates for Isolation
- 2.4: Simple Stain
- 2.5: Gram Stain
- 2.6: Endospore Stain
- 2.7: Capsule Stain
- 2.8: Acid-Fast Stain
- 2.9: Introduction to Micropipetting

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2.1: Required Materials

Cultures needed

- TSB tubes of
 - *E. coli*
 - *Serratia marcescens*
 - *E. coli* and *S. marcescens* mix
 - *Staphylococcus epidermidis*
 - *Bacillus subtilis*
 - *Enterobacter aerogenes*
 - *B. subtilis* (endo) – Grow at 37 C for 72 h

Aseptic Technique

- TSB tubes
- TSA Slant tubes
- TSA Deep tubes
- TSA Plates

Plating on Petri Plates for Isolation

- TSA Plates
- Simple Stain
- Slides
- Bibulous paper
- Methylene blue

Gram Stain

- Slides
- Bibulous paper
- Methylene blue
- Crystal violet
- Iodine
- Alcohol
- Safranin

Endospore Stain

Malachite green

- Methylene green
- Beakers
- Wire rack for beakers
- Hot plate
- Paper towel
- Safranin

Capsule Stain

Acid-fast Stain

Introduction to Micropipetting

- Micropipettes
- Micropipette tips

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2.2: Aseptic Technique

Learning Objectives

- Differentiate between the types of microbiological media.
- Define aseptic, aseptic technique, pure culture, contamination, sterilization, autoclave, disinfectant, and antiseptic.
- Successfully use aseptic technique in microbiology transfers.
- Describe good aseptic technique in microbiology transfers.
- Recognize examples of good and bad aseptic technique and possible sources of contamination.

Microbiological Media

To study bacteria and other microorganisms, it is necessary to grow them in controlled conditions. Microbes are grown in substances that provide the nutrients necessary to sustain their metabolic activities and reproduction called "growth media" or simply "media" (singular is "medium"). Growth media can be either liquid or solid.

A liquid medium is called a **broth**. Broths can be used to determine growth patterns in a liquid medium, and for certain types of inoculations and metabolic tests. They are also the method of choice for growing large quantities of bacteria.

Solid growth media usually contains **agar**, which is a mixture of polysaccharides derived from red algae. It is used as a solidification agent because it (1) is not broken down by bacteria, (2) contains no nutrients that can be used by bacteria and (3) melts at high temperatures, and yet is solid at temperatures used for most bacterial growth. Solid growth media is used in the following forms: **agar plates**, **agar slants** and **agar deeps**. Making solid media is similar to making Jell-O, where a powder is mixed into water and heated to fully dissolve the powder. When the solution cools it solidifies. Melted agar is poured into a test tube and then allowed to solidify vertically for an agar deep, or at an angle for an agar slant. **Agar plates** are made by pouring melted agar into a petri dish. (Petersen, 2016)

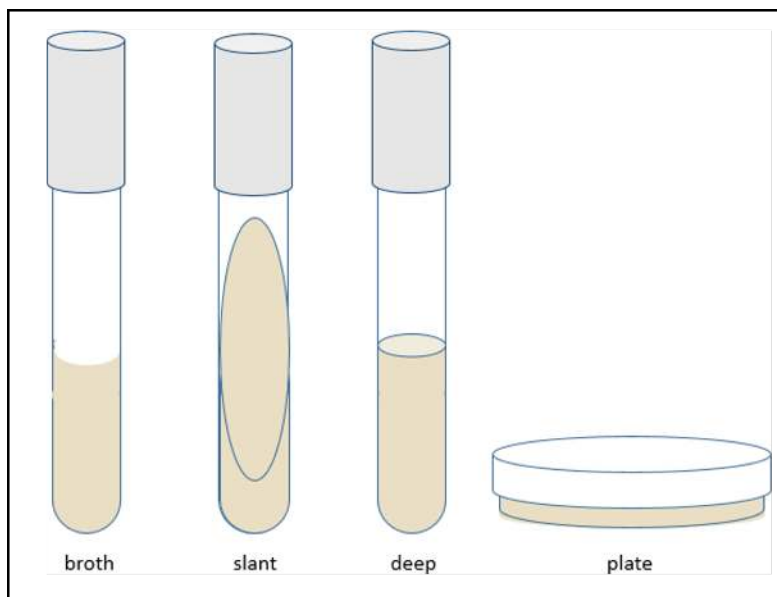


Figure 1: Illustration showing the different forms microbiological media can be prepared as.

Because of the relatively small tube opening (less opportunity to dry out or become contaminated) and the surface area available for growth, agar slants are commonly used to culture and store bacteria for intermediate periods of time (weeks). These types of cultures are called **stocks**. Deepes are often used to for certain differential metabolic tests.

In contrast to deepes and slants, agar plates have a large surface area for growth. Bacterial cells can be spread out over the surface so that they form discrete colonies which can be characterized. In a few weeks, you will be using a series of plate cultures to separate two different microbes from a mixture. In addition, specialized media in plate form is used for certain biochemical tests. (Petersen, 2016)

Media Contamination

Microbiologists generally study the organisms in **pure culture**, a culture that contains a single microbial species. If an unintended microorganism is introduced into a pure culture, the culture becomes **contaminated**. **Aseptic technique** is the collection of procedures and techniques designed to prevent the introduction of unwanted organisms into a pure culture or into the laboratory environment.

The term “aseptic” literally means “without contamination.” These procedures are as important for the experimenter’s safety as they are for maintaining culture purity.

Sterilization is the complete removal all vegetative cells, endospores, and viruses from an item (OpenStax CNX, 2018). Sterilization is all or none; something is either sterile or it is not sterile. In this course, all **media**, the substance in which the cells are grown, is sterilized by autoclave.

An **autoclave** uses moist heat (steam) under pressure to destroy all life forms. Whereas most vegetative cells can be killed at temperatures between 60 and 80°C, bacterial spores require temperatures above boiling (>100°C) for destruction. With a pressure of 15-20 lbs./in², the autoclave can achieve a temperature of 121-132°C. Media under these conditions for at least 20 minutes will kill all spores as well as vegetative cells. Larger volumes require longer exposure times to ensure sufficient heat transfer to the materials being sterilized. The steam must directly contact the liquids or dry materials being sterilized, so containers are left loosely closed and instruments are loosely wrapped in paper or foil. The key to autoclaving is achieving a temperature high enough to kill spores for complete sterilization (OpenStax CNX, 2018).

Disinfection is the killing or growth inhibition of vegetative microbes. Generally, spores and some hearty cells will survive disinfection. Chemical disinfectants, such as chlorine bleach or products containing chlorine, are used to clean nonliving surfaces such as laboratory benches, clinical surfaces, and bathroom sinks (OpenStax CNX, 2018). We will use a chlorine-based disinfectant to clean our work surfaces and to clean up any culture spills. Note that sterilization and disinfection are not interchangeable! (Why?) Spraying your bench top with disinfectant does not make it sterile.

Antiseptics are antimicrobial chemicals safe for use on living skin or tissues. Examples include hydrogen peroxide and isopropyl alcohol (OpenStax CNX, 2018).

When working in a microbiology laboratory, you must always remember that bacteria are present on all surfaces in the lab, as well as on your own hands and clothing. Aseptic techniques are designed to prevent the transfer of bacteria from the surrounding environment into a culture medium and from a culture to the environment. These techniques require care, concentration and practice. (Petersen, 2016)

Transfer Procedures

Because these procedures are completely new to most students, I strongly recommend that you watch the video at least twice. Keep in mind the following principles. (Some of these have been covered in the Laboratory Safety Exercise. They bear repeating because they are very important to keep you safe.)

Always begin by preparing your work area and making the necessary labels. Make sure you are clear about what transfers need to be made. The incinerator should be turned on HI for at least 20 minutes prior to using.

A transfer can be thought of in two parts, obtaining the cells (inoculum) from the source/parent culture and inoculating the new sterile tube or plate. Transfers, with very few exceptions, are performed by a single individual. You should not be holding the tube while your partner inoculates it.

Tutorial of Basic Aseptic Technique (slant to slant)



Before you Start

1. Culture media must initially be sterile. Inspect your media before you start. If a culture medium appears cloudy or you observe unwanted growth, consult with your TA or instructor to be sure it is not contaminated before using it.
2. Label your tubes white label tape. Masking tape is provided for other uses.
3. Label plates on the bottom.
4. Inspect the parent cultures. If the cells have fallen to the bottom, be sure to re-suspend them by flicking the tube gently to mix. Never shake a tube.
5. Disinfect the lab bench.
6. Light the Bunsen burner. The flame should be blue and a moderate height (not too tall, not too short).



Sterilizing the Inoculating Loop or Needle

1. Hold the inoculating loop in your dominant hand like a pencil. To sterilize, place it in the Bunsen burner **for at least 10 seconds**. The entire wire must be heated red hot. Use the center blue region of the flame (not the top or bottom of the flame). Watch the clock for the time. Students tend to count too fast.
2. Do not let the loop sit in the incinerator more than 15 seconds.
3. Hold the instrument in the air allowing the wire to cool for about 15 seconds before making any transfers. Please do not wave it around to cool it.
4. The wire is now sterile. If at this time, you set it down on the bench top, which is not sterile, it must be incinerated again before going into any culture. If a sterile instrument is touched to anything not sterile including your hand, sleeve, the outside of a tube or plate, a slide or the bench top, it becomes contaminated and cannot be used in an aseptic transfer.

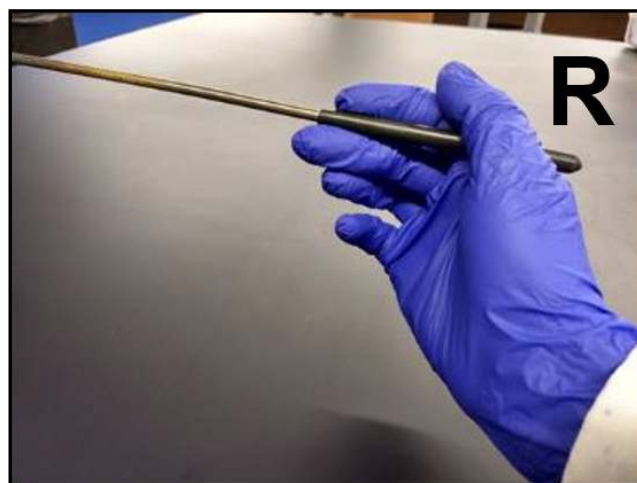
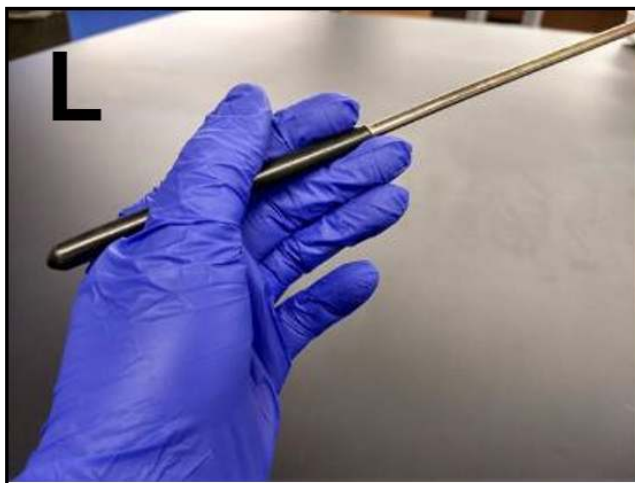


Figure 2: How to hold an inoculation loop or needle for left-handed people (L) and for right-handed people.

Obtaining the Inoculum from a Tube Culture

1. With your non-dominate hand, pick up the parent tube by grasping the tube just below the cap and lifting it out of the rack.
2. Grasp the cap with the pinky and ring finger of your dominate hand and gently twist the tube out of the cap keeping your dominate hand still. See Figures 3. The cap is kept in your hand and never placed on the bench top.
3. Heat the mouth of the open tube by passing it through the flame of the Bunsen burner. Heating creates convection currents, which carry airborne particles away from the mouth of the tube, preventing contamination of the culture or medium within.
4. For a broth parent culture: Place the cooled loop into the broth and remove making sure that you have a thin film of liquid filling the loop. Jiggling the loop in the broth is not needed and can result in the formation of tiny aerosol droplets. Please do not jiggle the wire.
5. For a slant parent culture: Touch the cooled loop to the growth. Do not break the agar surface. Refrain from “swiping” a large mass of cells. You do not need to see cells on the loop to have millions!
6. Again, heat the mouth of the tube after withdrawing the transfer instrument. This step incinerates any microbes that may have been deposited on the lip of the tube during the transfer.
7. Replace the cap and set the parent tube back in the test tube rack.
8. Keep the inoculating instrument in your hand.



Figure 3: Grasping, removing, and hold a test tube cap while holding an inoculation loop or needle. The cap should never be placed on the bench top and the open end of the cap should not touch anything to avoid contamination.

To obtain the inoculum from a plate culture

1. Turn the culture plate with bacteria growing on it right side up.

2. Lift the lid a short distance, with your non-dominate hand, so that the lid acts at a shield protecting the agar surface from falling microbes in the air. See Figure 4.
3. Touch the cooled loop to the growth. Do not breath the agar surface. Refrain from “swiping” a large mass of cells. You do not need to see cells on the loop to have millions!
4. Replace the lid immediately after withdrawing the transfer instrument and turn the plate upside-down again.
5. Keep the inoculating instrument in your hand.



Figure 4: Whenever transferring bacteria to or from a petri plate, the lid should only be lifted just enough to allow the loop to reach the agar. The lid of the petri plate should never be set down on the bench and should never touch anything to avoid contamination.

Inoculating a slant

1. With your non-dominate hand, pick up the parent tube by grasping the tube just below the cap and lifting it out of the rack.
2. Grasp the cap with the pinky and ring finger of your dominate hand and gently twist the tube out of the cap. Keeping your dominate hand still is especially important because there are cells on the loop at this point. Keep the cap in your hand.
3. Pass the mouth of the tube through the flame.
4. Insert the loop all the way to the bottom of the slant surface.
5. Drag the loop on the agar “snaking” your way up the slant creating a “fishtail pattern.” This is called a **fishtail inoculation**. See Figure 5.
6. Again, heat the mouth of the tube after withdrawing the transfer instrument. Replace the cap and set the parent tube back in the test tube rack.
7. Immediately flame the inoculating loop and wire for a full 10 seconds before setting it down.

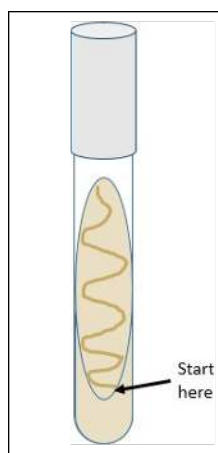


Figure 5: Inoculating a slant. Begin with the loop at the bottom of the slant you are transferring bacteria to and snake the loop up the surface of the slant.

Inoculating a broth

1. With your non-dominant hand, pick up the parent tube by grasping the tube just below the cap and lifting it out of the rack.
2. Grasp the cap with the pinky and ring finger of your dominant hand and gently twist the tube out of the cap. Keeping your dominant hand still is especially important because there are cells on the loop at this point.
3. Pass the mouth of the tube through the flame.
4. Insert the loop to the bottom of the broth liquid and then remove the loop. Jiggling is not necessary to dislodge cells.
5. Again, heat the mouth of the tube after withdrawing the transfer instrument. Replace the cap and set the parent tube back in the test tube rack.
6. Immediately flame the inoculating loop and wire for a full 10 seconds before setting it down.

After all inoculations

1. Cultures to be incubated should be placed in the designated area for culture incubation. Otherwise, a student's culture may be disposed of accidentally.
2. Be sure to turn it off the Bunsen burner when you are finished with it.

Because there is so much to remember, the first time you make transfers many of the above actions are repeated in context. After a few weeks practice, the repetition will no longer be necessary and it will be assumed that you will adhere to the procedures above without reminder.

Practice Aseptic Technique

1. Aseptically transfer a loop of sterile TSB to another test tube of sterile TSB:
 1. Flame loop.
 2. Remove cap from one test tube of sterile TSB and hold it in your hand (don't put it down and don't touch the open end).
 3. Pass the mouth of the test tube through the flame.
 4. Insert the flamed loop into the sterile TSB.
 5. There should be a film of liquid across the loop (similar to how a bubble wand will have a film across it).
 6. Pull the loop out of the test tube.
 7. Pass the mouth of the test tube through the flame.
 8. Put the cap back onto the test tube.
 9. Remove cap from the other test tube of sterile TSB and hold it in your hand (don't put it down and don't touch the open end).
 10. Pass the mouth of the test tube through the flame.
 11. Insert the loop with the film of sterile TSB.
 12. Pass the mouth of the test tube through the flame.
 13. Put the cap back onto the test tube.
 14. Flame the loop.
 15. Let each person in your group repeat with the same two test tubes.
 16. If every member of the group successfully transferred the TSB aseptically, there will be no growth (no turbidity) next class.
2. Put labels on both culture tubes with your name and "aseptic."
3. Check the culture tubes next class for turbidity to determine whether or not your aseptic transfer was successful. A successful transfer would result in both tubes being clear (no growth).

Aseptic Technique Results

1. Observe your results for each inoculated media. Did sterile media remain sterile? Did inoculated media exhibit signs of growth?
2. If you observed growth in the TSB culture tubes, what might have gone wrong? If you were successful in keeping both sterile, what are some possible sources of error that could cause contamination?

CARMEN QUESTIONS

3. Explain the importance of aseptic technique in microbiology.
4. What is the purpose of flaming in aseptic technique?
5. What are some signs of growth in a liquid medium?

Works Cited

- Petersen, J. a. (2016). Laboratory Excercises in Microbiology: Discovering the Unseen World Through Hands-On Investigation. CUNY Academic Works. Retrieved from http://academicworks.cuny.edu/qb_oers/16

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2.3: Plating on Petri Plates for Isolation

Learning Objectives

- Define "colony" and describe how colonies arise.
- Successfully identify isolated colonies.
- Characterize colony appearances and distinguish different microbial species based on colony appearance.
- Successfully execute a zig-zag streak plate and describe how and when this approach is used.
- Successfully execute a quadrant streak plate and describe how and when this approach is used.
- Describe and interpret how streak plate approaches result in the petri plate growth patterns.

Bacterial Colonies

If a single bacterial cell is placed on the surface of a TSA agar plate and allowed to multiply for 24 to 48 hours, it would grow into a mass of cells visible to the human eye called a **colony**. Colonies formed by the same microbial species growing on the same medium will all look alike. This is because the cell shape, pigmentation, division plane, rate of cell division and other characteristics of the organism result in the progeny cells stacking on one another in a pattern resulting in a characteristic colony form.

When identifying unknown microorganisms, to separate a mixture of microbial species, an **isolated colony** must be obtained. Since a single colony began with the growth of a single cell, all of the cells in that colony should be of a single species. Therefore, an isolated colony can be transferred to a sterile medium to isolate the species at the beginning of the identification process. Isolated colonies are found on their own away from other growth. A colony that is touching other colonies is not considered isolated (see Figure 1).

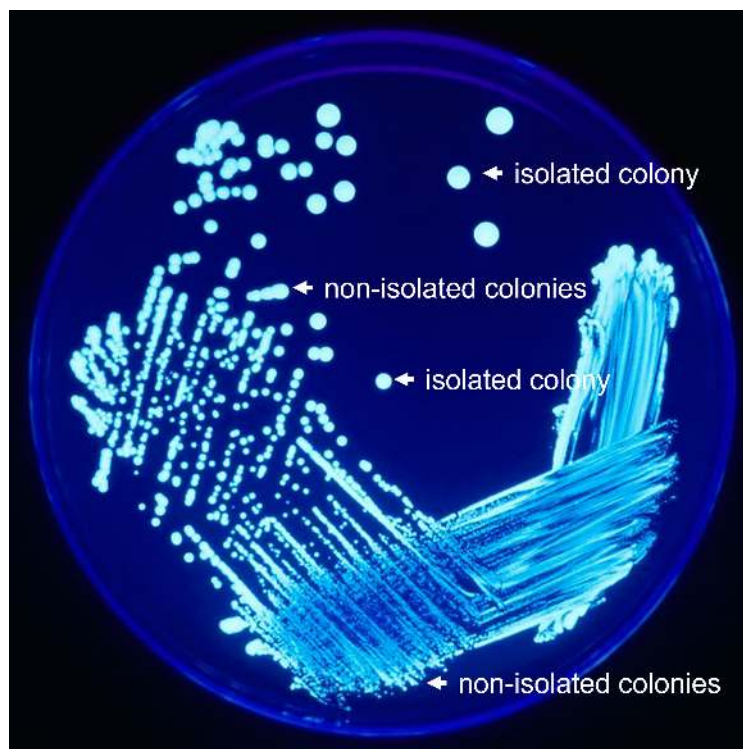


Figure 1: A petri plate with bacterial growth showing the distinction between isolated colonies and non-isolated colonies.

Characteristics of Colonies on a Petri Plates

Even on general purpose growth media, bacteria can exhibit characteristic patterns of growth. Some examples are shown below. While these growth patterns are an important piece of information when identifying a bacterial species, they are not sufficient for a positive identification. Staining procedures and metabolic tests must be used for a definitive identification.

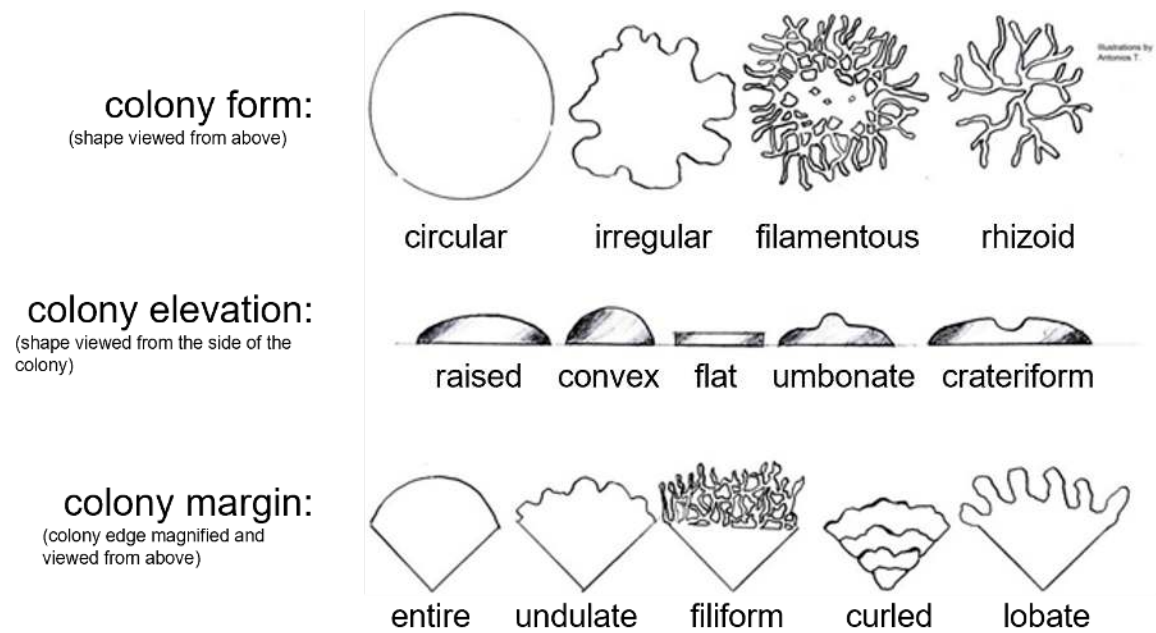


Figure 2: A guide for classifying colony characteristics. Colony form is the overall shape of the colony (circular, irregular, filamentous, or rhizoid) when viewed from above. Colony elevation examines the height and shape of the growth above the surface of the petri plate agar (raised, convex, flat, umbonate, or crateriform) and is best determined by viewing the colony from its side. Colony margin examines the shape of the edge of the colony when magnified (entire, undulate, filiform, curled, or lobate).

Zig Zag Streak Plate

If you swab a door handle, where bacteria are likely to be present, and then pass the swab across the surface of a TSA plate, cells would be deposited onto the surface from the swab. Initially, the swab may have a fairly high concentration of cells and the area touched by it will have lots of different cell types placed close together. After these grow up, the cells' progeny will crowd together and overlap with other cells' progeny forming areas called confluent growth.

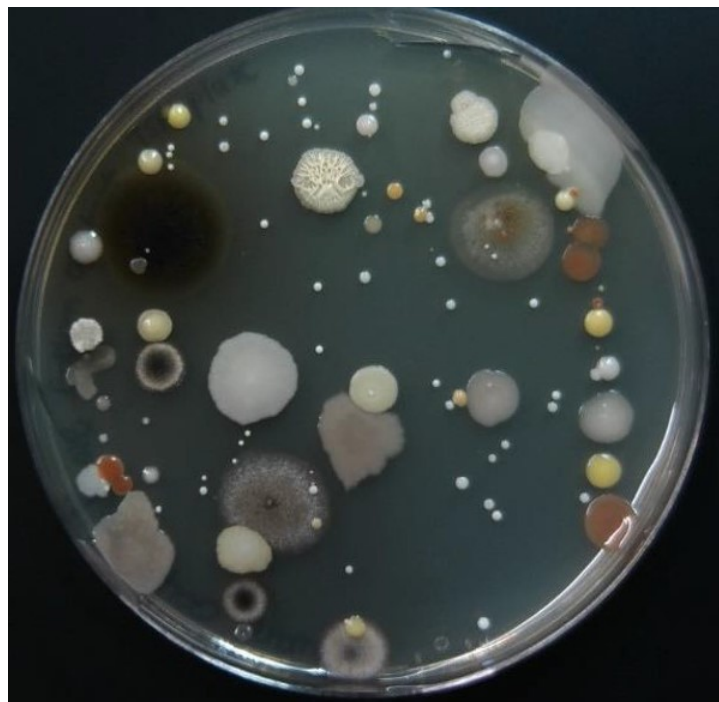


Figure 3: Zig zag streak plate of an environmental sample. The colonies have diverse colors, textures, and forms. This diversity is because different microbial species produce different colony characteristics. Environmental samples will contain mixtures of microbial species.

As the swab moves across the agar leaving cells on the agar in a zig zag pattern as shown in Figure 4, regions touched later in the process will have fewer and fewer cells. Individual cells are far enough apart that each one would grow into a discrete **isolated colony**. The result may look something like Figure 3. Because the door handle likely has a variety of microbes on it, there are numerous colony forms. This technique is called a **zig zag streak plate** and is one type of **isolation streak** method.

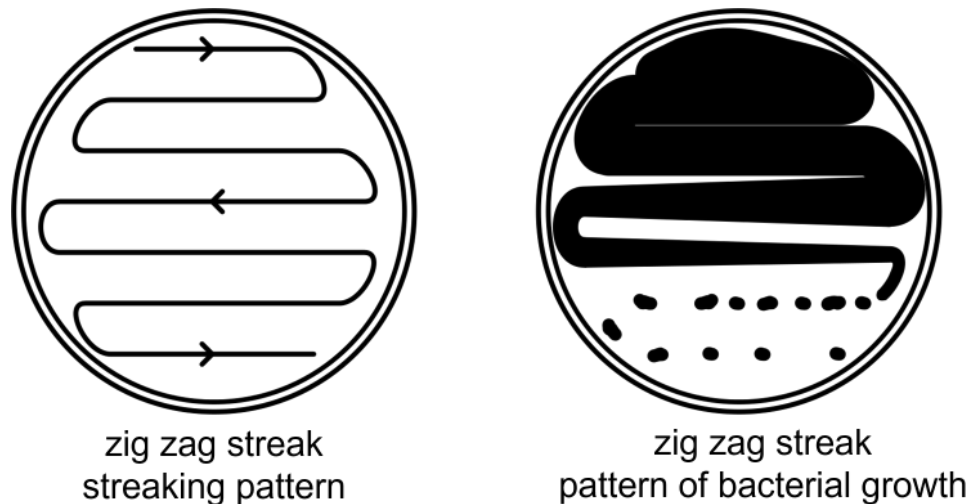


Figure 4: The zig zag streaking pattern uses streak directions moving left to right, then right to left by snaking across the petri plate. The entire petri plate surface is used. The result is bacterial growth that is dense (confluent) at the location of the first "zig" and becomes successively less dense as the streak snakes across the petri plate. Ideally, individual colonies are present at the end of the streaking pattern.

The steps of the zig zag streak plate are:

1. Aseptically obtain a sample using an inoculating loop or sterile cotton swab.
2. Turn the agar plate right-side-up.
3. Hold the plate lid so that it acts as a shield protecting the agar surface from microbes falling from the air.
4. Starting the streak on the side of the plate farthest from your dominant hand, pass the loop on the surface of the agar in a zig zag pattern filling the surface of the plate. See Figure 4.
5. Replace the lid, and immediately incinerate the loop or dispose of the cotton swab.
6. Place the plate upside-down for incubation.

Some tips for a good zigzag streak: Use as much of the agar surface as you can. Make broad strokes that span the width of the plate.

Quadrant Streak Plate

Now consider streaking a sample from a pure TSA broth culture prepared for you. If there is visible turbidity, there will be a high density of cells. If you used the same zig zag streaking pattern, the cells would never be reduced in concentration such that you could get isolated colonies. In order to reduce the cell density on the surface of the plate, we can use a **quadrant streak plate**.

The quadrant streak plate reduces or dilutes the number of cells using successive streaking zones on the petri plate and flaming the inoculation loop between zones. The petri plate is divided into four sections or zones. Bacteria are deposited in the first section at full strength from the source. Then the inoculating loop is sterilized. From this point on, no additional cells are added to the agar surface. The sterile loop is used to spread out cells that have already been placed on the plate. After spreading cells from the first area to the second, the loop is sterilized again. This eliminates extra cells from the loop. The sterile loop is used to spread some cells from the second area into the third area diluting them further. Cells are spread into a fourth area from the third area after the loop is sterilized. After all the regions have been inoculated, the hope is that in the last section cells are far enough apart so that they grow up into isolated colonies.



Figure 5: Bacteria growth on a petri plate streaked using the quadrant streak plate technique. Four zones of growth are apparent with successively less growth in each area/zone. Isolated colonies are present in the fourth area/zone streaked.

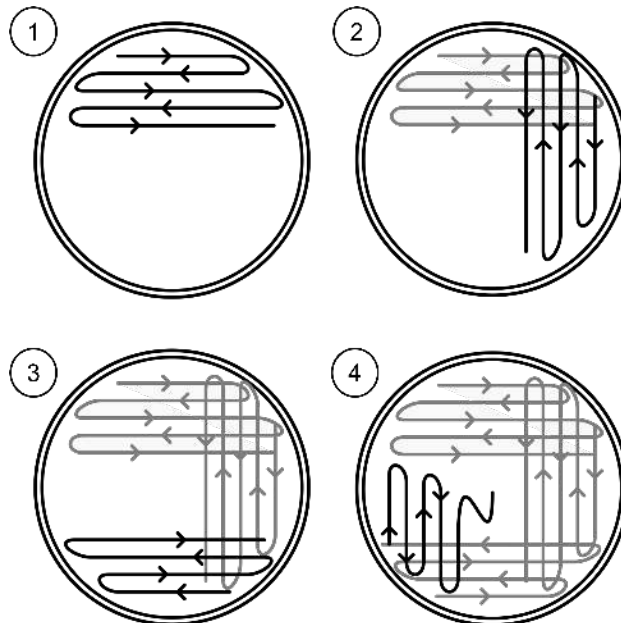


Figure 6: Quadrant streak plate technique to create four areas/zones with successively less growth. (1) Zone one occupies one fourth of the petri plate with the inoculating loop snaking back and forth in that quadrant. After zone one is completed, the inoculating loop is flamed to kill all microbes. (2) Zone two occupies a separate quarter of the petri plate and takes bacteria streaked from the first area/zone rather than collecting more bacteria from the original culture. After zone two is completed, the inoculating loop is flamed to kill all microbes. (3) Zone three occupies a separate quarter of the petri plate and takes bacteria streaked from the second area/zone. After zone three is completed, the inoculating loop is flamed to kill all microbes. (4) Zone four pulls bacteria from the third zone.

This quadrant streak plate technique allows one to observe isolated colonies and characterize them and determine if your observations are consistent with our expectations for the organism you are working with. If you are working with a pure culture, you would expect that all the colonies would look the same, similar size, color, shape etc. One or more different looking colonies indicates your culture was contaminated or you created contamination by poor aseptic technique.

The steps of the quadrant streak plate technique are as follows:

1. Aseptically obtain a loopful of the culture and set the tube back in a rack.
2. Turn the plate right-side up and place it on a piece of white scratch paper so that the lines can be seen.
3. Rotate the plate so that area/zone 1 is farthest from your dominate hand.
4. Holding the plate lid so that it acts as a shield protecting the agar surface from microbes falling from the air, pass the loop on the surface of the agar in area 1 in the pattern shown.
5. Replace the plate lid on the petri plate.
6. Flame the loop and allow it to cool.
7. Rotate the petri plate.
8. Spread the cells already on the petri plate agar surface in area/zone 1 with the sterile loop by streaking in the pattern shown.
This step pulls highly concentrated cells from area/zone 1 into area/zone 2 spreading them out.
9. Replace the plate lid on the petri plate.
10. Flame the loop and allow it to cool.
11. Rotate the petri plate.
12. Again, spread some of the cells from area/zone 2 into area/zone 3 by streaking in the pattern shown.
13. Replace the plate lid on the petri plate.
14. Flame the loop and allow it to cool.
15. Rotate the petri plate.
16. Again, spread some of the cells from area/zone 3 into area/zone 4 by streaking in the pattern shown.
17. Replace the plate lid and flame the loop again before setting it down.
18. Turn the plate upside-down for incubation and storage.

In this technique it is essential that you flame the loop after each area is inoculated. This is what reduces the cell density because you are spreading cells already on the plate. You are not adding additional cells by using a loop with cells on it. You eliminate additional cells by flaming. After incubation, the goal is at least 3 well isolated colonies.

Keep in mind that you want to use as much of the agar surface as possible. Your streaks should span the width of the plate. If you keep touching the previous high density streak, you will pull too many cells into the next area and will not reduce the number enough to get isolated colonies. If you do not cross over the previous area enough, you will not have enough cells in the next one.

If you choose to cool your loop in the agar, always use a spot close to the edge and away from any previous streak. The resulting growth pattern should be dense growth in area/zone 1, more diffuse in area/zone 2 and less growth in area/zone 3, and the least growth in area/zone 4.



Lab Instructions

1. Each person will label 1 TSA plate with their name, “quadrant streak plate,” and with the bacterial sample they will be streaking (one of the following - divide the following three cultures so that each person in your group does a streak plate of a different culture):
 1. *Escherichia coli*
 2. *Serratia marcescens*
 3. *Escherichia coli* & *Serratia marcescens* (mixture)
2. Follow the instructions above for the quadrant streak plate technique.
3. Invert the Petri plate and incubate at 30 °C.
4. Next class, examine the colonies and characterize the colony form, elevation, margin, diameter, and color.
5. Either make a drawing of the plates of your group or take photos of the plates.

CARMEN QUESTIONS - Streak Plate Results

1. For each plate, identify:
 1. colony form
 2. colony elevation
 3. colony margin
 4. colony color
 5. colony diameter (mm)
2. Did you obtain isolated colonies on each of the plates?

Escherichia coli:

Serratia marcescens:

Escherichia coli & *Serratia marcescens* (mixture):
3. Why can a single colony on a plate be used to start a pure culture?

Works Cited

Franklund, C. (n.d.). Microbiology Laboratory Manual, Observing and Recording the Microbial World. Farris State University. Retrieved 2018, from <https://github.com/WeeBeasties/microbiology-laboratory-manual>

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2.4: Simple Stain

Learning Objectives

- Define simple stain.
- Tell the purpose of simple staining and what bacterial feature(s) can and cannot be ascertained when using a simple stain.
- Prepare a bacterial smear and properly heat fix the smear.
- Tell the importance of fixation in preparing a bacterial sample for staining.
- Describe how bacterial smears are prepared differently when using a liquid culture versus using a solid culture.
- Prepare a simple stain.
- Examine the bacterial smear with a microscope and make an accurate illustration of the bacterial cells.
- Use the microscope to identify bacterial features (cell shape and cell arrangements).
- Identify bacterial cell shapes and cell arrangements given examples.
- Identify flaws in a simple stain and ascertain ways to improve future stains.

Simple Staining

Most types of cells do not have much natural pigment and are therefore difficult to see under the light microscope unless they are stained. Several types of stains can be used to make bacterial cells more visible. Some staining techniques can be used to determine the cells' biochemical or structural properties, such as cell wall type and presence or absence of endospores. This type of information can help scientists identify and classify microorganisms, and can be used by health care providers to diagnose the cause of a bacterial infection.

One type of staining procedure that can be used is the **simple stain**, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope. Some stains commonly used for simple staining include crystal violet, safranin, and methylene blue. Simple stains can be used to determine a bacterial species' morphology (cell shape) and arrangement (single, chains, clusters, etc.), but they do not give any additional information. Other staining approaches utilize more than one stain (**differential stains**) and can be used to determine other differences of between different bacterial species (e.g. cell wall structures, presence or absence of endospores, presence or absence of capsules, presence or absence and arrangement of flagella, etc.). These features cannot be distinguished using a simple stain.

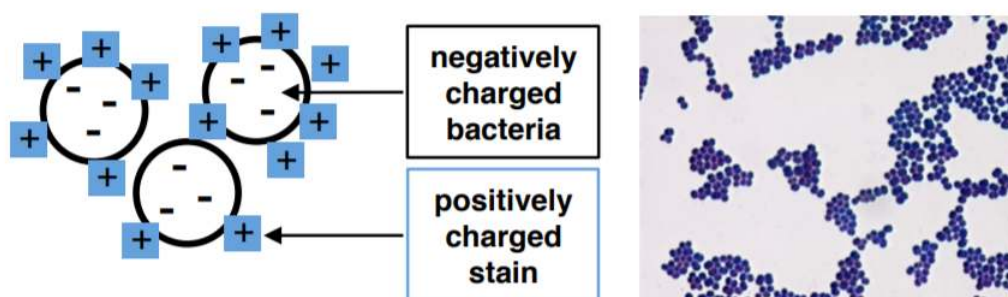
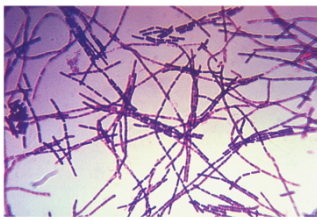


Figure 1: Methylene blue is a simple and direct stain used for determining bacterial morphology (shape and arrangement). It is a cationic dye (positive charge) which stains the cell a blue color. The presence of negatively charged molecules in the cell (like DNA & RNA) causes the cell to stain blue. The image to the right shows that the bacterial cell morphology is **cocci** (round) and the arrangement is in clusters or bunches, often distinguished with the prefix **staphylo-** (putting these two terms together, these bacteria can be described as **staphylococci** indicating they are round-shaped bacteria that occur in clusters or bunches).

Table 1: Common simple stain types include basic stains (high pH stains), acidic stains (low pH stains), and negative stains (stains the surroundings of cells instead of the cells themselves). Basic stains include methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, and safranin. Acidic stains include eosin, acid fuchsin, rose bengal, and congo red. Basic stains are attracted to negatively charged molecules in the cell including nucleic acids (DNA and RNA) and some proteins. Acidic stains can be positive stains (stain the cells) or they can be negative stains (stain the background and not the cells). Acidic stains can stain positively charged molecules in cells including some proteins. Negative stains include India ink and nigrosin. Negative stains

produce a darker background and cells appear light and unstained. When a simple stain is done, only one stain is applied to the cells to better see the cells using the microscope.

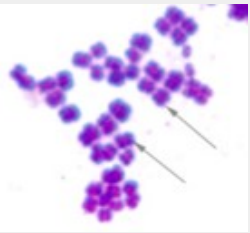
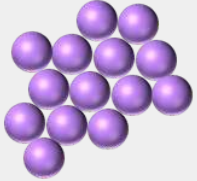
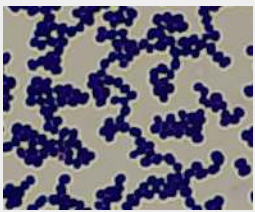
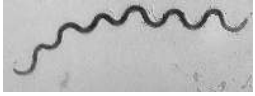
SIMPLE STAINS				
Stain Type	Specific Dyes	Purpose	Outcome	Sample Images
Basic stains	Methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	
Acidic stains	Eosin, acid fuchsin, rose bengal, Congo red	Stain positively charged molecules and structures, such as proteins	Can be either a positive or negative stain, depending on the cell's chemistry.	
Negative stains	India ink, nigrosin	Stains background, not specimen	Dark background with light specimen	

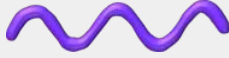
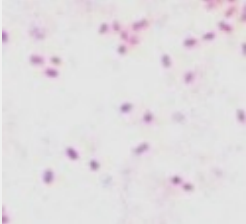
A good stained smear should be somewhat difficult to see with the naked eye on the surface of a microscope slide. If there is a dark spot of color visible, it means that the bacteria are stacked too densely for adequate evaluation. This is a common mistake made by students learning to make bacterial smears.

Bacterial Cell Morphology & Arrangement

Two characteristics that can be used to distinguish different types of bacteria is their microscopic shapes and their microscopic arrangements. Identification of these features can lead to a diagnosis and aid in treatment of a patient. The cell shape describes the overall shape of a single bacterial cell. Arrangements describe how bacterial cells are grouped (or not grouped) with each other. Both of these characteristics must be determined using a microscope and typically occurs after staining the cells.

Table 2: Summary of the most common bacterial cell shapes and bacterial cell arrangements with cartoon-like illustrations of each, descriptions, and microscopic images.

Cell Shape (singular / plural)	Cell Arrangement (singular / plural)	Illustration	Description	Image (color of cells is based on the type of stain used)
coccus / cocci	single or solitary		Cells are alone and round or spherical.	
coccus / cocci	diplococcus / diplococci		Cells are in pairs and round or spherical.	
coccus / cocci	tetrad		Cells are grouped in fours and are round or spherical.	
coccus / cocci	sarcina		Cells are group in eights as two groups of four on top of each other. Cells are round.	
coccus / cocci	streptococcus / streptococci		Cells are in a chain and are round or spherical.	
coccus / cocci	staphylococcus / staphylococci		Cells are in bunches or clusters and are round or spherical.	
spirochete / spirochetes	single or solitary		Cells are alone and long and thin with a three-dimensional corkscrew forming a helical shape.	

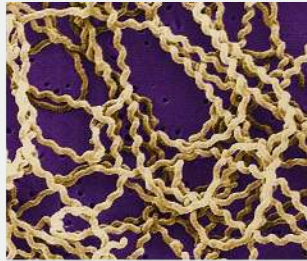
Cell Shape (singular / plural)	Cell Arrangement (singular / plural)	Illustration	Description	Image (color of cells is based on the type of stain used)
spirillum / spirilla	single or solitary		Cells are alone and long and thin with a wavy appearance but are not helical in shape.	
vibrio / vibrios	single or solitary		Cells are alone and have a rod shape with an arch or curve.	
bacillus / bacilli	single or solitary		Cells are alone and rod shaped.	
bacillus / bacilli	diplobacillus / diplobacilli		Cells are in pairs and are rod shaped.	
bacillus / bacilli	streptobacillus / streptobacilli		Cells are in chains and are rod shaped.	
bacillus / bacilli	palisades		Cells are rod shaped and lined up with each other long-ways resembling the pickets in a picket fence.	
coccobacillus / coccobacilli	single or solitary		Cells are alone and are short rods.	

Note

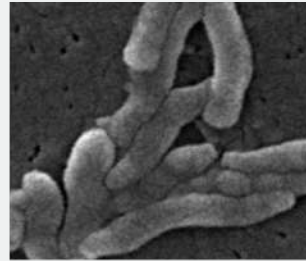
There are other less common bacterial morphologies (e.g., filamentous, squares, etc.) that are not shown here.

Note

It may be difficult to tell the difference between spirochetes and spirilla using a common light microscope. The difference is more obvious with an electron microscope (see images below) or using other types of light microscopes. Please note that spirilla typically have one flagellum at each end or pole of the cell. The spirilla image does not show flagella.



spirochetes
(electron micrograph)



spirilla
(electron micrograph)

To learn more about comparing spirilla and spirochetes, see this article [Difference Between Spirilla and Spirochetes](#).

Preparing Bacteria for Staining (Bacterial Smear and Fixation)

Bacterial Smear

Prior to staining bacterial cells they first must be applied to the microscope slide in what is called a **bacterial smear**. If bacteria are transferred from a liquid culture, a loopful of culture can be aseptically removed from the culture and then spread on the surface of the microscope slide. If bacteria are transferred from a solid culture such as a slant or a petri plate, a single drop of DI or distilled water is added to a microscope slide and then a small amount of growth is obtained aseptically with a loop. The loop is then used to spread the water drop around the slide so it becomes a thin layer of water containing bacteria.

Note

Chunks of bacteria on the microscope slide surface means that too much bacterial growth was transferred to the slide and it will be difficult to distinguish individual cells when viewing the sample with a microscope.

After bacteria have been applied to a microscope slide, the smear is dried thoroughly before fixation. The smear can be allowed to air dry or can be put on a slide warmer to speed up the drying process.

Warning

After preparation of the smear you must **fix** the sample (see the **Fixation** section below). The bacteria will wash off the slide during staining if you do not fix them to the slide.

Fixation

Before cells are stained with a dye, they must be fixed to the slide so that they do not wash away with the excess stain. The “fixing” of a sample refers to the process of attaching cells to a slide. In addition to adhering the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation.



Figure 2: A microscope slide with a bacterial smear is being heat fixed. The underside of the microscope slide is passed briefly into the flame of a Bunsen burner to heat fix. For safety, the person who is heat fixing the smear is holding a wooden clip that grips the microscope slide. This way the person heat fixing can keep their hands away from the flame and are not at risk of being burned from the hot microscope slide.

Fixation is often achieved either by heating (**heat fixing**) or chemically treating the specimen. To heat-fix a sample, after a thin layer of the specimen is spread on the slide (called a **smear** or **emulsion**), and the slide is then briefly heated over a heat source. Done correctly, this will attach cells to the microscope slide and kill the cells.

Note

A heat-fixed slide can be stained immediately or kept for months. If you wish to keep heat-fixed, unstained slides for further work, you can wrap the slides in a paper towel or put them in a slide box and store them in your lab drawer.

Laboratory Instructions

Prepare a Bacterial Smear

Note

You may find it helpful to draw a circle (wax pencil is best) on the opposite side of the slide where you will spread your smear. This will help you later in locating the smear. The wax pencil is better than a marker because it will not wash off easily from the glass.

Note

If you are using a liquid culture, gently mix the culture until you get an even, cloudy mixture (it should look somewhat like skim milk). If you mix too aggressively, you will lose the bacterial morphology.

1. Prepare a bacterial smear on the slide:
 - If you are taking bacteria from a solid culture (slant or petri plate), place a small drop (only 1 drop) of saline, deionized (DI) water, or distilled water onto a microscope slide and use a loop to aseptically add bacteria to the water (avoid taking a large chunk of bacteria since the cells will be too dense to view individual cells). Use the loop to spread the bacteria in the water drop and to spread the water drop out to make it thinner (it will dry faster).
 - If you are taking a bacteria from a a liquid culture (broth), place 1 or 2 loopfuls of bacteria directly onto a microscope slide (no saline or water added).
2. Allow the slide(s) to air dry on the slide warmer (or air dry if a slide warmer is not available).

Heat Fix the Bacterial Smear

Once the liquid has completely evaporated on the surface of the slide, heat fix by passing the slide:

1. Attach a wooden clip to the microscope slide to hold it.
2. Pass the underside of the microscope slide through a flame three times.
3. Allow the slide to cool and then continue with your staining protocol.

Note

If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them.

Simple Stain

1. Use the slide(s) that you already prepared when creating bacterial smears and heat fixing (see above).
2. Grip the microscope slide in wood clip over a waste container bucket.
3. Add methylene blue stain to the heat-fixed smear. There is no reason to cover the entire slide with stain. Just make sure to cover the smear with stain.
4. Set a timer for 1.5 or 2 minutes. The stain will remain on the smear during this time.
5. Angle the slide and gently rinse the slide with DI or distilled water using a squirt bottle. Water is not applied directly to the smear. Instead, apply the water to the area of the slide just above the smear so that water will rinse across the smear and into the waste bucket. Continue rinsing until water running off the slide appears clear.
6. Blot gently with bibulous paper. Do not wipe. Just gently blot with bibulous paper to get rid of excess water.
7. Place the stained slide on a microscope and examine. Be sure to begin at the lowest power (look for the blue color of the methylene blue dye). Focus at lowest power and then increase one objective magnification at a time.

CARMEN QUESTIONS - Simple Stain

1. What microbial characteristics can one ascertain from a simple stain?
2. What were the shapes and arrangement of the cells you observed? Use microbiological terminology.

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2.5: Gram Stain

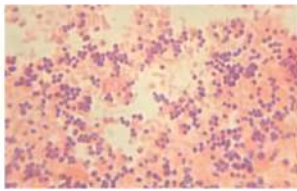
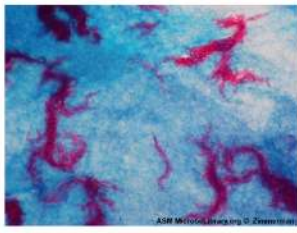
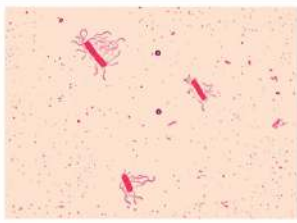
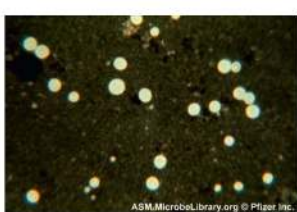
Learning Objectives

- Explain the importance of Gram stains in health care and microbiology.
- Define "differential stain" and contrast with "simple stain."
- Identify the Gram stain as a type of differential stain
- Tell what the Gram stain tells us about different species of bacteria.
- Examine Gram-stained cells and interpret whether the cells are Gram-positive or Gram-negative.
- Identify cell morphology of bacteria.
- Describe the structure of the cell walls of Gram-positive cells.
- Describe the structure of the cell walls of Gram-negative cells.
- Identify structures of Gram-positive cell walls and Gram-negative cell walls in diagrams models of cell walls.
- Successfully conduct a Gram stain and differentiate cells as Gram-positive and Gram-negative.
- Name each stain/reagent of the Gram stain and explain its function and how it will interact with Gram-positive and Gram-negative cell walls during the Gram stain procedure.
- Troubleshoot unsuccessful Gram stains and explain how errors might be fixed.

Differential Stains

Microbiologists commonly perform **differential stains**, as this allows them to gather additional information about the bacteria they are working with. Differential stains use more than one stain, and cells of different bacterial species can have different appearances based on their chemical or structural properties. Some examples of differential stains are the Gram stain, acid-fast stain, and endospore stain.

Table 1: Summary of some common differential stains used in microbiology. The Gram stain uses the following dyes/reagents: crystal violet, Gram's iodine, ethanol, and safranin. The Gram stain distinguishes cells by cell wall type (Gram-positive or Gram negative). Gram-positive cells stain purple/violet. Gram-negative cells stain pink. The acid fast stain uses the following dyes: basic fuchsin and methylene blue. The acid fast stain is used to distinguish acid fast bacteria (reveals the cell wall structure of certain types of bacteria beyond Gram-positive and Gram-negative). Acid fast bacteria appear red and non-acid fast bacteria appear blue. The endospore stain uses the following: heat, malachite green, and safranin. The endospore stain is used to distinguish between bacterial species that do and do not form endospores. Endospores (if present) appear bluish-green and all other cells/cell structures appear pink. The flagella stain uses the following dyes/reagents: tannic acid or potassium alum mordant, pararosaniline or basic fuchsin. The flagella stain is used to visualize flagella (if present). The capsule stain uses the following dyes: India ink or nigrosin (stains the background, not cells) and a counterstain. The result shows the background as dark, the cells the color of the counterstain, and the capsule (if present) appearing clear. The capsule stain determines if a bacterial species has capsules or not. Capsules appear as transparent halos around dyed cells.

DIFFERENTIAL STAINS				
Stain Type	Specific Dyes	Purpose	Outcome	Sample Images
Gram stain	Uses crystal violet, Gram's iodine, ethanol (decolorizer), and safranin	Used to distinguish cells by cell-wall type (gram-positive, gram-negative)	Gram-positive cells stain purple/violet. Gram-negative cells stain pink.	
Acid-fast stain	After staining with basic fuchsin, acid-fast bacteria resist decolorization by acid-alcohol. Non acid-fast bacteria are counterstained with methylene blue.	Used to distinguish acid-fast bacteria such as <i>M. tuberculosis</i> , from non-acid-fast cells	Acid-fast bacteria are red; non-acid-fast cells are blue.	
Endospore stain	Uses heat to stain endospores with malachite green (Schaeffer-Fulton procedure), then cell is washed and counterstained with safranin.	Used to distinguish organisms with endospores from those without; used to study the endospore.	Endospores appear bluish-green; other structures appear pink to red.	
Flagella stain	Flagella are coated with a tannic acid or potassium alum mordant, then stained using either pararosaniline or basic fuchsin.	Used to view and study flagella in bacteria that have them.	Flagella are visible if present.	
Capsule stain	Negative staining with India ink or nigrosin is used to stain the background, leaving a clear area of the cell and the capsule. Counterstaining can be used to stain the cell while leaving the capsule clear.	Used to distinguish cells with capsules from those without.	Capsules appear clear or as halos if present.	

The Gram Stain Shows Differences in Cell Wall Structures

The Gram stain, developed by Christian Gram in 1884, is the most widely used differential stain in bacteriology. Most bacteria are divided into two major groups- Gram-positive bacteria and Gram-negative bacteria based on the cell wall composition. Knowing the Gram reaction of a clinical isolate (isolated bacterial species from a patient) can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.

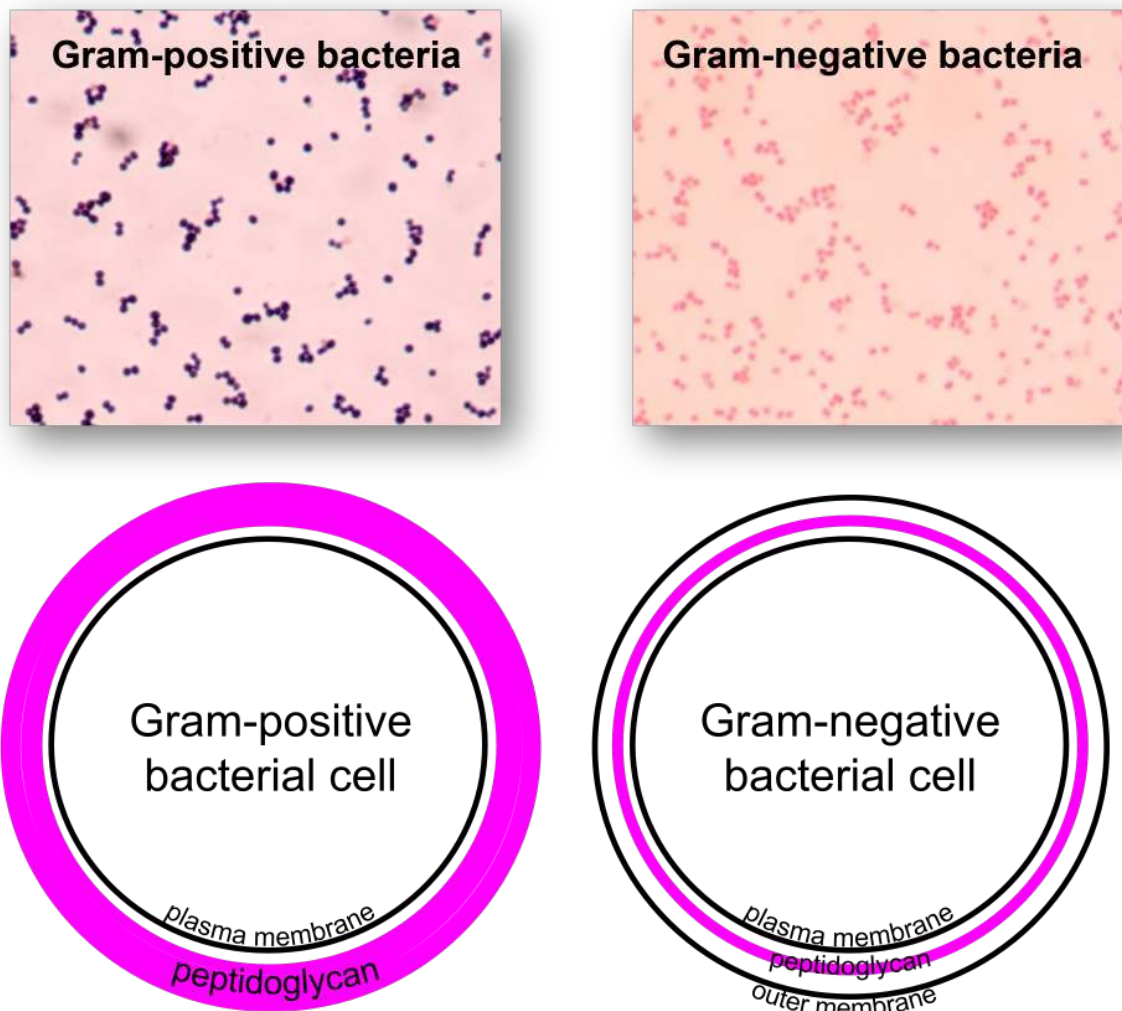


Figure 1: (Top) Microscopic images of bacteria stained with the Gram stain. (Top right) Gram-positive bacterial species stain purple with the Gram stain. (Top left) Gram-negative bacterial species stain pink with the Gram stain. (Bottom) Diagrams illustrating major structural components of Gram-positive bacterial cell walls and Gram-negative bacterial cell walls. (Bottom right) Gram-positive bacterial species have a thick layer of peptidoglycan outside of the plasma membrane. (Bottom left) Gram-negative bacterial species have a thin layer of peptidoglycan outside of the plasma membrane with an outer membrane outside of the peptidoglycan layer. Embedded in the outer membrane of Gram-negative cell walls are lipopolysaccharides (LPS) that are not shown in this diagram.

The results of the Gram stain reflect differences in cell wall composition. **Gram-positive** cells have thick layers of peptidoglycan (a substance composed of carbohydrates and protein subunits) in their cell walls. **Gram-negative** bacteria have very little peptidoglycan. Gram-positive bacteria also have teichoic acids, whereas Gram-negative bacteria do not. Gram-negative cells have an outer membrane that resembles the phospholipid bilayer of the plasma membrane. The outer membrane contains **lipopolysaccharides (LPS)**, which are released as **endotoxins** when Gram-negative cells die. This can be of concern to a person with an infection caused by a Gram-negative organism.

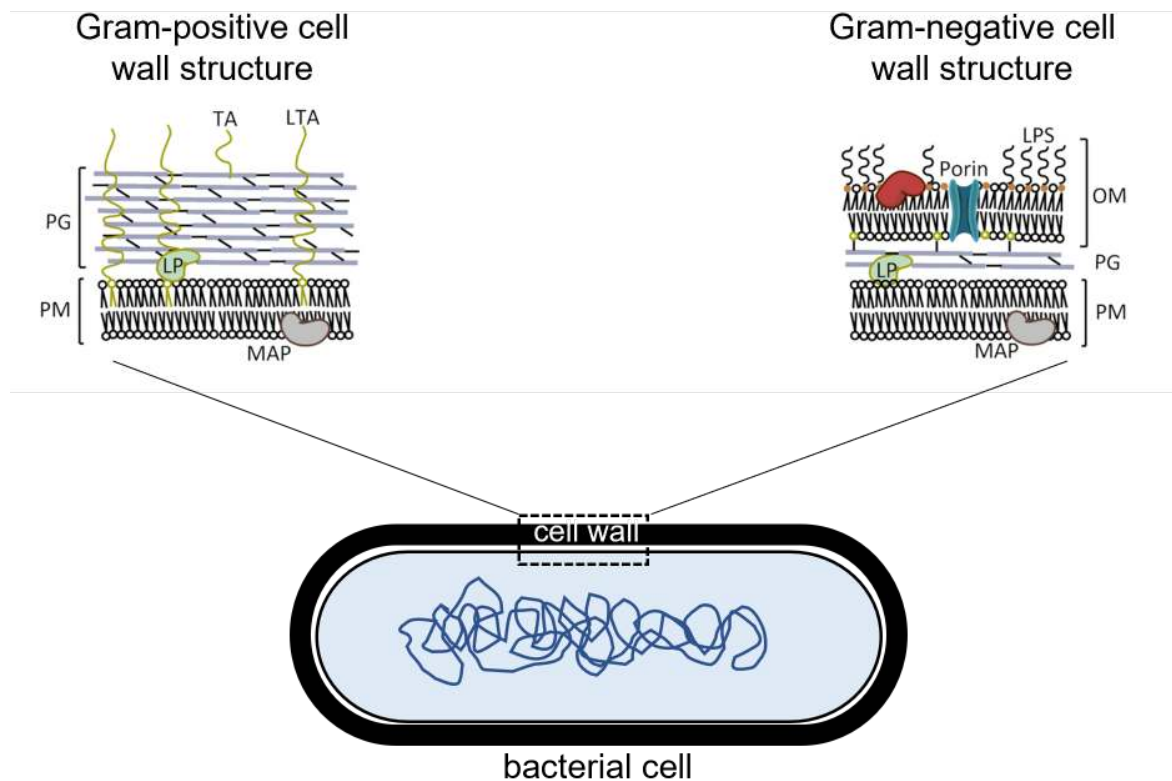


Figure 2: Detailed view of the structure of bacterial cell walls for Gram-positive species and for Gram-negative species. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across the peptidoglycan layer. (Right) Gram-negative cell walls consist of a thin layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM). Gram-negative cell walls have an outer membrane (OM) beyond the peptidoglycan layer containing lipopolysaccharide (LPS) as well as porin channels that enable some materials to pass across the outer membrane. In Gram-positive and Gram-negative cell wall types lipoprotein (LP) is also found.

Gram stains are best performed on fresh culture. Older cells may have damaged cell walls and not yield the correct Gram reaction. Also, some species are known as **Gram-variable**, and so both Gram-positive and Gram-negative reactions may be visible on your slide.

Although the vast majority of bacteria are either Gram-positive or Gram-negative, it is important to remember that not all bacteria can be stained with this procedure (for example, *Mycoplasma* sp., which have no cell wall, stain poorly with the Gram stain).

How the Gram Stain Works

The Gram stain uses four stains/reagents: crystal violet, Gram's iodine, ethanol, and safranin. Crystal violet (the **primary stain**), enters the peptidoglycan of all bacteria giving them a purple color. The next stain is Gram's iodine, the **mordant**, which combines with the crystal violet to make a bigger complex in the peptidoglycan wall. The next step is the most critical. Ethanol is used as a decolorizer will remove the crystal violet/iodine complexes from the thin peptidoglycan layers of Gram-negative cell walls, but not the thick peptidoglycan layers of Gram-positive cell walls. The length of time given for the decolorization step with ethanol will be important in obtaining the best results from the Gram stain. After the decolorization step, Gram-positive cells will still hold onto the purple crystal violet color, but Gram-negative cells will lose the purple color and be transparent. We still want to see the Gram-negative cells and to be able to distinguish them from the Gram-positive cells. Safranin is called a counterstain since it is used to stain the now colorless Gram-negative cells a pink color.

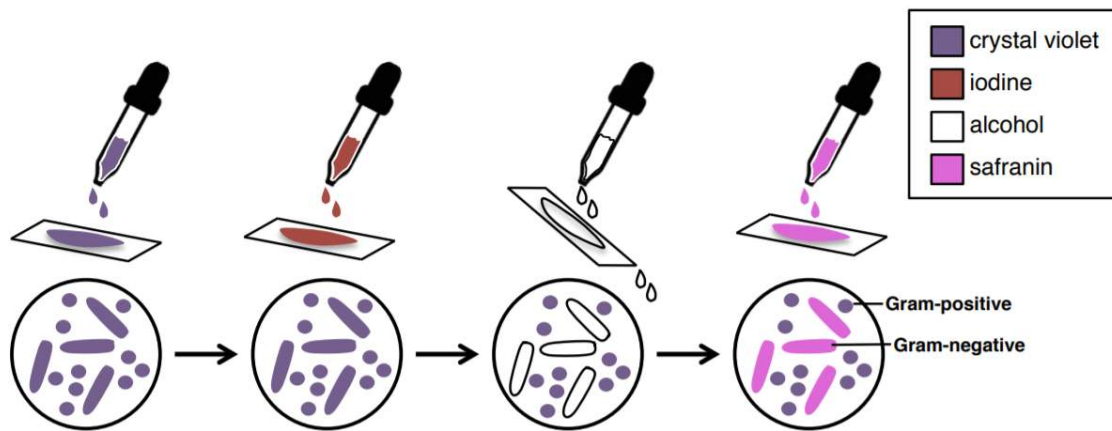


Figure 3: Overview of the Gram stain procedure and how each step changes cell color. (1) Applying crystal violet turns all cells purple. (2) Applying Gram's iodine does not change cell colors (all cells stay purple), but it will cause the crystal violet to firmly adhere to Gram-positive cell walls, but not Gram-negative cell walls. (3) The alcohol (ethanol) step is a decolorization step. Ethanol will remove crystal violet from Gram-negative cells, but not Gram-positive cells. After this step, Gram-positive cells will remain purple and Gram-negative cells will be transparent. (4) Safranin is a reddish-pink stain that will stain transparent cells (Gram-negative cells) pink. After this step, Gram-positive cells will still be purple and Gram-negative cells will be pink.

? Exercise 10.1

You conduct a Gram stain and when you observe the bacterial cells with the microscope, you observe pink colored cells. What does this tell you?

Answer

? Exercise 10.2

You conduct a Gram stain and when you observe the bacterial cells with the microscope, you observe purple colored cells. What does this tell you?

Answer

? Exercise 10.3

You conduct a Gram stain and when you observe the bacterial cells with the microscope, you observe purple colored cells and pink colored cells mixed together. What does this tell you?

Answer

The Gram stain has proven to be very useful in the identification of bacteria and in predicting which antibiotics are most likely to be effective to kill bacteria. There are some problems with the technique, however. If the procedure is not performed properly, the results may be erroneous. Thus, you will need to practice the technique until your results are satisfactory. There are some bacterial species, such as *Mycobacterium tuberculosis* or *Legionella pneumophila* that usually do not stain with the Gram stain at all. Nevertheless, this technique has become one that microbiologists rely heavily upon.

Table 2: Stains and reagents used during the Gram stain.

Stain/Reagent use in the Gram stain	Color of the Stain/Reagent	Role of the Stain/Reagent	What the Stain/Reagent Does
crystal violet	purple	primary stain	stains all cells purple
Gram's iodine	amber	mordant	attaches to the crystal violet to form larger complexes that prevent crystal violet from leaving thick peptidoglycan layers in Gram-positive cell walls
ethanol	clear	decolorizer	removes crystal violet from thin peptidoglycan layers in Gram-negative cell walls leaving Gram-negative cells appearing transparent
safranin	red/pink	counterstain	colors transparent Gram-negative cells pink

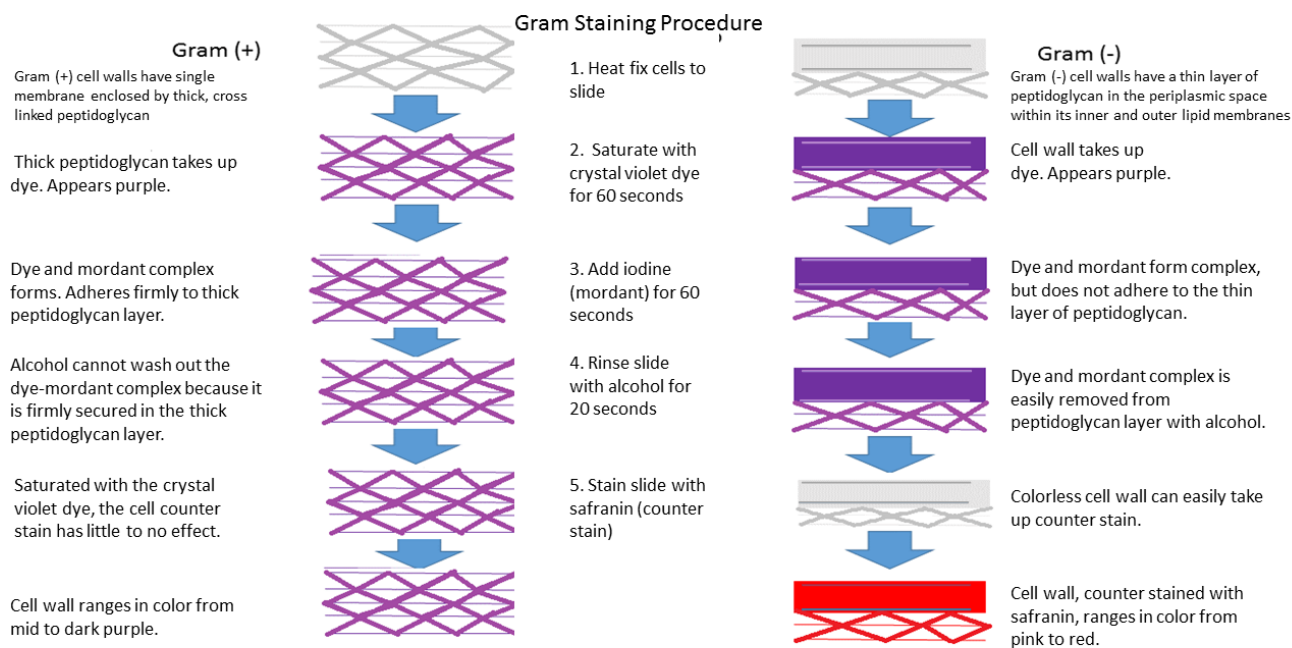
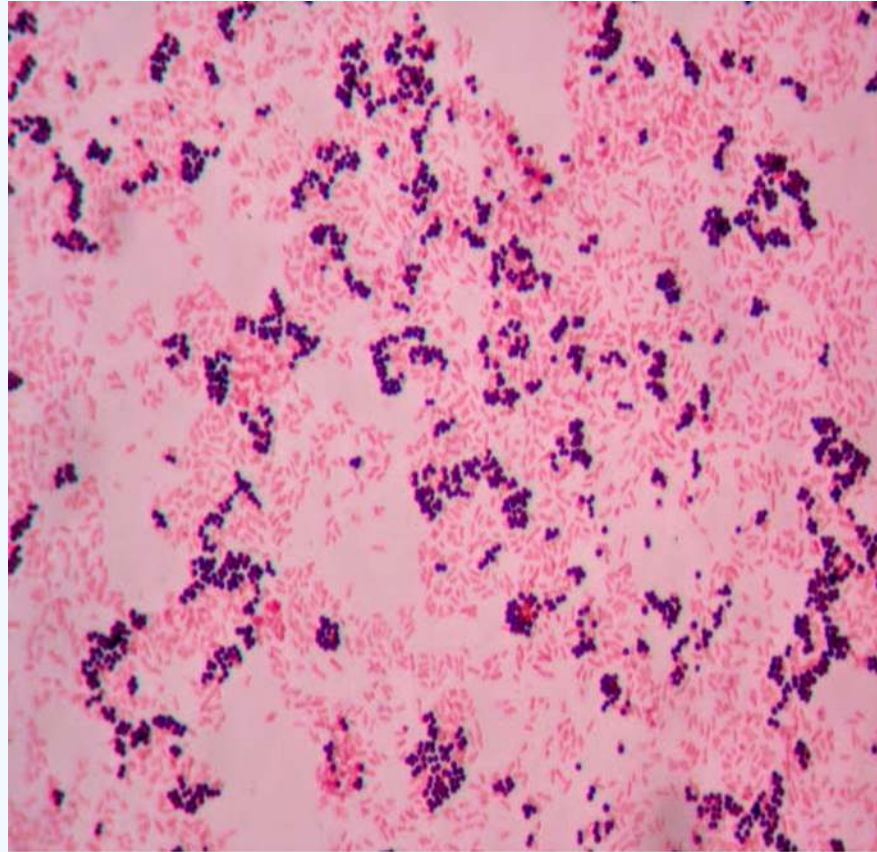


Figure 4: Gram-positive cell walls have a single membrane (the plasma membrane) enclosed by a thick, cross-linked peptidoglycan layer. Gram-negative cell walls have a thin layer of peptidoglycan between the plasma membrane and an outer membrane. When the Gram-positive cells and Gram-negative cells are saturated with crystal violet (first step of the Gram stain), they become purple. When Gram's iodine is applied to the cells, it causes the crystal violet to adhere firmly in the thick peptidoglycan layer of Gram-positive cell walls. In Gram-negative cell walls, the mordant does not firmly adhere the crystal violet within the thin peptidoglycan layer (even though iodine and crystal violet still react and form large complexes). In the third step of the Gram stain, the decolorizer, ethanol, is added to the cells. The ethanol removes the crystal violet from Gram-negative cells since crystal violet did not adhere strongly to the thin peptidoglycan layer and the cells become transparent. The crystal violet is not removed from the Gram-positive cells since the iodine-crystal violet complexes adhere to the thick peptidoglycan layer. Gram-

positive cells remain purple. In the last step of the Gram stain, safranin is used as a counterstain to stain transparent cells (Gram-negative cells) pink. The end result is Gram-positive cells that are purple and Gram-negative cells that are pink.

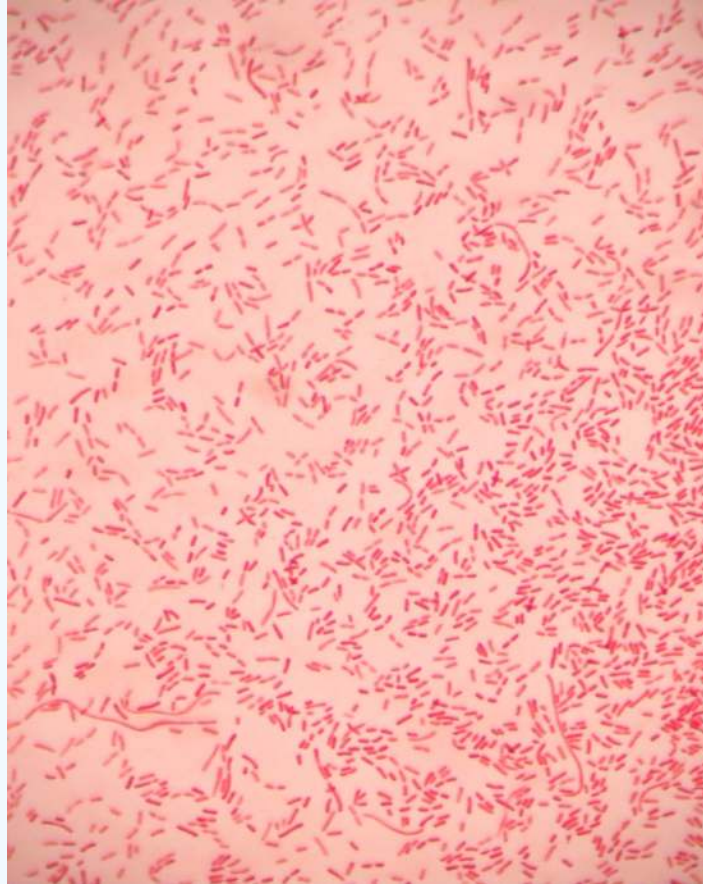
? Exercise 10.4



1. How many species can you see in this Gram stain?
2. What is the Gram (positive or negative) of the cells?
3. What is the cell shape of the cells (see [Bacterial Cell Morphology & Arrangement](#) for assistance)?

Answer

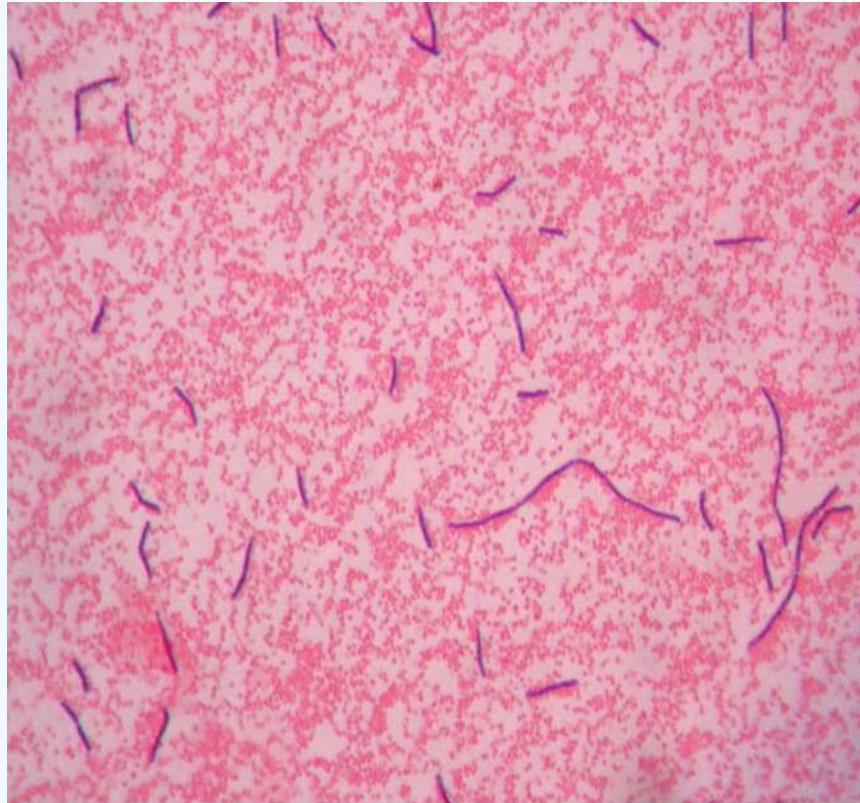
? Exercise 10.5



1. How many species can you see in this Gram stain?
2. What is the Gram (positive or negative) of the cells?
3. What is the cell shape of the cells (see [Bacterial Cell Morphology & Arrangement](#) for assistance)?

Answer

? Exercise 10.6



1. How many species can you see in this Gram stain?
2. What is the Gram (positive or negative) of the cells?
3. What is the cell shape of the cells (see [Bacterial Cell Morphology & Arrangement](#) for assistance)?

Answer

Improving and Troubleshooting your Gram Stain Approach

Gram staining requires practice. It is an art as much as a science. If your results do not come out as they should, adjust your procedure to correct the problem for future stains.

common mistakes

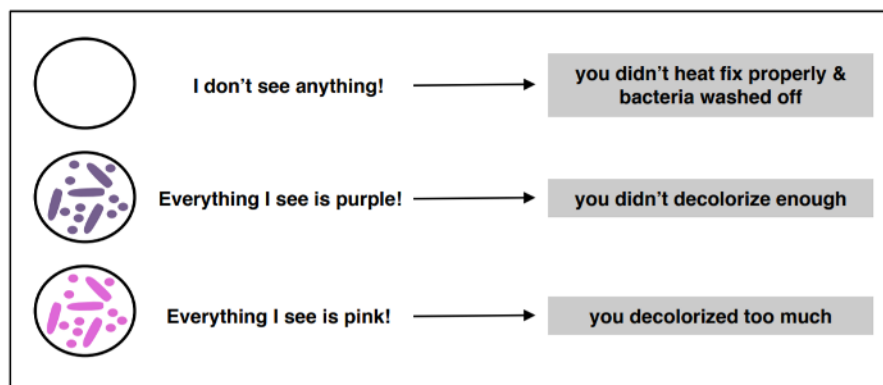


Figure 5: If you are staining a mixture of Gram-negative and Gram-positive bacteria and you do not see cells, see all cells purple, or see all cells pink, it is likely due to one of these common errors. (1) If you do not see anything (no cells are visible), it is likely

because the smear was not properly heat-fixed and the bacteria washed off the slide during staining. (2) If you see all cells appear purple, it indicates that the decolorization step was too short. The next time you do a Gram stain, increase the time for decolorization. (3) If you see all cells appear pink, it indicates that the decolorization step was too long. The next time you do a Gram stain, decrease the time for decolorization.

Factors to control to obtain a proper Gram stain result:

1. Smear preparation. Beginning students tend to make the smears too thick by putting too much inoculum on the slide. This will make it harder to properly decolorize the bacteria. Always put saline or water on the slide before or after adding bacteria when using a culture from solid medium (slant or plate). The amount of inoculum from a solid media should be a “pinpoint” amount. You should just barely touch the organisms you want to stain with your loop. NOTE: You do not need to put saline or water on the slide when making smears from a liquid media.
2. Allow the smear to air dry. This means the slide must sit until it has no longer appears moist. Alternatively, you may use a slide warmer to dry your smear. The slide warmer will dry the slide faster. When using the slide warmer, do not take your eyes off the smear! As soon as the slide is dry, remove it from the slide warmer. If too much heat is used, the cell wall of the organisms may be distorted or rupture and release the crystal violet dye when it should retain it. Extreme overheating may cause the entire cell to lyse, and you will see nothing under the microscope.
3. Heat fixing will adhere the bacteria to the slide and kill the bacteria. If you do not have any cells on the slide, likely the heat fixing step was skipped or not enough to fix the cells to the slide.
4. Freshness and concentration of reagents. Reagents must be fresh, and concentrations must be accurate.
5. Failure to drain slides before adding next reagent. After you wash a slide with water and before you add the next reagent, you must be sure to tilt the slide to allow excess water to drain off it. If you leave a bubble of water over the bacteria, the reagent you add may never reach the bacteria or it will be greatly diluted by the water present on the slide.
6. Age of the bacterial culture. Older Gram-positive organisms lose their ability to retain the violet dye as they move out of the exponential growth phase.
7. Timing of the decolorizing step. This is the most critical stage of the procedure. If the decolorizer is left on too long, Gram-positive organisms will appear Gram-negative. If the decolorizer is not left on long enough, Gram-negative organisms will appear Gram-positive. The thickness of the smear will dictate how long you will need to decolorize. It is impossible to give an exact time. You must decolorize one smear at a time and watch it closely. When the color begins to lift off the smear, you should wash off the decolorizer.
- 8.

Note

If you mix too aggressively, you will lose the bacterial morphology.

Note

If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them

Laboratory Instructions

Prepare a Bacterial Smear

Note

You may find it helpful to draw a circle (wax pencil is best) on the opposite side of the slide where you will spread your smear. This will help you later in locating the smear. The wax pencil is better than a marker because it will not wash off easily from the glass.

 Note

If you are using a liquid culture, gently mix the culture until you get an even, cloudy mixture (it should look somewhat like skim milk). If you mix too aggressively, you will lose the bacterial morphology.

1. Prepare a bacterial smear on the slide:
 - If you are taking bacteria from a solid culture (slant or petri plate), place a small drop (only 1 drop) of saline, deionized (DI) water, or distilled water onto a microscope slide and use a loop to aseptically add bacteria to the water (avoid taking a large chunk of bacteria since the cells will be too dense to view individual cells). Use the loop to spread the bacteria in the water drop and to spread the water drop out to make it thinner (it will dry faster).
 - If you are taking a bacteria from a liquid culture (broth), place 1 or 2 loopfuls of bacteria directly onto a microscope slide (no saline or water added).
2. Allow the slide(s) to air dry on the slide warmer (or air dry if a slide warmer is not available).

Heat Fix the Bacterial Smear

Once the liquid has completely evaporated on the surface of the slide, heat fix by passing the slide:

1. Attach a wooden clip to the microscope slide to hold it.
2. Pass the underside of the microscope slide through a flame three times.
3. Allow the slide to cool and then continue with your staining protocol.

 Note

If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them.

Gram Stain

1. Use the slide(s) that you already prepared when creating bacterial smears and heat fixing (see above).
2. Grip the microscope slide in wood clip over a waste container bucket.
3. **Primary stain:** Add crystal violet (primary stain) onto the smear (there is no reason to cover the entire slide with stain). Allow stain to remain on the smear for 1 minute.
4. Rinse the smear with deionized (DI) water or distilled water.
5. Shake excess water off the smear.
6. **Mordant:** Add Gram's iodine onto the smear (there is no reason to cover the entire slide with iodine). Leave for 1 minute.
7. Rinse with deionized (DI) water or distilled water.
8. Shake excess water off the smear.
9. **Decolorizer:** Tilt the slide at a 45° angle and let the ethanol run over surface of slide until no more crystal violet color comes out of the smear (time varies — no more than 5-15 seconds).
10. Tilt the slide at a 45° angle and rinse the smear with deionized (DI) water or distilled water.
11. Shake excess water off the smear.
12. **Counterstain:** Add safranin onto the smear (there is no reason to cover the entire slide with stain) for 1 minute.
13. Rinse with deionized water.
14. Shake excess water off the smear.
15. *Gently blot* (do not wipe) the excess water from the slide using bibulous paper.
16. Place the stained slide on a microscope and examine. Be sure to begin at the lowest power (look for the purple and pink colors of the stains). Focus at lowest power and then increase one objective magnification at a time.

CARMEN QUESTIONS - Gram Stain

1. What was the cell shape of the Gram-positive cells you observed (see [Bacterial Cell Morphology & Arrangement](#) for assistance)?
2. What was the cell shape of the Gram-negative cells you observed (see [Bacterial Cell Morphology & Arrangement](#) for assistance)?

3. What does being "Gram-positive" mean about the cell wall structure? What does being "Gram-negative" mean about the cell wall structure?
4. What might occur if the heat fixation step is skipped?
5. What might occur if decolorization is too long? Too short?
6. Is the Gram stain a simple stain or a differential stain? What is the difference?

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2.6: Endospore Stain

Learning Objectives

- Describe what an endospore/spore is and why they are important for the bacterial species that form them.
- Identify/give examples of environmental conditions that can stimulate spore formation.
- Tell that *Bacillus* species and *Clostridium* species can be clinically important endospore-forming species.
- Compare and contrast "vegetative cell" and "spore."
- Successfully conduct an endospore stain.
- Interpret results of an endospore stain.
- Identify when endospores are terminal, subterminal, and central in microscopic images, diagrams, and descriptions.
- Tell how the endospore stain works including the stains involved and how the stains penetrate cells and do or do not wash out of cells.
- Apply the concept of bacterial endospores to healthcare settings and how spores can make treatment of infections by spore-forming species challenging.
- Tell that endospores are only produced by only some bacterial species, and therefore their presence/absence can be useful in identifying bacterial species.

Endospores

An **endospore** is a form of a bacterial cell. Only some species of bacteria can produce endospores. Endospores are made so the cell can survive poor growth or poor survival conditions. An endospore is a mainly inactive version of a cell that is analagous to a survival bunker. When environmental conditions are poor, or even deadly, bacteria that are able to will produce endospores to survive. When environmental conditions improve, endospores can produce the active cells again (active cells are called **vegetative cells**.) Bacterial endospores are the most resistant structures of all living organisms, and they can live in this dormant dehydrated state for hundreds of years (even some documented at thousands of years). The stimulus that triggers sporulation (formatio of spores) can vary and may include nutrient depletion, desiccation, chemicals, heat, etc.

Note

Endospores are not for reproduction. One spore forms inside of one vegetative cell (vegetative = metabolically active cell). When environmental conditions improve, one spore germinates to produce one vegetative cell.

Endospore production is a very important characteristic of some bacteria, allowing them to resist adverse environmental conditions such as desiccation, chemical exposure, extreme heat, etc. Endospores were first identified in the 1800s (John Tyndall developed a process for destroying them with an intermittent heating procedure), although the stain procedures to identify them did not develop until the early twentieth century.

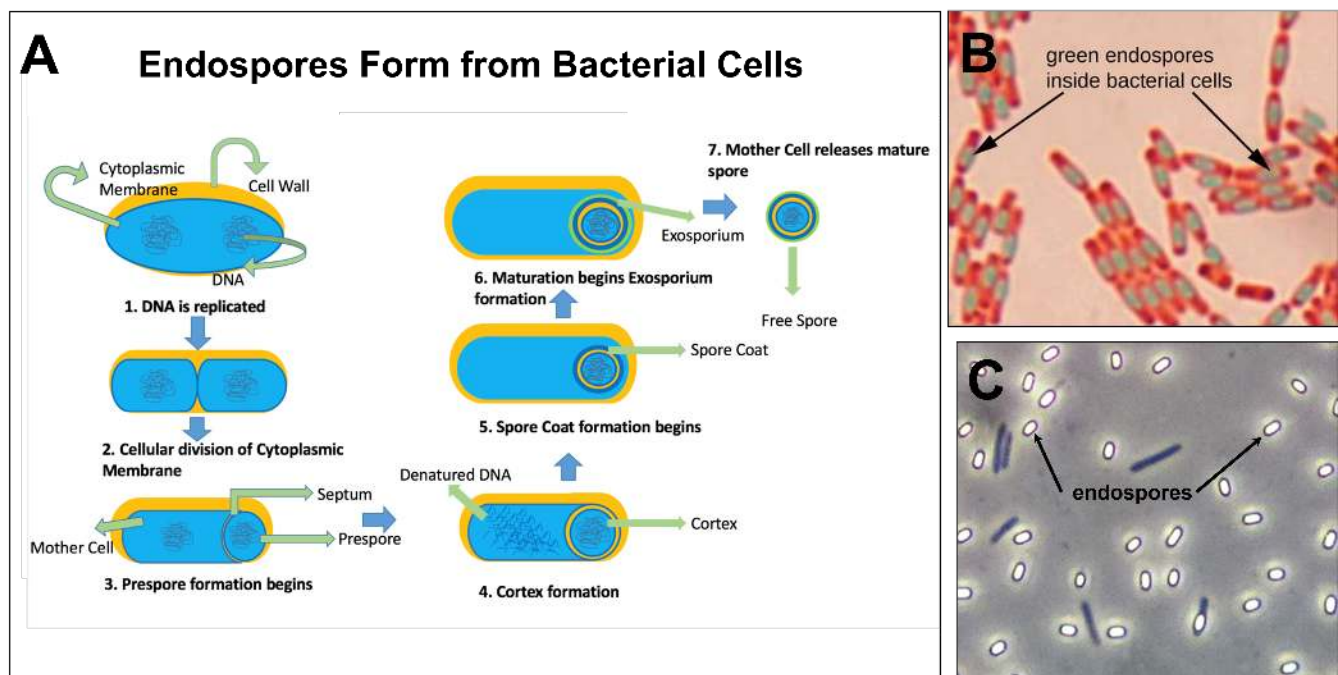


Figure 1: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore 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.

The identification of endospores is very important for the clinical microbiologist who is analyzing a patient's body fluid or tissue since there are not that many spore-forming **genera**. Knowing if the species of bacteria causing an infection forms spores or not helps to narrow down the possible bacterial species causing the infection. In fact, there are two major **pathogenic** (disease-causing) spore-forming genera: *Bacillus* and *Clostridium*. *Bacillus* and *Clostridium* species cause a number of dangerous and lethal diseases such as botulism, gangrene, tetanus, c-diff, and anthrax, to name a few.

Some bacteria have to be put into unfavorable situations (high cell density and starvation are two key triggers) to go into sporulation. Other species will make spores easily without much provocation (e.g. *Bacillus subtilis*). Vegetative cells that have not yet made spores may be in the process of making the spore or will not make them at all. The vegetative cell is metabolically active, whereas the spore is not. The location where an endospore is within a vegetative cell is also useful for distinguishing bacterial species. Endospores may be located in a **terminal** (end of the cell), **subterminal** (near the end of the cell), or **central** (middle of the cell) position. A particular species of the genus will form spores in a specific area, producing another useful taxonomic identification tool and therefore useful in identifying the species of bacteria.

Endospore Stain

The endospore stain is a differential stain that enables visualization of endospores and differentiation of spores from vegetative cells. The primary stain, malachite green, is a relatively weakly binding stain that attaches to the cell wall of vegetative cells and the spore wall of endospores and mature spores. In fact, if washed well with water, the stain comes right out of the cell walls of vegetative cells. In contrast, malachite green will not wash from the spore wall. Once the stain is in the spore wall, it is locked in and will not wash out. This is why there does not need to be a decolorizer in this stain procedure.

It is difficult to stain the tough spores. Heat is used to enable the malachite green to penetrate the low-permeability spore walls. A variety of chemicals make up the spore wall (keratin protein, calcium), but deeper in the wall is peptidoglycan. The keratin forming the outer portion of the endospore wall resists stain. Heating the cells will make the spore wall more permeable to the malachite green so it can then attach to the peptidoglycan. Once in, the malachite green will not come out because the overlying spore wall becomes less permeable when the smear cools.

When the malachite green is washed out of the vegetative cells they become transparent. A counterstain, safranin, is used to stain the vegetative cells pink. The result is endospores that appear green and vegetative cells that appear pink.

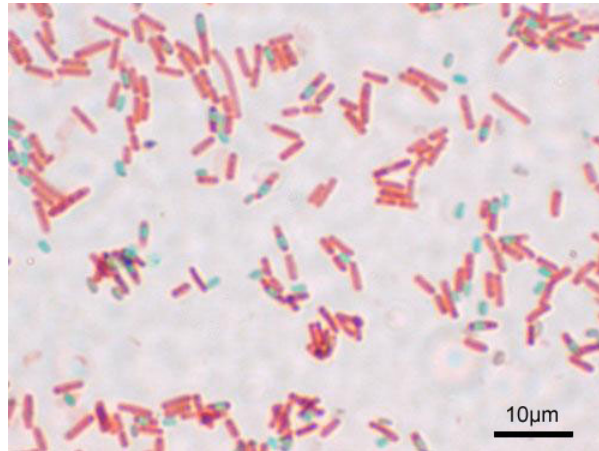


Figure 2: Bacterial cells that have undergone the endospore stain. This species, *Bacillus subtilis*, forms endospores that are visible in green. The vegetative cells are also present and appear pink. This image shows cells magnified by a microscope at 1000x.

Laboratory Instructions

Prepare a Bacterial Smear

Note

You may find it helpful to draw a circle (wax pencil is best) on the opposite side of the slide where you will spread your smear. This will help you later in locating the smear. The wax pencil is better than a marker because it will not wash off easily from the glass.

Note

If you are using a liquid culture, gently mix the culture until you get an even, cloudy mixture (it should look somewhat like skim milk). If you mix too aggressively, you will lose the bacterial morphology.

1. Prepare a bacterial smear on the slide:

- If you are taking bacteria from a solid culture (slant or petri plate), place a small drop (only 1 drop) of saline, deionized (DI) water, or distilled water onto a microscope slide and use a loop to aseptically add bacteria to the water (avoid taking a large chunk of bacteria since the cells will be too dense to view individual cells). Use the loop to spread the bacteria in the water drop and to spread the water drop out to make it thinner (it will dry faster).
- If you are taking a bacteria from a liquid culture (broth), place 1 or 2 loopfuls of bacteria directly onto a microscope slide (no saline or water added).

2. Allow the slide(s) to air dry on the slide warmer (or air dry if a slide warmer is not available).

Heat Fix the Bacterial Smear

Once the liquid has completely evaporated on the surface of the slide, heat fix by passing the slide:

Attach a wooden clip to the microscope slide to hold it.

1. Pass the underside of the microscope slide through a flame three times.
2. Allow the slide to cool and then continue with your staining protocol.

 Note

If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them.

Endospore Stain

1. Put a beaker of water on the hot plate and boil until steam is coming up from the water.
2. Turn the hot plate heat down so that the water is barely boiling.
3. Place a wire stain rack over the beaker. Steam should be coming up through the wire rack.
4. Cut a small piece of paper towel and place it on top of the smear on the slide. The towel will keep the dye from evaporating too quickly, thereby giving more contact time between the dye and the bacterial walls.
5. Flood the smear with the primary dye, malachite green, and leave for 5 minutes. Keep the paper towel moist with the malachite green. DO NOT let the dye dry on the towel.
6. Remove and discard the small paper towel piece.
7. Wash the slide really well with water.
8. Move the slide off the wire rack. The next steps are done without the steam of the hot water.
9. Use a wooden clip and attach it to the slide and put the slide over a small chemical waste bucket.
10. Apply safranin to the smear and leave for 1 minute.
11. Wash the slide well with water allowing the water to run off the slide into the bucket.
12. Blot the dry (do not wipe) with bibulous paper.
13. Place the stained slide on a microscope and examine. Be sure to begin at the lowest power (look for the pink and green colors of the stains). Focus at lowest power and then increase one objective magnification at a time.

CARMEN QUESTIONS - Endospore Stain

1. The species of bacteria you stained is *Bacillus subtilis*. Based on the results of your endospore stain, does *Bacillus subtilis* form endospores? How can you tell?
2. What color are the vegetative cells in the endospore stain?
3. Why might a species of bacteria form endospores? What is the advantage of forming spores for bacteria?
4. Why might infections with species of bacteria that produce spores be more difficult to treat in a healthcare setting than species of bacteria that do not produce spores?

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2.7: Capsule Stain

Learning Objectives

- Describe what bacterial capsules are and where they are found in bacterial cells.
- Tell how the capsule stain works.
- Give at least three ways bacterial capsules benefit bacterial cells.
- Successfully conduct a capsule stain.
- Examine, illustrate, label, and interpret results of a capsule stain.

Bacterial Capsules

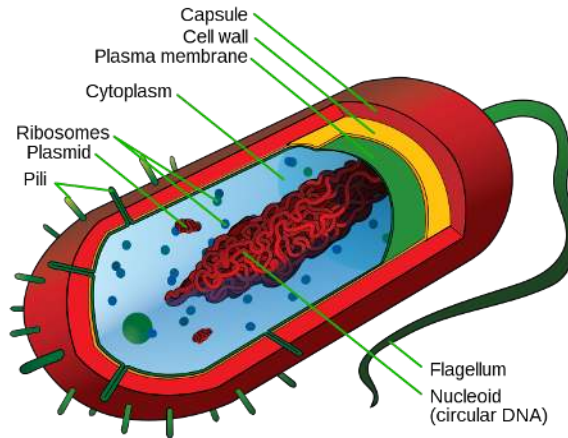


Figure 1: Diagram of a prokaryotic cell shows that capsules are a layer located outside of the cell wall.

Bacterial **capsules** are typically composed of a polysaccharide layer which is thick, detectable, and discrete layer outside the cell wall. Capsules do not stain well for microscopic examination. Because of this, to visualize bacterial capsules, stains/reagents are used in a way that will stain the environment surrounding the bacterial cells and stains the bacterial cell itself, but not the capsule. This creates a non-stained area that is the capsule. Capsules appear as uncolored halos surrounding the bacterial cell.

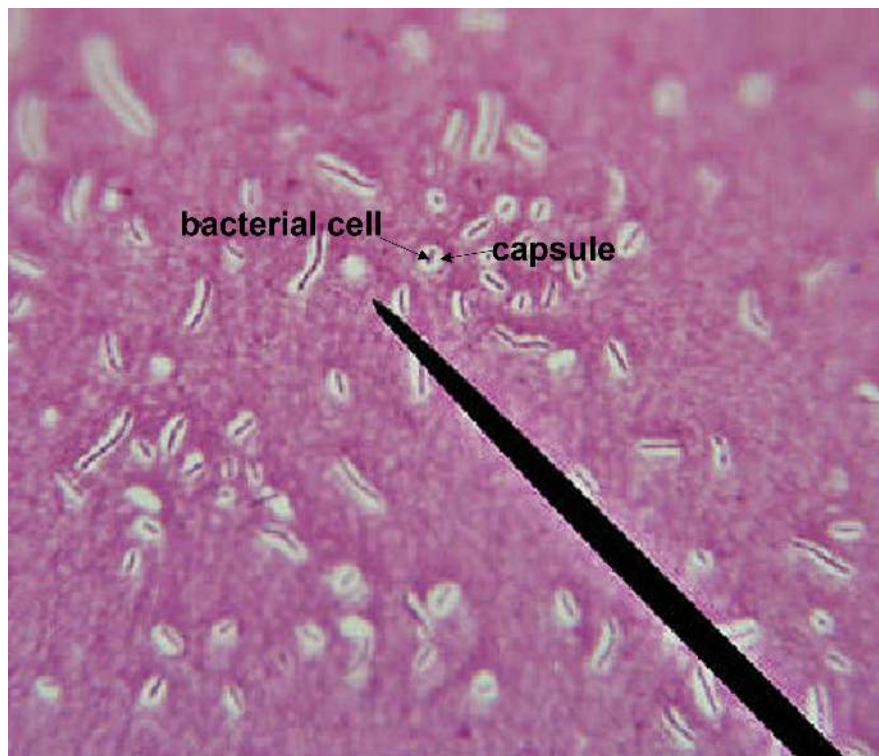


Figure 2: Microscopic image of bacteria stained with the capsule stain. The environment surrounding bacterial cells and the bacterial cells are stained pink/purple. The unstained region that appears white surrounding each bacterial cell is the capsule.

The ability to produce a capsule is coded in the DNA of the bacteria and can therefore be species-dependent or even strain-dependent. The capsule is not an absolutely essential cellular component for bacteria and some species and strains do not form capsules. Capsules are often produced only under specific growth conditions. The thickness of the capsule can vary depending on the bacterial species, its age, and the medium in which the bacterium is growing. Even though not essential for life, capsules can help bacteria to survive.

Capsules protect pathogenic bacteria from the phagocytic action of immune cells and allow pathogens to invade the body by enabling them to avoid being engulfed by white blood cells. If a pathogenic bacterium loses its ability to form capsules, it often ceases to be pathogenic. Some bacterial species of the microbiome also have capsules to protect them from phagocytosis. Environmental bacteria living in soil and water are protected by their capsules from being engulfed by free-living protozoa.

In addition to protection from phagocytosis, the capsule protects cells against desiccation (drying out). Capsules also enhances the ability of cells to attach. For example, pathogenic bacterial cells with capsules aid in attachment to host cells to establish their growth/infection. Further, capsules enable bacterial cells can attach to other bacteria in biofilms. Bacteria that establish the first layer of a biofilm may utilize their capsules to adhere to a surface and enable attachment of other bacterial cells for biofilm development.

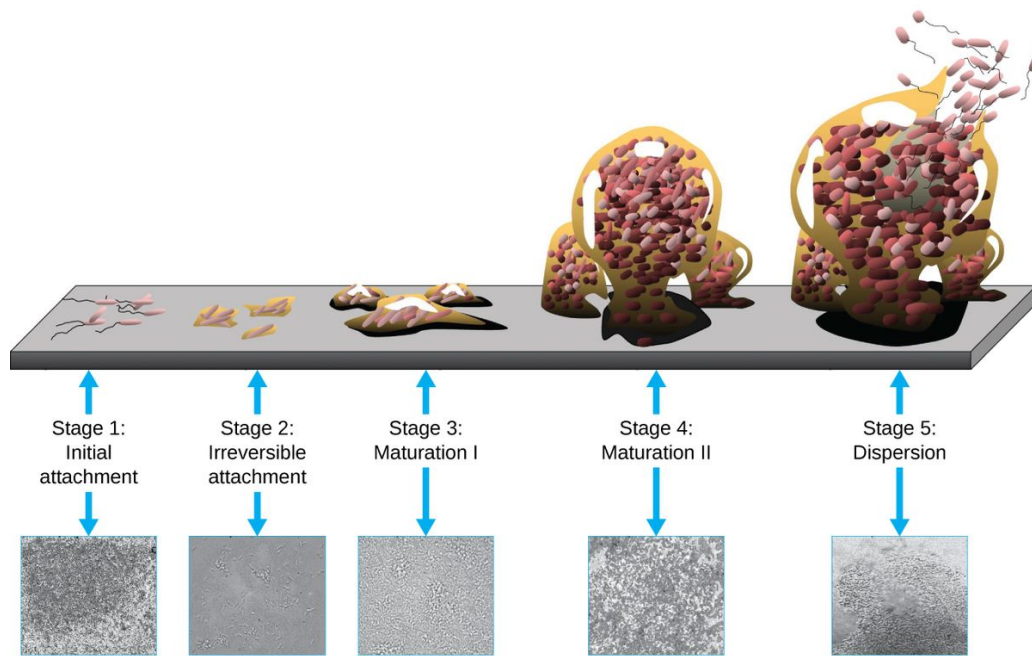


Diagram showing five stages of biofilm development of *Pseudomonas aeruginosa*. All photomicrographs are shown to same scale.

Figure 3: Process of biofilm development. Stage 1: initial attachment of bacteria to a surface. Stage 2: irreversible attachment. Stage 3: maturation I. Stage 4: maturation II. Stage 5: dispersion of cells out of the biofilm.

Laboratory Instructions

Capsule Stain (Anthony's Capsule Stain)

1. **Gently** stir the skim milk broth culture with your loop and place **2-3 loops** of *Enterobacter aerogenes* or *Serratia marcescens* (whichever is available) on a microscope slide.
2. Using your inoculating loop, spread the sample out to cover about one inch of the slide.
3. Let it completely air dry. **Do not heat fix or use the slide warmer.** Capsules stick well to glass, and heat may destroy the capsule.
4. Stain with 1% crystal violet for two minutes (**do not use the same crystal violet solution as the Gram stain**).
5. **Gently** wash off the excess crystal violet stain with 20% copper sulfate solution.
6. Gently shake off the excess copper sulfate solution and let air dry (**do not blot or use slide warmer**).
7. Examine the slide with the microscope. The bacterial cell and the milk dried on the slide will pick up the purple dye while the capsule will remain colorless.

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2.8: Acid-Fast Stain

Learning Objectives

- Describe the difference between acid-fast bacteria and non-acid-fast bacteria.
- Explain how the acid-fast stain works comparing acid-fast and non-acid fast bacteria.
- Identify the genera of bacteria that are acid-fast and two examples of diseases caused by these species.
- Differentiate the cell wall structures of acid-fast and non-acid fast bacteria.
- Successfully execute an acid-fast stain and interpret the results.

Acid Fast Stain

Acid fast stain is a differential stain used to identify acid-fast organisms such as members of the genus *Mycobacterium*. Acid-fast microorganisms are characterized by wax-like, nearly impermeable cell walls; they contain **mycolic acid** and large amounts of fatty acids, waxes, and complex lipids. This type of cell wall is resistant to most compounds, therefore acid-fast microorganisms require a special staining technique.

The ability of the bacteria to resist decolorization with acid-alcohol confers **acid-fastness** to the bacterium. Acid-fast bacteria, of which there are very few---the major genus *Mycobacterium*, have a high concentration of mycolic acid, a lipid, in their cell walls. Although difficult to stain, once the stain goes into the cell wall, the cell will not de-stain or decolorize easily. The ability of the bacteria to resist decolorization with acid-alcohol confers acid-fastness to the bacterium. The phenol in the carbol fuchsin facilitates the dye going into the waxy wall of the bacterium. Acid-fast bacteria stain poorly with the Gram stain procedure, appearing weakly Gram-positive or Gram-variable. They are usually characterized using the acid-fast staining procedure.

Steam is used to get the carbol fuchsin primary dye to go into the cell wall. Once in, it will not come out: But the acid-alcohol decolorizer will take it out of the non-acid fast cell walls since the primary dye does not bind strongly to the cell wall. Non-acid fast bacteria will also take up the carbol fuchsin, but the acid alcohol decolorizer will remove it from wall since the primary dye does not bind strongly to the cell wall.

The primary stain used in acid fast staining, **carbol fuchsin**, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the addition of heat in the form of heat (steam). Steam helps to loosen up the waxy layer and promotes entry of the primary stain inside the cell. The smear is then rinsed with a very strong decolorizer (acid-alcohol), which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counterstain, which in our case is methylene blue.

Acid-fastness is an uncommon characteristic shared by the genera *Mycobacterium* and *Nocardia* (weakly acid-fast). Because of this feature, this stain is extremely helpful in identification in diseases caused by acid-fast bacteria, particularly tuberculosis and leprosy. In addition, the stain is used to determine the presence of acid-fast bacteria from lung tissue in patients undergoing antibiotic therapy.

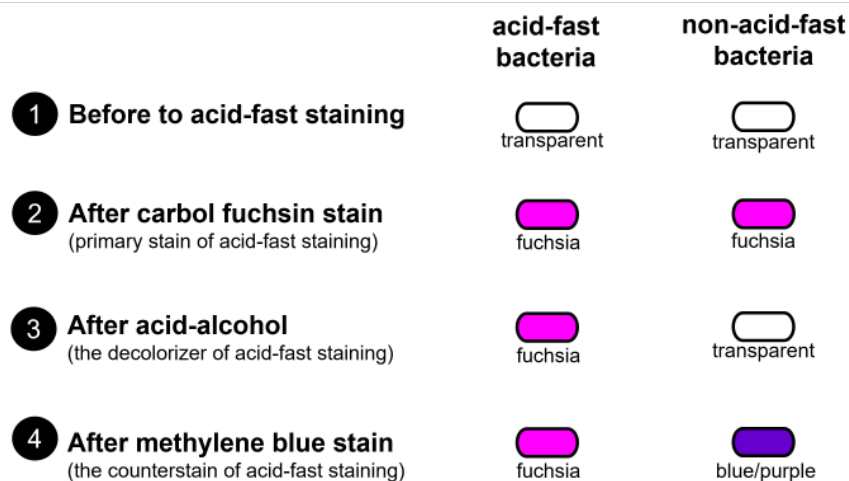


Figure 1: Diagram shows how cells change color during the steps of the acid-fast stain by comparing acid-fast bacteria and non-acid-fast bacteria. Acid-fast bacteria and non-acid-fast bacteria begin transparent. After staining with carbol fuchsin, acid-fast and non-acid-fast bacteria both appear the color fuchsia (a pinky-purple color). After the decolorization step, acid-fast bacteria retains the carbol fuchsin and remains fuchsia and the non-acid fast bacteria loses the color from the carbol fuchsin becoming mainly transparent. Methylene blue counterstain causes non-acid-fast bacteria to stain blue or bluish-purple, whereas acid-fast bacteria remain fuchsia.

Structure and Composition of the Acid-Fast Cell Wall

The mycobacterial cell wall resembles both the Gram-positive and Gram-negative cell walls by having a peptidoglycan layer nearly as thick as Gram-positive cell walls and an outer, waxy layer mimicking the outer membrane of Gram negative cell walls. The cell wall of mycobacteria plays a key role in intrinsic antibiotic resistance and virulence (Forrellad *et al.* 2013; Becker and Sander 2016).

Acid-fast bacteria are Gram-positive, but in addition to peptidoglycan, the outer membrane or envelope of the acid-fast cell wall of contains large amounts of glycolipids, especially mycolic acids that make up approximately 60% of the acid fast cell wall in the genus *Mycobacterium*.

The following is a list of the layers of the acid-fast cell wall beginning with the innermost layer (nearest to the plasma membrane):

- **Layer 1:** Layer of peptidoglycan
- **Layer 2:** Layer of arabinogalactan (a long carbohydrate composed of the sugars arabinose and galactose)
- **Layer 3:** Outer membrane containing mycolic acids
- **Layer 4:** Outer surface is studded with surface proteins that differ with the strain and species of the bacterium

Like the outer membrane of the Gram negative cell wall, porins are required to transport small hydrophilic molecules through the outer membrane of the acid-fast cell wall.

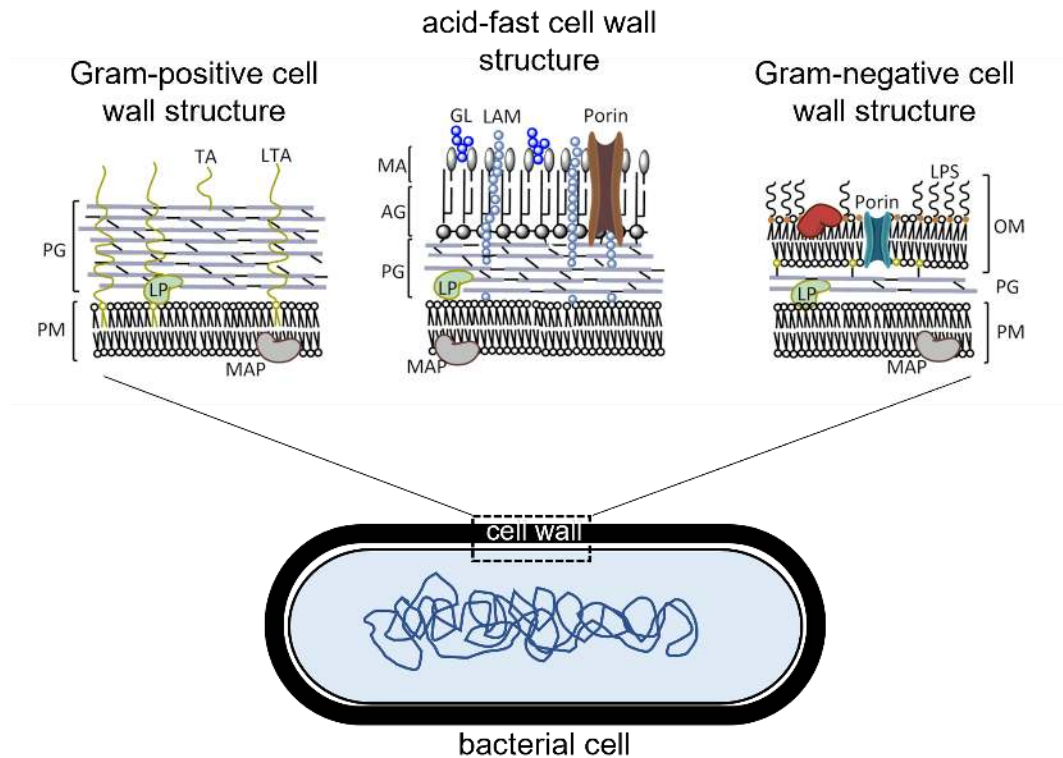


Figure 2: Comparison of the structures of different types of bacterial cell walls. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across the peptidoglycan layer. (Right) Gram-negative cell walls consist of a thin layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM). Gram-negative cell walls have an outer membrane (OM) beyond the peptidoglycan layer containing lipopolysaccharide (LPS) as well as porin channels that enable some materials to pass across the outer membrane. (Center) Acid-fast cell walls have a moderately thick layer of peptidoglycan (PG) outside of the plasma membrane. A layer of arabinogalactan (AG) is outside of the peptidoglycan layer and beyond the AG layer is mycolic acid (MA) containing lipoarabinomannan (LAM) and glycolipid (GL). In all three cell wall types, lipoprotein (LP) is also found.

Lab Instructions

1. Label a slide and draw a circle on the center of the slide with a wax pencil.
2. Prepare an emulsion on the slide with 4 loopfuls of *Staphylococcus epidermidis* from a broth culture onto the slide (these will be your non-acid-fast bacteria).
3. Then, add one loopful of *Mycobacterium chelonae* (these are your acid-fast bacteria) and mix the two bacteria together.
4. Allow the slide(s) to air dry on the slide warmers (while these slides are drying).
5. Once the liquid has completely evaporated, heat fix the bacteria by passing it through your flame three times.
6. Make sure the slide rack on top of your beaker is completely level. Then, bring your water to boil while the slides are drying.
You only need about 200 milliliters of water. If you add more, you will be waiting all lab period for your water to boil.
7. Once the water is boiling, place your slide on the slide rack above the boiling water.
8. Cover the area of your smear on the slide with a square piece of paper towel. Cut the paper towel to make sure none of the paper is hanging off the slide.
9. Carefully apply the carbol fuchsin stain to the paper towel. If a stain appears in the water you are boiling, please stop and discard the stained water in the liquid waste disposal. The fumes from carbol fuchsin can be toxic.
10. Steam with the stain on the slide for 7 minutes while continuously applying more stain so the paper square never dries out.
11. Gently remove the paper with forceps and discard it in the small waste paper cup that will be provided on your bench. Then, rinse the slide with water.
12. Put the slide over the staining basin and gently rinse with water.
13. Decolorize with 6 drops of acid-alcohol (not ethanol from the Gram stain kit), then rinse with water.
14. Counterstain with methylene blue for 2 minutes.

15. Rinse with water and blot dry with bibulous paper (do not use the slide warmer).
16. Examine under the 100x objective lens with oil immersion and record your results.

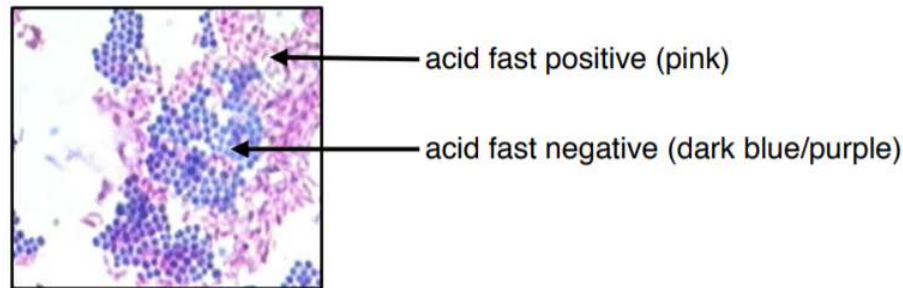


Figure 3: Microscopic image of two different species mixed together with acid fast stain. The acid-fast bacteria appears fuchsia or pinky-purple. The non-acid-fast bacteria appears dark blue or blue-purple. The colors in this image may be slightly off due to printing/copying.

CARMEN QUESTIONS - Acid Fast Stain

1. What color are the acid-fast bacteria?
2. What color are non-acid-fast negative bacteria?
3. Describe the structure of the cell walls of bacteria that appear fuchsia with the acid-fast stain.
4. Describe what you know about the structure of the cell walls of bacteria that appear blue/purple with the acid-fast stain.

Works Cited

- Becker K, Sander P. *Mycobacterium tuberculosis* lipoproteins in virulence and immunity—fighting with a double-edged sword. *FEBS Lett.* 2016;590:3800–19.
- Forrellad MA, Klepp LI, Gioffré A et al. . Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence.* 2013;4:3–66.

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2.9: Introduction to Micropipetting

When scientists need to transfer small volumes of liquid, they use a piece of equipment known as a micropipettor (or pipettor). There are a few different types of micropipettors based on the minimum and maximum volumes of liquid they are designed to transfer. Today you will learn how to use a micropipettor to a) transfer 1 ml (1000 μ l) volumes of liquid in the preparation of serial dilutions, and 2) transfer 0.1 ml (100 μ l) of these dilutions onto agar plates.

A. How to set the micropipettor for the appropriate volume:

The picture of the micropipettor shown below is similar to the one you will be using. As you can see, this micropipettor is designed to transfer volumes between 100 μ l -1000 μ l (this type of micropipettor is sometimes called a P1000). The display window reads “1000” when the pipettor is set to its maximum volume (1000 μ l or 1 ml). If we were using this same pipettor to transfer 500 μ l (or 0.5 ml) the window would read 500.

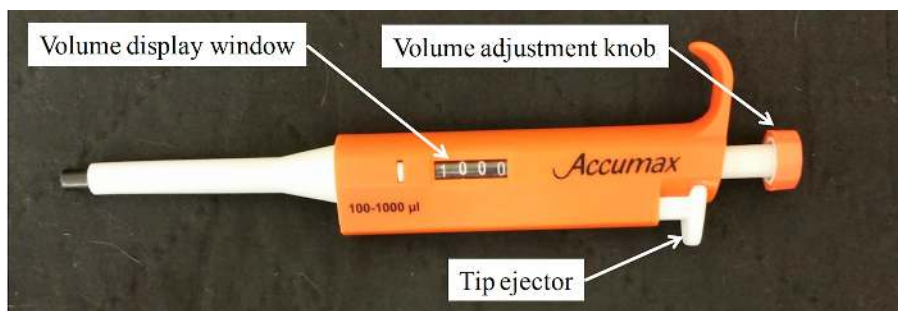


Figure 1: P1000 micropipettor

Similarly, the pipettor shown below (a P200) is designed to transfer volumes between 20- 200 μ l (0.02- 0.2 ml) It is set at 50 μ l —to pipet 100 μ l you would set the display to read ‘100’.



Figure 2: P200 micropipettor

The volume is set by turning the knob at the top of the pipettor until the correct volume is reached.

Note

Never try to turn the knob above the minimum or maximum volumes or you may damage the micropipettor!

B. How to attach a pipet tip:

After setting the appropriate volume, you will be attaching a pipet tip to the narrow end of the micropipettor. The P1000 uses blue tips; P200's use clear or yellow tips. The tips have been sterilized in the autoclave so as not to introduce any contaminating microbes into your sample. To pick up a tip:

1. Open the box of tips and insert the end of the pipettor into the end of a pipet tip.
2. Press down gently to ensure that the tip stays attached to the micropipettor.
3. Close the box to prevent the other tips from getting contaminated.

C. To transfer liquid using the pipettor:

 Note

Follow these steps very carefully!

1. With your thumb, depress the plunger on the top of the pipettor until you first feel resistance.

 Note

It is important not to go past the first stop. If you do, you will pick up too much liquid!

2. While holding the plunger in the depressed position, place the pipet tip into the liquid you will be transferring.

 Note

If you depress the plunger after placing the tip in the liquid you will be blowing air into the liquid!

3. Slowly release the plunger to allow the tip to pick up the liquid.

 Note

Be sure to keep the tip in the liquid the entire time so that you don't get air bubbles.

4. Place the tip into the tube (or plate) where you will be transferring the liquid to.
5. Slowly press the plunger down again until all the liquid leaves the pipet tip.
6. Withdraw the tip from the tube before releasing your thumb from the plunger.
7. Use the tip ejector to discard the tip in the container as instructed by your professor.

 Note

Pipet tips NEVER get disposed of in the regular garbage.

Other important things to remember:

1. It is important not to let the pipet tip touch anything other than the liquid you are transferring.
2. Always keep the pipettor in the upright position when in use. If you tilt it too much the liquid in the tip will make contact with the pipettor and will no longer be sterile (this could also damage the pipettor).

Practice:

Set your P200 pipettor to 100 μ l and practice pipetting by transferring the liquid onto a piece of parafilm. Let your lab partners try as well. All droplets should be the same size (and the same size as your instructor's). Try transferring different volumes as instructed until you feel comfortable using the pipettor.

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CHAPTER OVERVIEW

3: Lab 3

[3.1: Required Materials](#)

[3.2: Bacterial Colony Morphology](#)

[3.3: Determination of Bacterial Numbers](#)

[3.4: Unknown Bacteria Identification Project](#)

[3.5: Unknown Bacteria Identification Project Report](#)

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3.1: Required Materials

Cultures Needed

- TSA plates of
 - *Pseudomonas*
 - *Micrococcus luteus*
 - *B. subtilis*
 - *Streptomyces*
 - *Streptococcus*
- TSB cultures of
 - *E. coli*
- Mixed 1% saline cultures of unknowns

Bacterial Colony Morphology

- Plates from previous labs

Determination of Bacterial Numbers

- Spectrophotometer
- Microcentrifuge Tubes
- Sterile water
- Microcentrifuge tubes
- TSA plates

Unknown Bacteria

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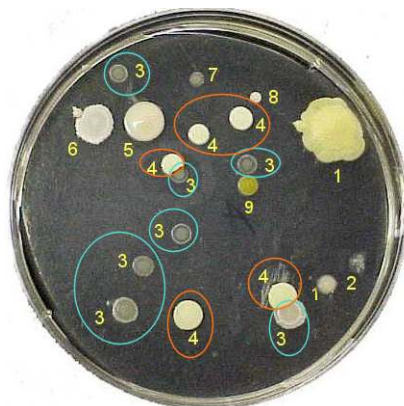
3.2: Bacterial Colony Morphology

Objectives

- Describe features of colonies.
- See variations in colonial morphology among various species of bacteria.

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell, therefore a colony constitutes a clone of bacteria all genetically alike.

In the identification of bacteria and fungi much weight is placed on how the organism grows in or on media. This exercise will help you identify the cultural characteristics of a bacterium on an agar plate - called **colony morphology**. Although one might not necessarily see the importance of colonial morphology at first, it really can be important when identifying the bacterium. Features of the colonies may help to pinpoint the identity of the bacterium. Different species of bacteria can produce very different colonies.



In the above picture of a mixed culture, an agar plate that has been exposed to the air and many different colony morphologies can be identified. Nine obviously different colonies are numbered: some colony types recur in various areas of the plate (note # 3 and # 4). Not only are pigment differences seen, but also size, edge, pattern, opacity, and shine. Two circles have been drawn around merging colonies, where the species of the 2 colonies are different. Trying to pick a bit of one of those adjacent colonies increases the chances of picking up another mixed culture, consisting of the 2 species that were merged together. ALWAYS pick a well-isolated colony when subculturing.

WHOLE SHAPE OF COLONY

Varies from round to irregular to filamentous and rhizoid (root-like)

SIZE OF COLONY

Can vary from large colonies to tiny colonies less than 1 mm = punctiform (pin-point). **Measure with a millimeter rule.**

EDGE/MARGIN OF COLONY

Magnified edge shape (use a dissecting microscope to see the margin edge well)

CHROMOGENESIS

- Color of colonies, pigmentation: white, buff, red, purple, etc.
- Some pigments are water-soluble, others are not.
- If you take a large inoculum and place it in a tube of water or saline, do you see color?
- Do you see any pigment if the organism is growing in a broth medium?
- Does incubation temperature affect the color?
- Does the entire colony have the color, or is it more like a bull's eye?

OPACITY OF COLONY

Is the colony transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision—like looking through frosted glass), iridescent (changing colors in reflected light)?

ELEVATION OF COLONY

How much does the colony rise above the agar (turn the plate on end to determine height)?

SURFACE OF COLONY

Smooth, glistening, rough, dull (opposite of glistening), rugose (wrinkled)

CONSISTENCY or TEXTURE

Butyrous (buttery), viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like)

MATERIALS NEEDED

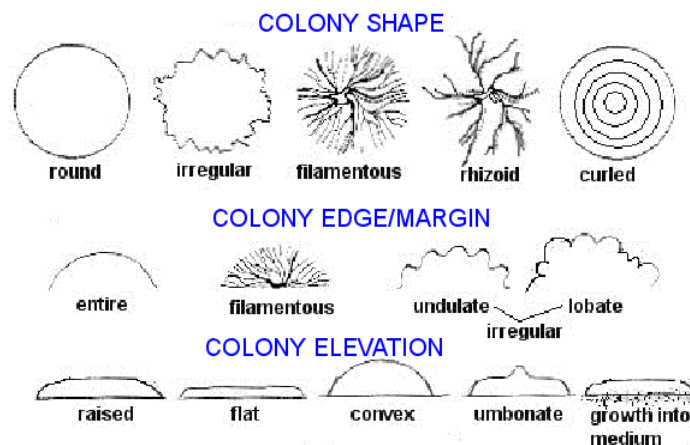
- agar plates of various bacteria (examples = *Pseudomonas*, *Chromobacterium*, *Micrococcus*, *Bacillus*, *Streptomyces*, *Streptococcus*, and *Neisseria*)

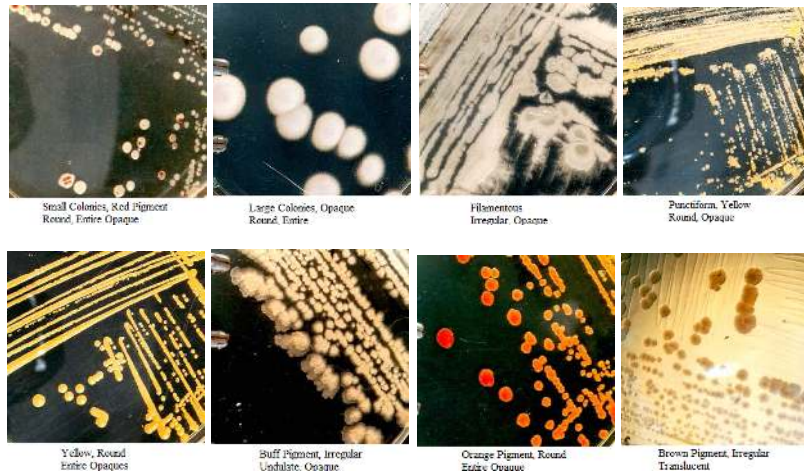
THE PROCEDURE

1. Use a plate which has well-isolated colonies. Look at the largest colonies with the naked eye to determine general shape and chromogenesis.
2. Use a dissecting/stereoscopic microscope for more detail. Place the plate **RIGHTSIDE UP** on the stage, leaving the petri dish cover **ON** (Otherwise, your culture will become contaminated.) There are 2 lenses on our scopes—10X and 20X - the black lens knob is on the right side of the head of the microscope. The magnification is especially helpful for the study of elevation, surface, opacity, size, and edge. There are 2 lights on these microscopes that you might find helpful, either using one at a time, or both, or even sometimes without them. Two small black rotating knobs on either side of the base control the 2 lights, one light from above and one light from below the stage.
3. Or you may want to use the Quebec colony counter since it has a magnifying glass, and a light behind the plate stage. Make sure that the dish is right-side up.
4. If you see water condensation on the lid cover, take a KimWipe and carefully remove the water from the cover, then quickly replacing the cover on the dish.
5. In order to determine **CONSISTENCY**, you need to use your inoculating loop or needle to pick up the colony and determine the consistency of the inoculum material as the loop leaves the agar medium.

CARMEN QUESTIONS - Colony Morphology

1. List the name of the specimens examined and characterize colony.





Images of Bacterial Colonies

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3.3: Determination of Bacterial Numbers

Learning Objectives

- Give at least three real-world examples why determining bacterial numbers is an important technique.
- Explain how the standard plate count approach works.
- Calculate CFU of an original sample.
- Explain how absorbance can be used as a measure of sample turbidity and cell numbers.
- Tell what a spectrophotometer is and how it works.
- Compare and contrast the standard plate count approach and absorbance measurements as it relates to determining cell density in a sample and the type of information these techniques provide about the cells.
- Describe why a standard line correlating CFU and absorbance is important and how it can be used.
- Use a standard line as well as a standard line equation to determine CFU of a sample from absorbance readings.
- Successfully conduct plate counts and absorbance readings.
- Graph CFU vs. absorbance and create a standard line.
- Calculate CFU for samples and compare with safety standards to make conclusions about the safety of the samples.

What is the Determination of Bacterial Numbers Approach and What is it Used for?



Figure 1: Professionals in government and industry measure the concentration of bacteria to maintain human safety and environmental health including: wastewater treatment (top left), food processing and food safety (top right), water recreation in oceans, lakes, ponds, rivers, and streams (bottom left), safety in swimming pools (bottom center), and safety of drinking water (bottom right). If treated wastewater still has too many bacteria, the water will undergo further treatment prior to being released into the environment. Foods that have unsafe levels of bacteria are disposed of and protocols enacted to fix the bacterial overgrowth in the facility. Natural waters that have unsafe levels of bacteria may be closed to recreation until they are safe again. Swimming pools may be closed if unsafe. Boil advisories are issued if drinking water has unsafe numbers of bacteria. This means that water used for drinking, cooking, and bathing must be boiled first to kill bacteria. These measures help to make sure that humans will not get sick and that the environment is kept safe.

Many microbiological studies require determining the concentration of viable bacterial cells within a sample. In addition to microbiological research, the concentration of viable bacterial cells is also important to examine to:

- make sure water is safe to drink

- make sure treated municipal wastewater is safe to release into the environment
- make sure water is safe to swim in (ocean, lakes, ponds, pools etc.)
- make sure food products are safe

Professionals in government and industry frequently test the safety of water and foods using the same type of approach described below to ascertain bacterial concentrations.

How does the Determination of Bacterial Numbers Approach Work?

The determination of bacterial numbers approach is used to answer one main question: what is the concentration of viable bacterial cells in this sample? This approach utilizes petri plates to conduct a standard plate count. The plate count will make it possible to determine cell concentrations, but it will take about 24 hours before we have those results.

Pairing the standard plate count technique with a faster method for estimating cell concentrations, absorbance measurements, can provide accurate cell concentrations within a couple minutes (rather than waiting 24 hours for cultures to grow). This involves creating a standard curve using plate count results and absorbance readings. Once a standard curve is produced, the number of bacteria in a sample can be determined using only absorbance readings and the standard curve (without the need to grow petri plate cultures for 24 hours).

Plate Count

Microbiologists use a technique called the **standard plate count** to estimate the population density of bacteria in a sample by plating a small and dilute portion of the sample and counting the number of bacteria colonies.

Note

A single bacterial colony originates from a single bacterial cell. When a single cell is deposited on a petri plate, it divides to form a colony that is visible with the naked eye. Therefore, when we count then number of isolated bacterial colonies on a petri plate, we are counting the number of cells present in that sample.

We use serial dilutions to create decreasing concentrations of the original sample that are then plated so that a plate will be created with a low enough number of bacteria that we can count individual colonies. From that number, we can calculate the original cell density in the broth. A turbid broth culture of bacteria can have millions or billions of bacterial cells in a single milliliter! If we transferred bacteria directly from that tube to a petri plate, it would be impossible to count colonies since there would be an overwhelming amount of growth on the petri plate (often called a **lawn** of bacteria).

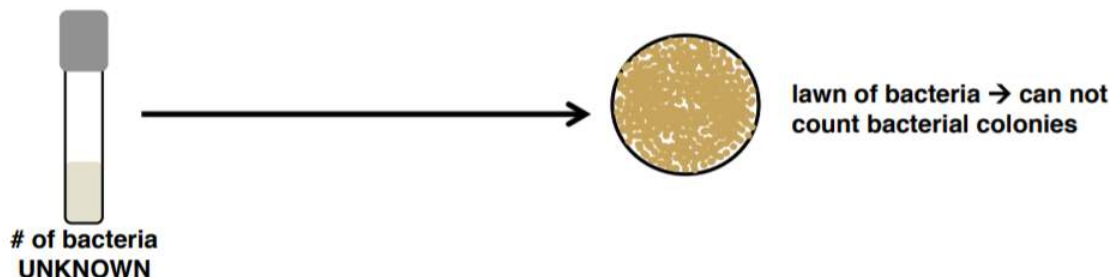


Figure 2: A bacterial culture can contain millions or billions of cells per milliliter! If even a small amount of that culture is plated directly onto a petri plate undiluted, it will form a "lawn" of bacteria where the colonies cannot be distinguished from each other since there are just so many of them growing together. This is why the standard plate count technique dilutes samples and conducts multiple dilutions to make sure that a countable number of bacterial colonies (25-250 colonies) is obtained on at least one of the petri plates.

Since we do not know how many bacteria are in a given sample or culture, we do not know how much we need to dilute the culture in order to grow a petri plate where the number of bacterial colonies is "countable" (we count a plate that has between 25-250 bacterial colonies). Therefore, we do a dilution series to create a number of plates, with a range of dilutions, in the hopes we will produce a countable plate. The number of colonies we count on a petri plate enables us to calculate the **CFU** or **colony forming units**. CFU is a measure of the concentration of the live, viable bacterial cells capable of reproducing when grown on a petri plate in cells per milliliter (cells/mL).

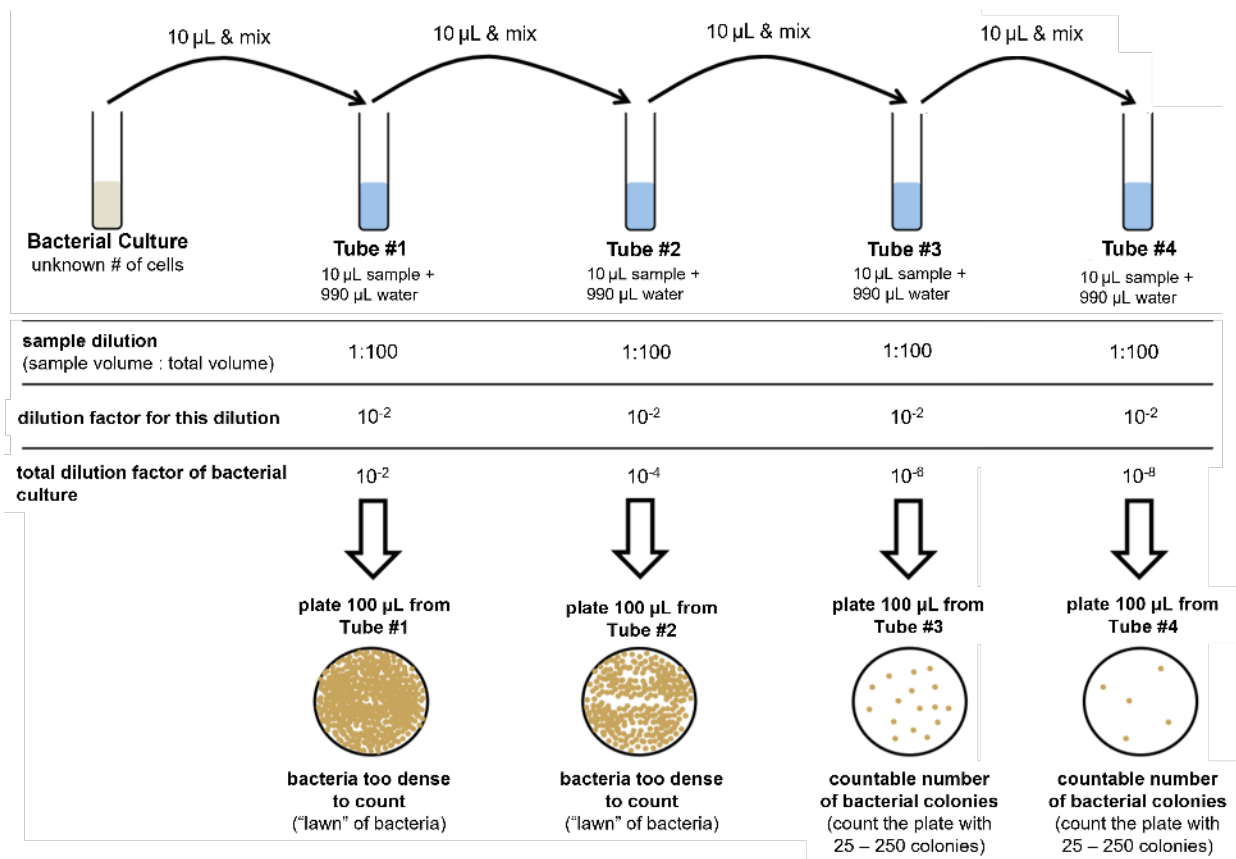


Figure 3: (Top) The process of diluting a bacterial culture in a dilution series is shown. In this process, 10 µL of the culture is diluted in 990 µL of water and mixed. This is a 1:100 dilution, or 10^{-2} dilution. A sample from this first dilution is plated onto a petri plate. The first dilution is further diluted and plated three more times in a similar manner. From the 1:100 dilution, 10 µL is removed and put into another 990 µL of water and mixed. This process is repeated to produce a total of four dilutions and four petri plates. After incubation of the petri plates, at least one plate should have countable colonies (between 25 and 250 colonies). From the counted colonies, the CFU can be calculated.

To Calculate CFU (cells/mL)

Step 1: Determine the concentration of cells in the diluted sample:

$$(\text{\# of colonies counted on the petri plate}) \div (\text{amount of diluted sample added to the petri plate in mL}) = \text{CFU in diluted sample (cells/mL)}$$

Note: 100 µL = 0.1 mL; 200 µL = 0.2 mL

Step 2: Determine the concentration of cells in the original sample:

$$(\text{CFU in diluted sample}) \div (\text{dilution of the petri plate counted}) = \text{CFU in original sample (cells/mL)}$$

✓ Example of CFU calculation

You conduct a standard plate count where 200 µL of each dilution is added to each petri plate. The petri plates diluted to 10^{-2} and 10^{-4} are lawns and cannot be counted. The 10^{-6} diluted petri plate results in 129 colonies. The 10^{-8} diluted petri plate has 12 colonies on it (so it is not used for the CFU calculation since it is not within 25-250 colonies). Calculate the CFU in cells/mL of the original sample based on these results.

Solution

- # of colonies counted = 129
- amount of diluted sample added to the petri plate in mL = 200 µL = 0.2 mL
- dilution of the petri plate counted = 10^{-6}

Step 1: Determine the concentration of cells in the diluted sample:

$$(\text{\# of colonies counted on the petri plate}) \div (\text{amount of diluted sample added to the petri plate in mL}) = \text{CFU in diluted sample (cells/mL)}$$

$$(129 \text{ colonies}) \div (0.2 \text{ mL diluted sample added to petri plate}) = 645 \text{ cells/mL in the diluted sample}$$

Step 2: Determine the concentration of cells in the original sample:

$$(\text{CFU in diluted sample}) \div (\text{dilution of the petri plate counted}) = \text{CFU in original sample (cells/mL)}$$

$$(645 \text{ cells/mL in diluted sample}) \div (10^{-6} \text{ dilution of the petri plate counted}) = 645,000,000 \text{ cells/mL in original sample}$$

$$\text{CFU} = 645,000,000 \text{ cells/mL in original sample}$$

? Exercise 15.1

You conduct a standard plate count where 100 μL of each dilution is added to each petri plate. The petri plates diluted to 10^{-2} and 10^{-4} and 10^{-6} are lawns and cannot be counted. The 10^{-8} diluted petri plate results in 211 colonies. Calculate the CFU in cells/mL of the original sample based on these results.

[Answer](#)

? Exercise 15.2

You conduct a standard plate count where 50 μL of each dilution is added to each petri plate. The petri plates diluted to 10^{-2} is a lawn and cannot be counted. The 10^{-4} diluted petri plate results in 162 colonies. The 10^{-6} and 10^{-8} diluted petri plates both had less than 25 colonies on it (so it is not used for the CFU calculation since it is not within 25-250 colonies). Calculate the CFU in cells/mL of the original sample based on these results.

[Answer](#)

? Exercise 15.3

You conduct a standard plate count where 250 μL of each dilution is added to each petri plate. The petri plates diluted to 10^{-2} and 10^{-4} are lawns and cannot be counted. The 10^{-6} diluted petri plate results in 89 colonies. The 10^{-8} diluted petri plate has 3 colonies on it (so it is not used for the CFU calculation since it is not within 25-250 colonies). Calculate the CFU in cells/mL of the original sample based on these results.

[Answer](#)

Absorbance Readings

Cells can absorb, transmit, and/or reflect light. **Spectrophotometry** is a technique where the amount of light a substance absorbs, or the amount of light that transmits through the sample, is measured.

A **spectrophotometer** is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution.

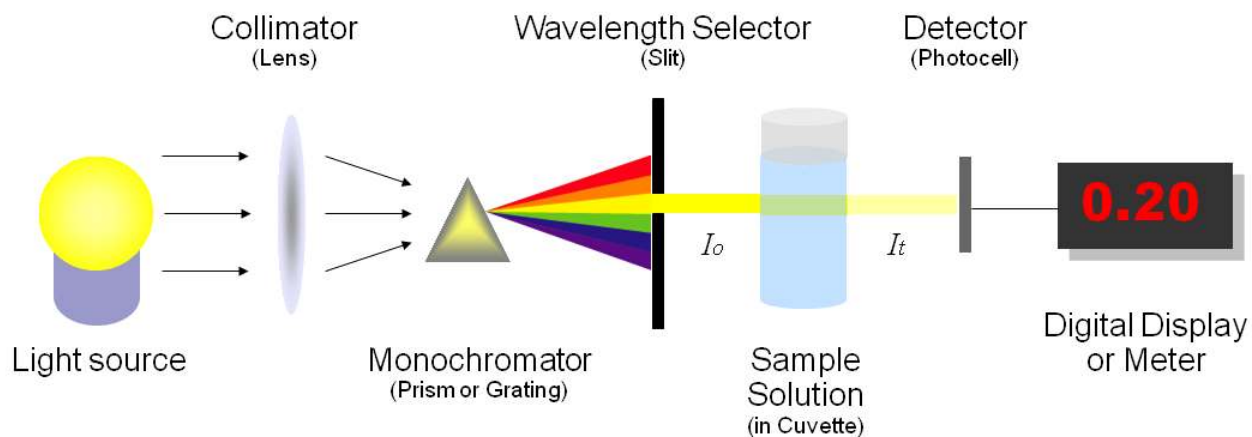


Figure 4: The diagram shows the components of a spectrophotometer. (Left) Light is emitted from a light source. (Second from the left) The wavelength of light is narrowed to a specific wavelength of light (accomplished by the monochromator) that is selected by the person using the spectrophotometer. (Second from the right) The light is passed through a sample in a container called a cuvette. The amount of light that reaches the other side of the sample is measured by a detector. (Right) The spectrophotometer displays the absorbance of light that occurred by the sample. The higher the absorbance, the more light did not pass through the sample. Therefore, the more cells in a sample, the higher the absorbance reading and the less light passing through the sample. A spectrophotometer can also be set to measure transmittance, which is the opposite of absorbance. Transmittance tells the person using a spectrophotometer how much light *did* pass through the sample. Therefore, a sample with a lot of cells would have a lower transmittance reading than a sample with fewer cells.

Spectrophotometric (turbidimetric) analysis can be used to determine the quantity of cells in a sample based on the amount of light that is absorbed by the sample. It measures how turbid (cloudy) the solution is. When photons of light hit a cell, the light is absorbed, reflected, or diverted in a different direction. Therefore, the amount of light passing through a sample can be used to measure cell density in a sample.

Although using spectrophotometry to measure cell density in a sample can be quite effective, it will yield results that can be different from plate counts. For example, the standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead **and** alive.

Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, the amount of transmitted light decreases as the cell population increases. The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading, called **absorbance or optical density**, indirectly reflects the number of bacteria. This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of 10^7 cells/mL or greater. Samples can be diluted to obtain accurate results when samples contain more than 10^7 cells/mL or more.

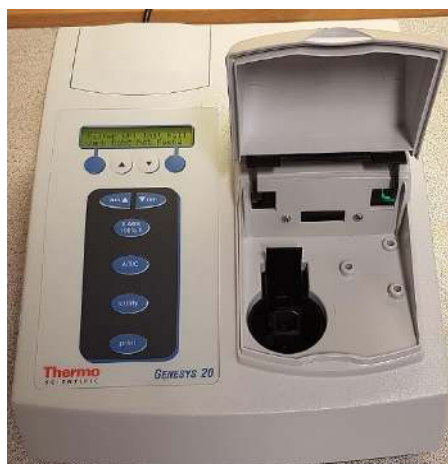


Figure 5: This image shows one type of spectrophotometer. The dark circle toward the right is where a sample is placed inside of the spectrophotometer. The lid is closed to allow light to pass through the sample and collect an absorbance or transmittance reading. The person using the spectrophotometer can select settings using the buttons on the left.

Putting it Together: Using Absorbance as a Quick Way to Determine Cell Concentrations

The CFU concentration determined in the standard plate count can be correlated with absorbance readings. This is done by setting up a graph with absorbance readings of different dilutions on one axis and cell concentrations of that culture at those dilutions on the other axis. Then the CFU is calculated different dilutions and correlated with the corresponding absorbance readings at those same dilutions. These data points are plotted on a graph and a line is drawn the best fits these data. This line, called a trend line or standard line, can be used to determine the cell concentrations of samples with unknown cell concentrations using absorbance only. This enables a microbiologist to quickly determine cell concentrations within a couple minutes rather than taking the time and materials to conduct plate counts for every sample.

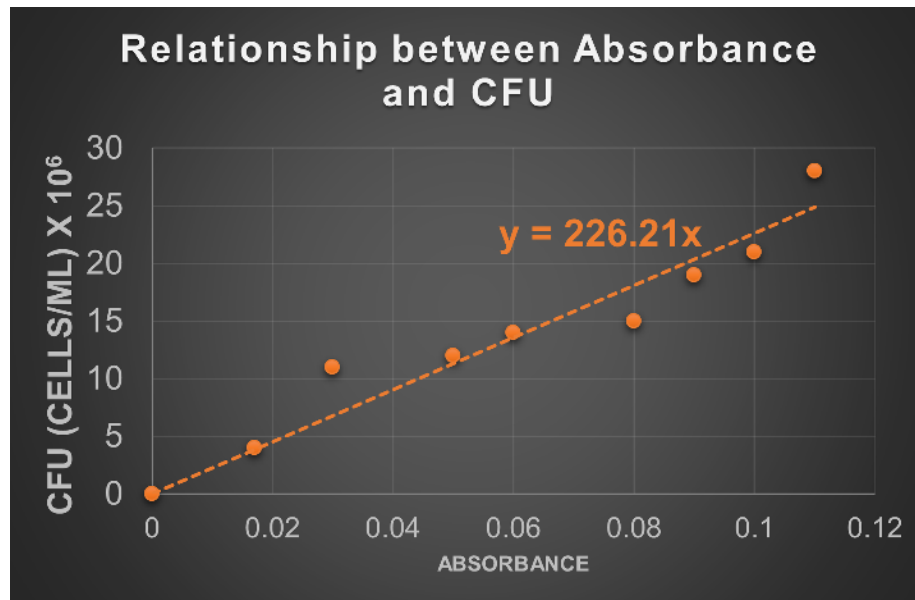


Figure 6: Absorbance data for a sample that has undergone serial dilutions is plotted against the CFU (based on standard plate count data) calculated at those dilutions. The result is a series of data points that can be used to plot a trend line. That trend line may be used to determine the CFU of samples without completing the standard plate count.

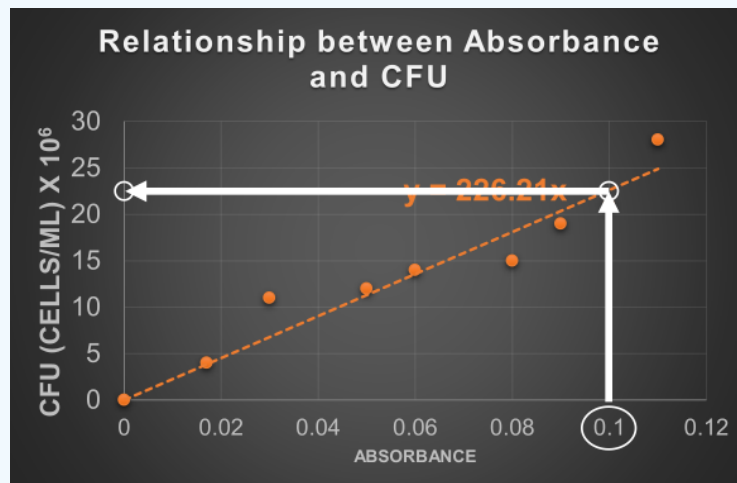
✓ Example of Using Position of Standard Line to Determine CFU of a Sample

You have a liquid sample containing the same species of bacteria used to make the standard line in the graph above. You want to find out the cell concentration of the sample in CFU without conducting a standard plate count because you need an answer right away (and not in 24-48 hours). You read the absorbance of the sample in the spectrophotometer. The absorbance reading is 0.1. Use the standard line above to determine the CFU.

Answer

The CFU at 0.1 absorbance is approximately 22.5×10^6 cells/mL **or** 2.3×10^7 cells/mL

Solution



1. Find the number corresponding to the absorbance on the graph's axis (in this case 0.1 on the x-axis).
2. On the graph, draw a straight line along the absorbance reading on the axis until it reaches the standard line. In this case, draw straight up from 0.1 until it hits the dotted standard line.
3. On the graph, draw a straight line from the point identified in step 2. to the other axis (in this case away toward the y-axis).
4. Where you intersect the other axis, determine the value of this point on the graph. This will be the approximate CFU at this absorbance. At 0.1 absorbance, the standard line is about half way between 25 and 20 (22.5 is halfway between 25 and 20), but the unit given on the graph indicates these numbers should be multiplied by 10^6 . This is how 22.5×10^6 cells/mL was determined as the CFU at this absorbance. We may adjust how we present the number to 2.3×10^7 cells/mL.

? Exercise 15.4

You have a liquid sample containing the same species of bacteria used to make the standard line in **Figure 6**. The absorbance reading of the sample is 0.04. Determine the cell concentration of the sample in CFU without conducting a standard plate count.

Answer

An alternate way, and more accurate way, to determine the CFU from a standard line is to use the equation for the standard line. The equation for the standard line on the graph above is $y = 226.21x$. The way this graph is set up is with CFU on the y-axis, so the y in this equation is equal to CFU. The way this graph is set up is with absorbance on the x-axis, so the x in this equation is equal to absorbance. If we have an absorbance value, we just plug that number into the location where the x is and calculate what y is. y will be the CFU (but in this case because of the label on the y-axis, the answer will be $\times 10^6$)

✓ Example of Using Standard Line Equation to Determine CFU of a Sample

The absorbance of a sample is measured at 0.2. Use the equation from the standard line created from data in **Figure 6**.

Solution

- **Equation for standard line:** $y = 226.21x$
- **The absorbance (which is x):** 0.2
- **Set up the equation:** $y = (226.21) \times (0.2)$
- **Calculate y:** 45.242
- **Recall that the graph axis indicates y is $\times 10^6$, so the final answer is:** 45.242×10^6 cells/mL or 4.5×10^7 cells/mL

? Exercise 15.5

The absorbance of a sample is measured at 0.31. Use the equation from the standard line created from data in **Figure 6**.

Answer

? Exercise 15.6

The absorbance of a sample is measured at 0.18. Use the equation from the standard line created from data in **Figure 6**.

Answer

Laboratory Instructions

Plate Counts of a Bacterial Culture for Determination of Bacterial Numbers

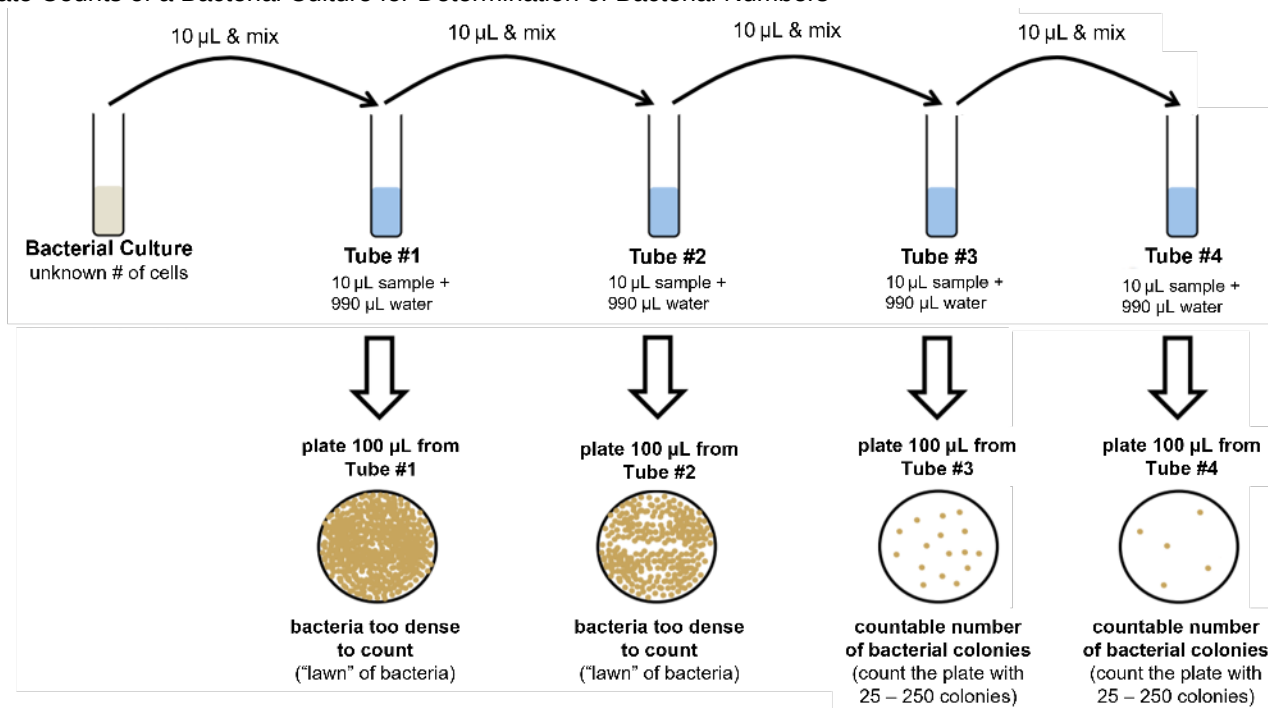


Figure 7: Summary of the serial dilution/dilution series process (top row) and how those dilutions are used to produce multiple petri plates for the standard plate count (center and bottom rows).

Dilution Series

1. Obtain 4 microcentrifuge tubes and label them #1-4.
2. Aseptically add 990 µL of sterile water to the tubes labeled #1-4.
3. Mix the broth culture, then aseptically add 10 µL of the culture to tube #1 and vortex.
4. Change the pipette tip. Aseptically transfer 10 µL from tube #1 to tube #2, close the lid and vortex.
5. Change the pipette tip. Aseptically transfer 10 µL from tube #2 to tube #3, close the lid and vortex.
6. Change the pipette tip. Aseptically transfer 10 µL from tube #3 to tube #4, close the lid and vortex.

Plate Culture Dilutions

1. Obtain 4 TSA plates and label them with the dilution of the sample you will add to the plate (10^{-2} , 10^{-4} , 10^{-6} , and 10^{-8}) as well as your group name/number.
2. Add 100 µL from tube #1 onto the center of the petri plate labeled 10^{-2} .
3. Change the pipette tip. Add 100 µL from tube #2 onto the center of the petri plate labeled 10^{-4} .

4. Change the pipette tip. Add 100 μL from tube #3 onto the center of the petri plate labeled 10^{-6} .
5. Change the pipette tip. Add 100 μL from tube #4 onto the center of the petri plate labeled 10^{-8} .
6. Put a small amount of ethanol in one half of an empty petri plate (not one of the ones you labeled).
7. Dip the spreader tool in the ethanol.
8. Wave the spreader through the flame of a Bunsen burner. You should see the ethanol burning off the spreader.
9. Pause a moment to allow the spreader tool to cool down.
10. Partially open the petri plate labeled 10^{-2} and touch the spreader to the agar in a location away from the 100 μL you added to the plate. This will help to cool the spreader and avoid frying the bacteria with the hot spreader.
11. Once cooled, use the spreader to evenly spread the 100 μL you added to the plate all over the surface of the plate. Allow the spreader to gently skid along the surface of the medium. Rotate the petri plate to make it easier to spread the liquid evenly over the surface.
12. Close the petri plate and set aside to dry.
13. Repeat steps 7-12 for each of the petri plates.
14. Put the spreader back in the ethanol to kill any bacteria on it.
15. Allow the petri plates to dry for 5-10 minutes.
16. Invert the petri plates and incubate at 37°C for 24 hours.
17. Count the colonies where appropriate (only between 25 and 250) and record results in the table below.
18. Calculate CFU for the original culture using the colony count that falls between 25 and 250.

Absorbance of a Bacterial Culture for Determination of Bacterial Numbers

Dilution Series

1. Place the original (non-diluted) culture in a test tube rack with four test tubes containing 5 mL of sterile TSB. Label the four test tubes of sterile media as "1/2," "1/4," "1/8," and "1/16."
2. Transfer 5 mL of the *original* (non-diluted culture), to the tube labeled 1/2. Thoroughly mix.
3. Transfer 5 mL from the 1/2 tube to the tube labeled 1/4. Thoroughly mix.
4. Transfer 5 mL from the 1/4 tube to the tube labeled 1/8. Thoroughly mix.
5. Transfer 5 mL from the 1/8 tube to the tube labeled 1/16. Thoroughly mix.
6. Record your values, along with the dilutions that they came from. Using the plate count data, calculate the colony-forming units per milliliter for each dilution.

Measure Absorbance of Diluted Samples

1. Set the spectrophotometer wavelength at 600 nm.
2. Transfer sterile TSB to a cuvette and place in the spectrophotometer and close the lid.
3. Zero the spectrophotometer.
4. Add 2 mL of the original (non-diluted) culture in a cuvette. Record the absorbance.
5. Add 2 mL of the 1/2 dilution to a cuvette. Record the absorbance.
6. Add 2 mL of the 1/4 dilution to a cuvette. Record the absorbance.
7. Add 2 mL of the 1/8 dilution to a cuvette. Record the absorbance.
8. Add 2 mL of the 1/16 dilution to a cuvette. Record the absorbance.

CARMEN QUESTIONS - Bacterial Enumeration

Plate Counts of a Bacterial Culture for Determination of Bacterial Numbers

Dilution Plated	Amount of the Dilution Plated	Number of Colonies
10^{-2}	100 μL	
10^{-4}	100 μL	
10^{-6}	100 μL	

Dilution Plated	Amount of the Dilution Plated	Number of Colonies
10^{-8}	100 μL	

1. Examine the petri plates. Count colonies where you can. If there are more than 250 colonies then simply write "250+." If you cannot distinguish individual colonies write "lawn." If there are less than 25 colonies then simply write "-25." Only plates with 25-250 colonies are used for determining bacterial numbers.
2. Calculate the original CFU in cells/mL in the original culture (before dilution).
3. A plate has 72 colonies with a total dilution factor of 10^{-7} . 100 μL was pipetted onto the plate. What was the CFU concentration the original sample?

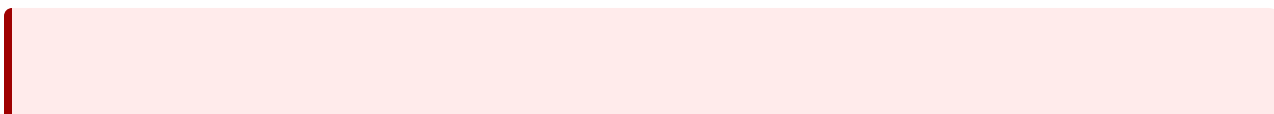
Absorbance of a Bacterial Culture for Determination of Bacterial Numbers

1. Record the absorbance (600 nm) readings for the 1 (undiluted), 1/2, 1/4, 1/8/, and 1/16 dilutions.
2. Examine any trends in the data. Finish this sentence: As the culture becomes more dilute, the absorbance _____.
3. What is an absorbance reading? What does it measure?
4. Briefly explain what a spectrophotometer is and what it does.
5. Describe how an absorbance can indicate the density of cells within a culture.
6. How is the information about the cells in a culture indicated by an absorbance reading different from the information about the cells in a culture indicated by a plate count?

Creating a Standard Line between Plate Counts and Absorbance

Culture Dilution	Absorbance (600 nm)	CFU (cells/mL)
1 (no dilution)		
1/2		
1/4		
1/8		
1/16		

1. Compare absorbance and CFU data:
 1. Transfer data from absorbance readings in the previous section to the "Absorbance (600 nm)" column in the table above.
 2. Transfer the CFU of the original culture calculated in the plate count section to the "CFU (cells/mL)" column in the dilution row that says "1 (no dilution)."
 3. Using the original culture CFU, calculate the CFU at a 1/2 dilution and put this value in the "CFU (cells/mL)" column in the dilution row that says 1/2: $(\text{CFU}) \times (1/2)$
 4. Using the original culture CFU, calculate the CFU at a 1/4 dilution and put this value in the "CFU (cells/mL)" column in the dilution row that says 1/4: $(\text{CFU}) \times (1/4)$
 5. Using the original culture CFU, calculate the CFU at a 1/8 dilution and put this value in the "CFU (cells/mL)" column in the dilution row that says 1/8: $(\text{CFU}) \times (1/8)$
 6. Using the original culture CFU, calculate the CFU at a 1/16 dilution and put this value in the "CFU (cells/mL)" column in the dilution row that says 1/4: $(\text{CFU}) \times (1/16)$
2. Create a graph with absorbance on the x-axis and CFU (cells/mL) on the y-axis. You can number the y-axis indicating that the number on the axis is $\times 10^6$, $\times 10^7$, or $\times 10^8$ as appropriate for your data. You may graph data using Excel or other graphing software or using graph paper. Follow the instruction of your instructor. Plot each data point as a dot on the graph.



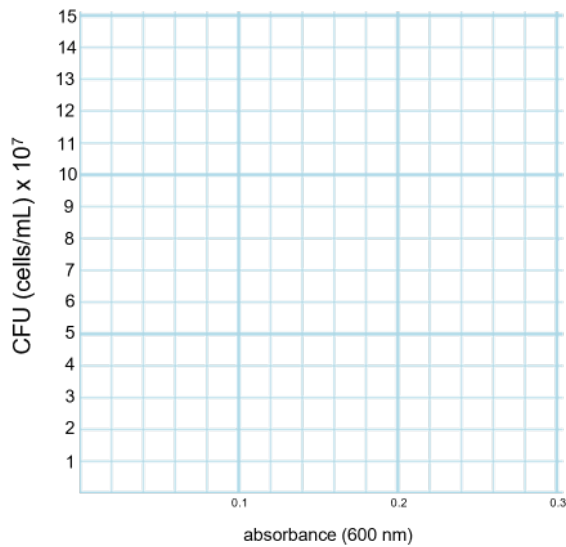
Important!

- If you are graphing using graph paper, make sure that when you label the axes that each line or box on the graph has a defined value and that every box on that axis counts as that same value. There examples given below showing how to number axes evenly so that each box has a certain unit and remains consistent for that entire side of the graph. In example 1, the y-axis has each box worth 1 and the x-axis has each box worth 0.02 (and only every five boxes are labeled). In example 2, the y-axis has each box worth 0.4 (and only every five boxes are labeled) and the x-axis has each box worth 0.01.

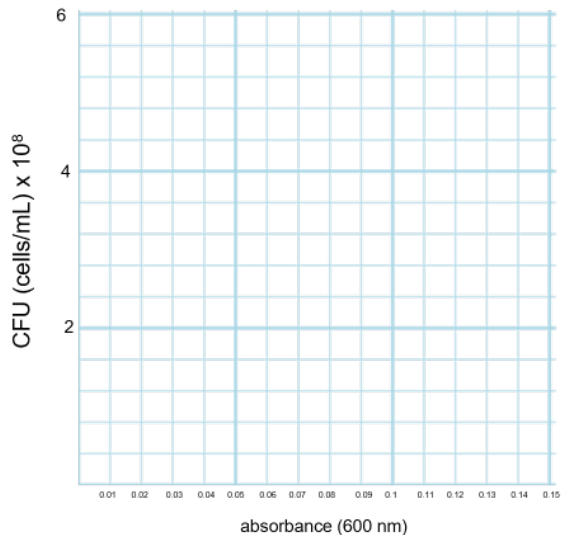
In the "Do not Graph This Way!" column below, an example of what not to do is shown. Notice that the boxes on the axes have been labeled with numbers, but each box does not have a defined unit and the numbering is not consistent. Do not do this.

Graph This Way (Every Box on the Axis has a Defined Value that is Consistent on that Axis)

Example 1:

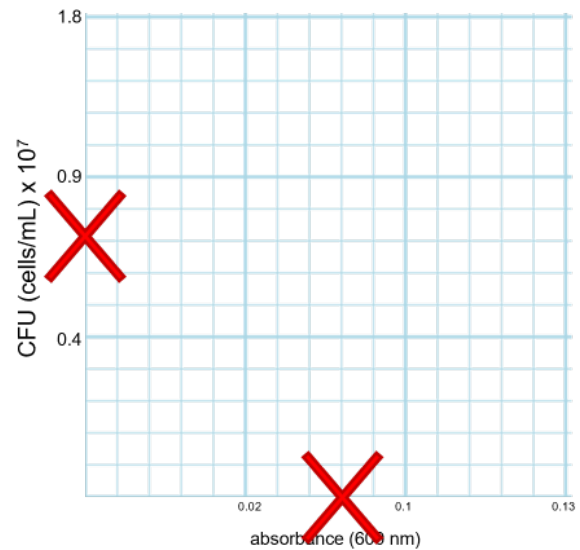


Example 2:



Do not Graph This Way! (Boxes on the Axis Do Not have a Defined Value)

Example of what NOT to do:



- If you used graphing software add a trend line to your data and set the y-intercept at zero. Also make sure to have the software place the equation for the line on the graph. If you are using graph paper, use a ruler and it place along the data points making

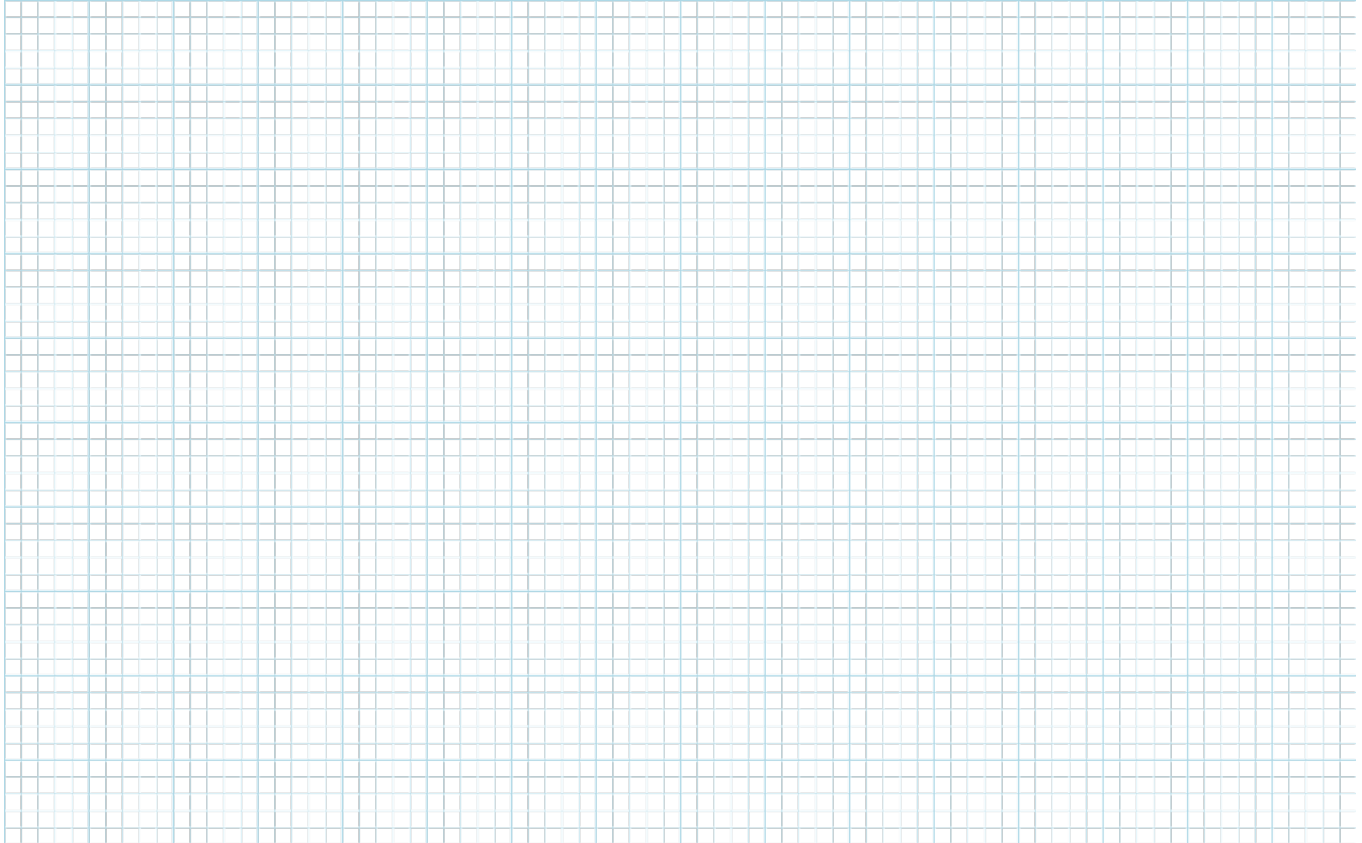
sure the ruler passes through the bottom left corner of the graph corresponding with zero on the x-axis and the y-axis. Draw a straight line using the ruler that best matches the data points. Only draw one straight line. This line does not have to touch every data point (and it probably will not). Do not connect each data point with the ruler with multiple lines. There should only be one straight line on the graph (see **Figure 6** for an example).

4. After creating the standard curve above you continue your research with this same bacterial species, but now instead of doing the plate counts (which took a lot of time and materials) you simply use a spectrophotometer to measure absorbance to determine CFU (cells/mL) using the standard curve. As you continue your research, you measure the bacterial samples using the spectrophotometer. Use the standard curve you created to determine the amount of CFUs in each sample in the table below based on the absorbance. If you used graphing software, determine CFUs using the trend line equation ([see example of using trend line equation to determine CFU from absorbance](#)). If you used graph paper, use the position of the trend line as it relates to the absorbance reading to determine CFUs ([see example of using position of the trend line to determine CFU from absorbance](#)).

1.	Absorbance (600 nm)	CFU (cells/mL)
	0.15	
	0.02	
	0.3	
	0.25	

5. Explain the purpose of constructing the calibration curve.
6. You count colonies on a plate where 100 μL was added of a 10^{-3} dilution. The colony count is 53. How many cells per milliliter are in your original sample?
7. You count colonies on plate where 100 μL was added of a 10^{-5} final dilution. Your count is 129. How many cells per milliliter are in your original sample?
8. You are testing a sample of water from a local swimming pool. An undiluted sample (10^0) where 100 μL was added to a petri plate produced 38 colonies. The safety standards for swimming pools state that the water cannot contain more than 200 CFU per milliliter. Is the pool you are testing safe to swim in? Why or why not?
9. As part of an ongoing recreational waters safety monitoring program, you are testing a sample of water from swimming beaches in Morro Bay, CA. An undiluted sample (10^0) where 100 μL was added to a petri plate produced 1 *Escherichia coli* colony. The EPA safety standards for recreational water cannot contain more than 235 *E. coli* per 100 mL. Is the beach safe to swim in? Why or why not?
10. You are testing a sample of milk from a local dairy that has been cited for some recent safety violations. A 10^{-1} dilution where 100 μL was added to a petri plate produced 201 colonies on it. The FDA safety standard for milk of the type you sampled is 3×10^4 per milliliter (above this threshold and the milk is unsafe). Is this milk sample safe to drink? Why or why not?

Graph Paper



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3.4: Unknown Bacteria Identification Project

Learning Objectives

- Apply microbiological tools to isolate and identify bacterial species of unknown identities.
- Carefully document results of microbiological tests.
- Effectively collaborate with a classmate.
- Successfully identify the unknown bacterial species.

Introduction

In this project you will experience the type of process that microbiologists have traditionally used to identify a bacterial species. This will involve:

1. Isolating bacteria (one species per culture - must begin with an isolated colony to insure that there is only one species) - if bacteria are not isolated, you cannot rely on the results of **any** of the other tests you do.
2. Conducting biochemical tests to narrow down the possible species of the unknown bacteria
3. Collaborating with a partner and contributing equally to the work
4. Writing a scientifically-written report detailing the project, its experiments, and its results

Important

In scientific research and throughout the professional world, collaboration occurs often and is an essential skill. Employers want you to be able to collaborate effectively with others. You are building your job and career skills here!

Important

In scientific research and throughout the professional world, being able to clearly communicate through writing is an essential skill. Employers want you to be able to communicate effectively with others. You are building your job and career skills here!

Instructions for Identifying Unknown Bacteria

1. Obtain a culture of unknown bacteria from your instructor. Your unknown culture will have a letter or a number. What letter or number is your unknown bacterial culture?
2. Your culture of unknown bacteria will have two different species in it. Follow the steps below **carefully** to identify these bacterial species.

Step 1: Isolate the Two Bacterial Species & Do Initial Gram Stain

1. Conduct eight (8) streak plates on TSA in order to obtain isolated colonies (each partner will conduct four). By doing eight streak plates, this increases the chances that you and your partner will obtain isolated colonies of both your bacterial species.
2. Make sure your names, unknown number/letter, and the date are on the Petri plates, invert the Petri plates, tape together, and put into the bin to be incubated.
3. Do a Gram stain to determine:
 - Whether the mixture of bacterial species contains a Gram positive and a Gram negative species, both Gram positive, or both Gram negative.
 - The cell shape of each bacterial species (i.e. coccus, bacillus, vibrio, spirillum, or spirochete).
 - The cell arrangement of each bacteria species (i.e. single, pairs, chains (strepto-), or bunches (staphylo-)).
4. Record the Gram stain, cell shape, and cell arrangement for each of your unknown species. Refer back to this data to make sure that the bacteria you isolate match the bacteria you saw in this Gram stain.

Note

Taking photos of results at every stage of the project will make a stand-out report! Include these photos in your report to provide visuals and evidence of your results. Make sure each photo is accompanied by a caption telling what the photo shows. If the photo is of a microscopic sample, make sure to indicate the magnification used when the photo was taken.

Step 2: Characterize Bacterial Colonies, Create Stock Cultures of Isolated Bacteria, & Do Gram Stain on Isolates

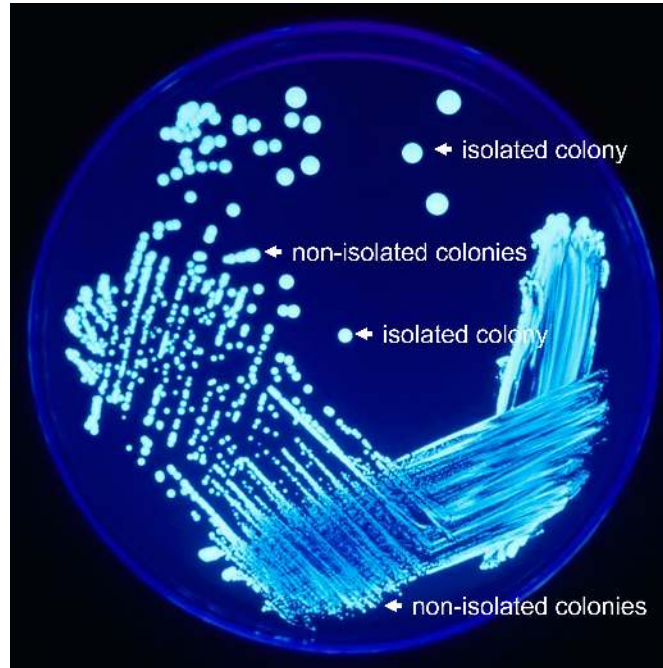


Figure 1: A quadrant streak plate after growth. Use this as a guide to help identify what are and are not isolated colonies. Isolated colonies originate from a single cell, and therefore should contain only one bacterial species. Therefore, growing a culture from an isolated colony is an effective way to separate different species.

Note

Taking photos of results at every stage of the project will make a stand-out report! Include these photos in your report to provide visuals and evidence of your results. Make sure each photo is accompanied by a caption telling what the photo shows. If the photo is of a microscopic sample, make sure to indicate the magnification used when the photo was taken.

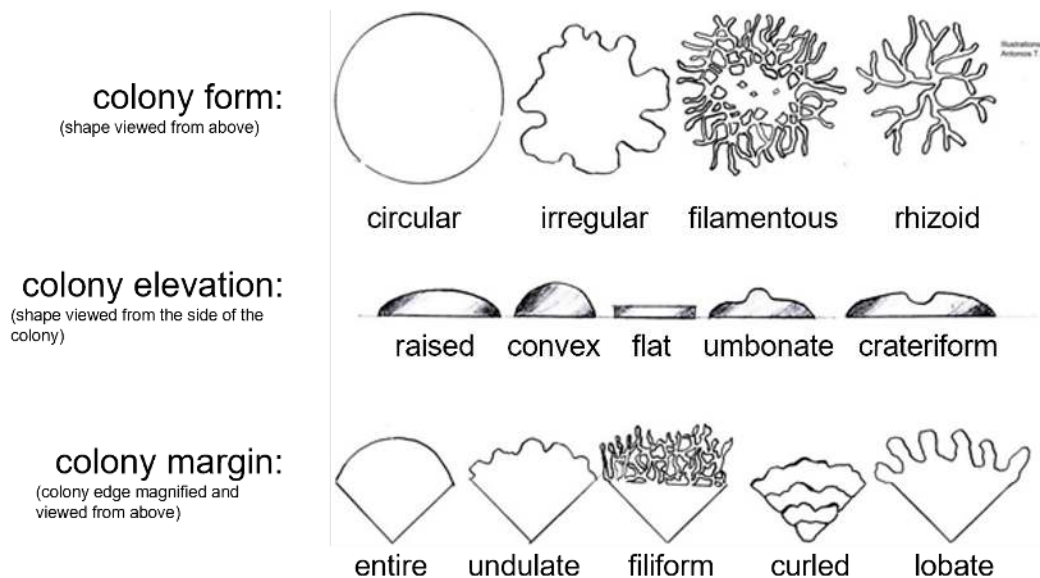


Figure 2: A guide for classifying colony characteristics. Colony form is the overall shape of the colony (circular, irregular, filamentous, or rhizoid) when viewed from above. Colony elevation examines the height and shape of the growth above the surface of the petri plate agar (raised, convex, flat, umbonate, or crateriform) and is best determined by viewing the colony from its side. Colony margin examines the shape of the edge of the colony when magnified (entire, undulate, filiform, curled, or lobate).

1. Carefully examine streak plates. Identify two bacterial colonies that have differences in appearance.
2. For the two different bacterial colonies you find, describe differences in the colony forms in the tables below. Do the best you can **keeping your Petri plate closed** to prevent contamination. It is essential that these bacterial colonies do not become contaminated with other bacteria or microbes floating in the air.
3. Label two TSA slants. Give each Unknown bacterial species a name (you can use names "A" and "B" or "1" and "2" or "Yessica" and "Yoli" - choose any names you and your partner would like - you will just need to keep straight which colony is which).
4. Transfer **one third (1/3)** of one of the isolated colonies to one of the TSA slants.
5. Transfer **one third (1/3)** of the other isolated colony to one of the TSA slants.
6. Use one **third (1/3)** of each of the colonies to make [separate bacterial smears on a slide and conduct a Gram stain](#).
- 7.
8. Compare results from the Gram stains. Did you obtain the same types of bacteria and separate them successfully? If you see Gram positive and Gram negative cells together, they are not isolated. If you see bacilli and cocci together, they are not isolated.
 1. If one or both bacterial species are not isolated (they are mixed with another species), re-examine the petri plates to see if there is another colony that appears different and create a new TSA slant, and create a new bacterial smear on a slide. Do the Gram stain on this if there is still time. If there is not time, save your bacterial smear for next class to see if the bacteria are now isolated.
 2. If you do not have better isolated colonies or continue to find that you have a mixture, conduct more streak plates to isolate bacterial colonies again.
9. When you have successfully isolated your bacteria, record results from the Gram stain in the tables below.

Step 3: Follow the Flow Chart to Identify Bacterial Species

Note

Taking photos of results at every stage of the project will make a stand-out report! Include these photos in your report to provide visuals and evidence of your results. Make sure each photo is accompanied by a caption telling what the photo shows. If the photo is of a microscopic sample, make sure to indicate the magnification used when the photo was taken.

Unknown Bacterial Species _____ (put name you chose in the blank space above)	
Gram	
cell shape	
cell arrangement	
colony form	
colony elevation	
colony margin	
colony color	
colony diameter (mm)	
test 1:	
test 2:	
test 3:	
bacterial species	

Unknown Bacterial Species _____ (put name you chose in the blank space above)	
Gram	
cell shape	
cell arrangement	
colony form	
colony elevation	
colony margin	
colony color	
colony diameter (mm)	
test 1:	
test 2:	
test 3:	
bacterial species	

- After your bacteria have been isolated and you have good results from your Gram stain, begin to follow the flow chart below.
 - For example, if your Bacteria "A" is Gram-positive, use your TSA stock slant to inoculate a starch plate to do a starch hydrolysis test.
 - For example, if your Bacteria "B" is Gram-negative, use your TSA stock slant to inoculate a SIM deep culture to test for H₂S production.
 - Use the results (positive or negative) from each test to determine the next test you should do on the flow chart.
 - Return to the appropriate chapters in this lab manual to assist with conducting each test:
 - starch hydrolysis
 - H₂S test

- [oxidase test](#)
- [coagulase test](#) and/or [mannitol salts agar](#)
- [fermentation test](#)

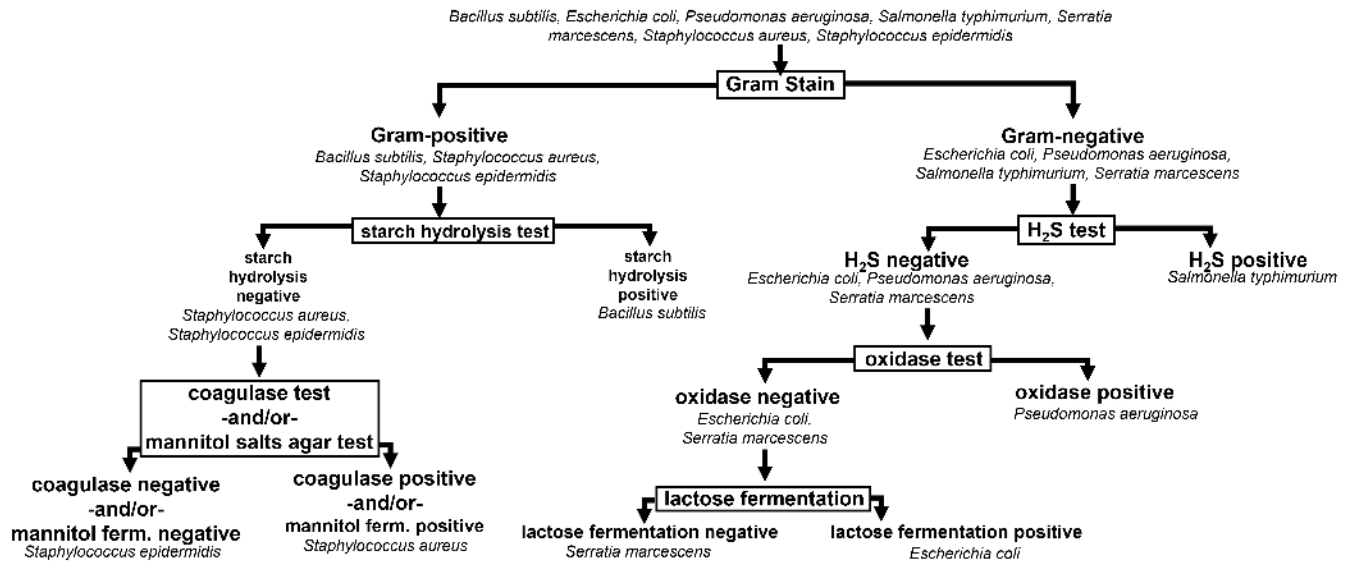


Figure 3: Unknown bacterial species identification flow chart. Once your bacterial species are isolated and you have good Gram stain results, begin to follow the flow chart for both of your unknown bacterial species. The tests that are a part of this project are in boxes. Use the results from each test to determine what the next test is or what your unknown bacterial species is.

Attributions

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- [Legionella Plate 01.png](#) by CDC/James Gathany is in the [public domain](#).

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3.5: Unknown Bacteria Identification Project Report

Learning Objectives

- Write an organized and well formatted scientific report detailing the Unknown Identification Project.
- Understand expectations of the Unknown Identification Project Report and grading guidelines.

Instructions for Writing Unknown Bacterial Species Identification Project Report

Originality

TurnItIn

The words in your report must be 100% your own. To make sure that the report is 100% your own, you will submit this report using TurnItIn. TurnItIn compares your report with websites, books, publications, and the work of other students who have submitted their documents to TurnItIn. For your report to be acceptable, the similarity rating between your work and other works will need to be below 20%.

Citing Sources

You may use outside resources to provide information that will help you to write the report (e.g. information about Gram stains, oxidase test, starch hydrolysis test, etc.), but **you must cite the sources of information you used**. The text must also be fully in your own words. Do not just change a couple words around. TurnItIn is smarter than this and will flag the text.

✓ Example 42.1

Example of scientific writing using information from an external source:

The initial Gram stain was unsuccessful the first time since all of the cells appeared purple, indicating that the decolorization step was too short (Pakpour and Horgan, 2021). As a result, the initial Gram stain was repeated using a decolorization step that was 10 seconds longer than in the previous attempt. This produced a Gram stain where Gram negative rods and Gram positive cocci were apparent.

More Information about this Example

In this example, the authors' last names are Pakpour and Horgan and the work is from 2021. A full reference would then be provided at the end of the report in a "Works Cited" section.

Works Cited Section

A works cited section is found at the end of a scientific report. It lists the full references for all external resources cited throughout the entire report. There are many different ways to format a Works Cited section. What matters most is:

- you use the same **professional formatting** for your references (i.e. consistency)
- references are complete (with title, year, author name(s), publisher/journal/book name, URL (if applicable), page numbers/name (if applicable), etc.)
- references are listed in alphabetical order

✓ Example 42.2

Works Cited

Hartline, 2022. Starch Hydrolysis. **Microbiology Laboratory Manual**. LibreText. Accessed on 10/30/2022 from:

Pakpour and Horgan, 2021. Lab 3: Simple, Negative, and Gram Stain. **General Microbiology Lab Manual (Pakpour & Horgan)**. LibreText. Accessed on

10/29/2022

from:

https://bio.libretexts.org/Learning_Objects/Laboratory_Experiments/Microbiology_Labs/Book%3A_General_Microbiol

Quoting Sources

Quoting another author in the sciences is rare. As a result, ***in this report, quoting another author is not allowed.*** Take information from texts and put it into your own words and cite the source. Don't just change one or two words (Turn It In will flag this). You need to write your own sentence using the ideas you read. Or even better, synthesize your own ideas and results together with ideas from another source and then cite the source.

This type of synthesis is shown in the example above discussing the Gram stain. Events and results from the laboratory were synthesized together into the same sentence with the idea in the cited source that the decolorization step was too short since all the cells appeared purple.

Working with Your Partner

Since you and your partner worked together and will have done the same steps and will have the same results, it may be tempting to share parts of your reports with each other. TurnItIn will recognize the similarities between your reports and flag it as plagiarism.

You must write your own report without sharing text with your partner. However, you are encouraged to get your partner to proofread your report and give feedback and comments to help improve the report before you submit it. The images in your reports may be the same since you will share the same results and will want to choose the best photos for the report.

Organization of the Report

The report should be broken into the following sections. Each section should have a bold header with larger font than the text in each section. Do you see how this document is separated into sections with bold headers? This is how your report should look too, except name each section as follows:

- **Introduction**
- **Methods**
- **Results & Discussion**
- **Conclusions**
- **Works Cited**
- **Activity Log**

✓ Example 42.3

Introduction

Introduction text / subsections here.

Methods

Methods subsections and text here.

Results & Discussion

Results and discussion subsections and text here.

Conclusions

Conclusion text here.

Works Cited

Full references here in alphabetical order.

Activity Log

Photos of activity log pages here.

Introduction

An introduction gives important background information to the reader. Don't write this report assuming that your instructor knows all of this information already and therefore you shouldn't include it. Instead, write the report as if you were writing it for a biology student who hasn't taken microbiology yet. Therefore, you will need to:

- Address the reasons why identifying bacterial species is important.
- Describe how (generally speaking) identifying bacterial species is done.
- Describe each of the microbiological tests and microbiological approaches that you used in this project including:
 - how each works
 - what each test tells about the bacteria
 - how you determine the results of a test
- Experimental Overview paragraph (see "Important" box below)

✓ Example 42.4

For example, if you identified your bacteria as *Bacillus subtilis* and *Escherichia coli*, you will have different paragraphs or subsections in the Introduction on the following topics:

- Streak plate
- Gram stain
- Starch hydrolysis
- H₂S production
- Oxidase test
- Lactose fermentation

In the paragraph introducing the Gram stain (for example), you will need to discuss:

- How the crystal violet stain will remain inside a thick layer of peptidoglycan
- How the crystal violet stain will be decolorized from a thin layer of peptidoglycan
- How cells with the thin peptidoglycan layer are stained with a different color
- The structure of the cell walls of Gram-positive bacteria
- The structure of the cell walls of Gram-negative bacteria
- The color Gram positive bacteria appear in the microscope after the Gram stain
- The color Gram negative bacteria appear in the microscope after the Gram stain

👋 Important

End the introduction with a paragraph that describes the basic premise and approach of this project and how this approach either resulted in the successful identification of both unknown bacteria, one identification was successful and the other unsuccessful, or that both were not correctly identified. ***Be sure to name the species you had and what species you identified them as.***

It may seem like this is spoiling the ending of the paper - ***and it is!*** This is common among scientific publications to end the Introduction with a paragraph that tells the reader what they can expect to read about in the rest of it.

Methods

Methods will tell the approaches you used to conduct each test. The Methods section **does not** discuss the concepts of each test, the meaning of each test, or the results of the tests. The Methods section describes what you did with your bacteria in the laboratory for each test and how you did the tests. The Methods is written in paragraph form (do no bulletpoint or number steps - do not copy from laboratory protocols). Separate the **Methods** section into subsections.

✓ Example 42.5

Continuing the example from above where you identified your bacteria as *Bacillus subtilis* and *Escherichia coli*...

Methods

Streak Plate

Text here about what you did to make your streak plates (e.g. using aseptic technique, using a Bunsen burner and loop, where bacteria were collected from [e.g. original Unknown culture, stock TSA slant, etc.], how the petri plate was streaked in quadrants while flaming loop in between, plate inverted and incubated at 37°C for 48 hours).

Gram Stain

Text here about what you did to make bacterial smears and conduct Gram stains.

Starch Hydrolysis Test

Text here about what you did to do the starch hydrolysis test.

H₂S Production Test

Text here about what you did to do the H₂S production test.

Oxidase Test

Text here about what you did to do the oxidase test.

Lactose Fermentation Test

Text here about what you did to do the lactose fermentation test.

Since you are only describing how you did a test (the steps you took in the laboratory to do the tests), each subsection in the Methods may be relatively short. The **Methods** section *should not include any results and is not the place to discuss the theory of each test*. It should simply state how each test/component was done.

✓ Example 42.6

Methods

H₂S Production

To test whether Unknown Bacteria A produces H₂S, Unknown Bacteria A was aseptically transferred from the stock TSA slant into a SIM deep using an inoculation needle. This culture was incubated at 37 °C for 48 hours before it was examined to determine test results.

Results & Discussion

Separate the **Results & Discussion** section into subsections.

✓ Example 42.7

Continuing the example from above where you identified your bacteria as *Bacillus subtilis* and *Escherichia coli*...

Results & Discussion

Initial Gram Stain of Mixed Culture

Text & figures here about streak plate results and discussion

Quadrant Streak Plate

Text & figures here about streak plate results and discussion

Identification of Unknown Bacterial Species #1

Gram Stain

Text & figures here about Gram stain results and discussion

Starch Hydrolysis Test

Text & figures here about starch hydrolysis results and discussion

Identification of Unknown Bacterial Species #2

H₂S Production Test

Text & figures here about H₂S production test results and discussion

Oxidase Test

Text & figures here about oxidase test results and discussion

Lactose Fermentation Test

Text & figures here about lactose fermentation test results and discussion

Important

Each Results & Discussion subsection should contain **both** results and discussion:

- **Results:** simply tells the observation(s) from each test.
- **Discussion:** interprets observations and the meanings of the results of each test.

✓ Example 42.8

Result for Gram stain Section: "The initial Gram stain showed a mixture of purple-colored cocci arranged in chains and pink-colored bacilli."

Discussion for Gram stain Section: "These results indicate that Unknown Culture #4 contains Gram-positive cocci, possibly *Streptococci*, and Gram-negative rods. The bacteria staining Gram-positive have a thick layer of peptidoglycan in their cell walls and lack an outer membrane. The Gram-negative bacterial species have cell walls with a thin layer of peptidoglycan and an outer membrane."

The Results and the Discussion should be synthesized together in paragraph form in each section.

✓ Example 42.9

Example of a Gram Stain subsection of the Results & Discussion:

"The initial Gram stain showed a mixture of purple-colored cocci arranged in chains and pink colored rods. This indicates that Unknown Culture #4 contains Gram-positive cocci, likely *Streptococci*, and Gram-negative rods. The bacteria staining Gram-positive have a thick layer of peptidoglycan in their cell walls and lack an outer membrane. The Gram-negative bacterial species have cell walls with a thin layer of peptidoglycan and an outer membrane.

"After these bacteria were isolated using the quadrant streak-plate technique, the subsequent Gram stains showed one isolate appeared as purple cocci and the other isolate were pink bacilli. There was no evidence of pink bacilli with the purple cocci or purple cocci with the pink rods. This indicates that the two species that were examined in the initial Gram stain were successfully isolated and separated into their own cultures. The purple-staining cocci are a Gram positive bacterial species and will hereafter be referred to as "Unknown Bacteria A." The pink-staining rods are a Gram negative bacterial species and designated as "Unknown Bacteria B" in the remainder of this report."

In the example text above, the description would be further supported with images of the initial Gram stain, the Gram stain of Unknown Bacteria A, and the Gram stain of Unknown Bacteria B. These images will require captions to indicate what the image is. Images can be placed as separate figures, each with their own caption, or arranged together as a single figure with a single caption.

✓ Example 42.10

This is an example of a single photo showing results with its caption:

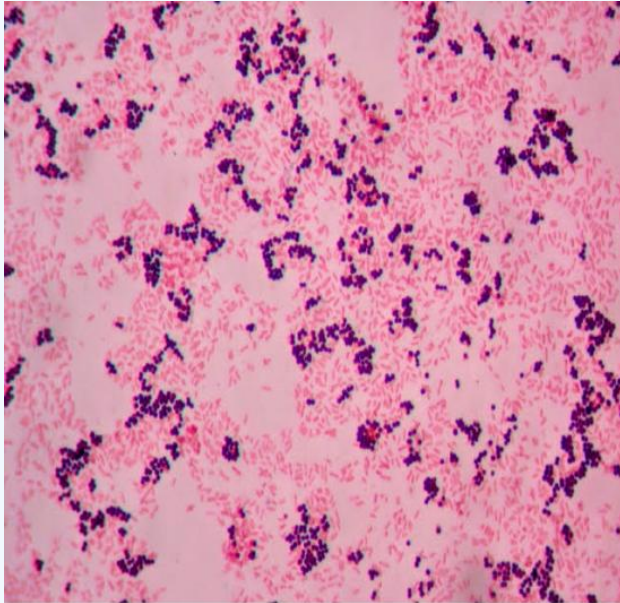


Figure 1: Results from initial Gram stain of the original Unknown Bacterial Culture showing both species mixed together in this culture.

✓ Example 42.11

This is an example of multiple photos that have been put together as a single figure with one caption:

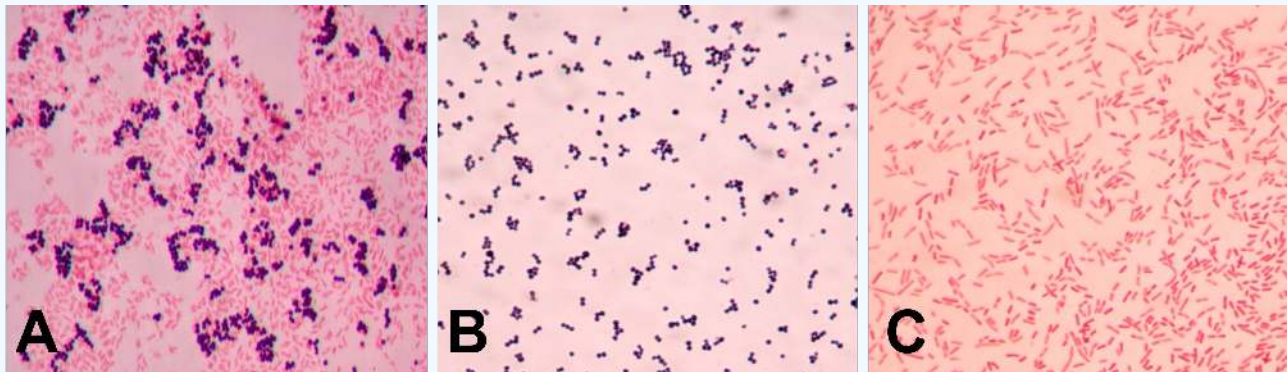


Figure 1: (A) Results from initial Gram stain of the original Unknown Bacterial Culture showing both species mixed together in this culture. (B) Gram stain of isolated unknown bacteria "A." (C) Gram stain of isolated unknown bacteria "B."

Conclusions

In the conclusions section of the report, including the following:

- Name the two bacterial species that you identified the species as from the laboratory tests you conducted.
- Tell if the bacterial species identified were in fact the species that you had in the unknown culture. If they were not what was identified, name the correct species.
- If there was a misidentification, tell what species you **did** have and reflect on and discuss sources of error or events during the project that might have led to misidentification.
- If you successfully identified the bacterial species, reflect on and discuss what likely contributed to your successful identification of the bacterial species.

Activity Log

Take clear photos of the activity log or scan these pages and include them in this section of the report.

Writing Style

Write in Third Person

Write in the third person. This means that you act as a narrator as if you were outside of the experiment and not involved in the experiment.

- **First person (don't write this way):** I examined my Gram stain and saw that the cells were pink.
- **First person (don't write this way):** We examined our Gram stain and saw that the cells were pink.
- **Second person (don't write this way):** When you examined the Gram stain, you saw that the cells were pink.
- **Third person (write this way):** Upon examining the Gram stain, pink cells were observed.

Write in Past Tense

Write in the past tense.

- **Future tense (don't write this way):** A Gram stain will be done.
- **Present tense (don't write this way):** A Gram stain is being done.
- **Past tense (write this way):** A Gram stain was done.

Proofread

Use Grammar and Spelling Checkers

Check all of your text for proper grammar and spelling. Make adjustments were necessary.

Write Early & Read Multiple Times Before Submitting

Complete your report at least a few days before submitting it. Read it a couple times after writing it (including on separate days). Make edits on each reading.

Have a Classmate Proofread and Give Feedback

Complete your report early, print out a copy or email it to a classmate and ask them to read it over and make notes to help you to improve your report. Make edits that you deem will improve your report as suggested by your classmate.

Instructions for Submitting the Report

Upload a file with your Unknown Identification Project Report in the **PROJECT: Unknown Identification Project Report** assignment in Canvas. This upload will use TurnItIn.

TurnItIn will:

- Electronically analyze and review the text in your document to insure your work is original.
- Compare your work to its student paper repository.
- Compare your work to current and archived web site content.
- Compare your work to periodicals, journals, and publications.
- Create an originality report of your work.
- Both you and your instructor will be able to see the originality report.

See how to upload your work to this assignment in this video guide:



Grading

Grading Rubric

	100% of points	85% of points	70% of points	50% of points	20% of points	0% of points
Introduction (40 points)	The Introduction section thoroughly and accurately addresses all expected components for the introduction. Excellent writing quality and style. Citations are included as appropriate.	The Introduction section addresses most of the expected components and most or all of the components are accurate. Writing style demonstrates quality and thoughtful development. Citations are included as appropriate.	The Introduction section addresses some of the expected components and most/some of the components are accurate. Writing and writing style may be good or may requires some improvement. Citations may or may not be included.	The Introduction section addresses a few of the expected components and some of the components are accurate. Writing and writing style requires additional development. Citations may or may not be included.	The Introduction section only slightly addresses the expected components. Writing and writing style requires additional development. Citations may or may not be included.	The Introduction section is absent or addresses almost none of the expected components. Writing and writing style requires a lot more development. Citations may or may not be included.
Methods (10 points)	The Methods section thoroughly and accurately addresses all expected components without including text better suited to the introduction or results & discussion sections. Excellent writing quality and style.	The Methods section addresses most of the expected components and most or all of the components are accurate without including text better suited to the introduction or results & discussion sections. Writing style demonstrates quality and thoughtful development.	The Methods section addresses some of the expected components and most/some of the components are accurate and may include text that would be better suited to the introduction or results & discussion sections. Writing and writing style requires additional development.	The Methods section addresses a few of the expected components and some of the components are accurate and may include text that would be better suited to the introduction or results & discussion sections. Writing and writing style requires additional development.	The Methods section addresses a few of the expected components and some of the components are accurate and may include text that would be better suited to the introduction or results & discussion sections. Writing and writing style requires additional development.	The Methods section is absent or addresses almost none of the expected components and may include text that would be better suited to the introduction or results & discussion sections. Writing and writing style requires a lot more development.
Results and Discussion (40 points)	The Results and Discussion section thoroughly and accurately addresses all expected components. Quality figures are provided to support results and have captions.	The Results and Discussion section addresses most of the expected components and most or all of the components are accurate. Figures are provided to support results and may or may not have	The Results and Discussion section addresses some of the expected components and most/some of the components are accurate. Figures are provided to support results and may or may not have	The Results and Discussion section addresses a few of the expected components and some of the components are accurate. Figures may or may not be provided to support results and may or may	The Results and Discussion section does not address most of the expected components. Figures may or may not be provided to support results and may or may not have captions. Writing	The Results and Discussion section is absent or addresses almost none of the expected components. Writing and writing style requires a lot more development. Citations may or

	100% of points	85% of points	70% of points	50% of points	20% of points	0% of points
	Excellent writing quality and style. Citations are included as appropriate.	captions. Writing style demonstrates quality and thoughtful development. Citations are included as appropriate.	captions. Writing and writing style requires additional development. Citations may or may not be included.	not have captions. Writing and writing style requires additional development.	and writing style requires additional development.	may not be included.
Conclusions (5 points)	Report conclusions are reflective, clearly states what species were identified through the project and whether or not these identifications were accurate, offers multiple thorough and thoughtful plausible explanations for each successful and unsuccessful identifications. Writing is excellent.	Report conclusions are reflective, clearly states what species were identified through the project and whether or not these identifications were accurate, and provides at least one thoughtful and plausible explanation for each successful and unsuccessful identifications. Writing style is good to excellent.	Report conclusions are reflective, clearly states what species were identified through the project and whether or not these identifications were accurate, and provides one explanation for each successful and unsuccessful identifications. Writing style may be good or may require additional development.	Report conclusions states what species the unknown species actually were, and may provide one explanation successful and unsuccessful identifications. Writing style requires additional development.	Report conclusions states what species the unknown species actually were, and does not provide any explanations for successful and unsuccessful identifications. Writing style requires lots of additional development.	Conclusions section is absent or fails to address appropriate content for this section.
Works Cited (5 points)	The Works Cited section has consistent formatting for all references. References are provided for each citation in the text. Information included is thorough. References are listed in alphabetical order.	The Works Cited section has consistent formatting for all references. References are provided for each citation in the text. Information included is mostly thorough, but some important information is missing. References are listed in	The Works Cited section has mostly consistent formatting for all references. References are provided for most of the citations in the text. Information included is mostly thorough, but some important information is missing. References order	The Works Cited section has inconsistent formatting. References are provided for most/some of the citations in the text. Information included is missing components. References order may or may not be alphabetical.	The Works Cited section lacks formatting. References are missing or most of the information required for full references are missing. References order may or may not be alphabetical.	Works Cited section is either missing or is just a list of titles or websites used without any attempt at formatting or organization.

	100% of points	85% of points	70% of points	50% of points	20% of points	0% of points
		alphabetical order.	may or may not be alphabetical.			

Sample Unknown Identification Report

To help you better grasp how all of these guidelines look in a cohesive report, a sample report has been developed for your reference. This report is to help you better understand:

- The overall formatting for the report
- The writing style for this type of report
- The type of content that is appropriate for each section of the report
- How citations may appear throughout the text
- Appropriate formatting for figures and captions

*****This sample report is NOT provided for you to copy *even a single sentence*. Even if you change some of the words in a sentence you copy, this is still plagiarism. The ideas can be the same in the reports, but the way they are worded MUST be completely your own way of writing.*****

[Click here to view the sample report.](#)

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CHAPTER OVERVIEW

4: Lab 4

- 4.1: Required Materials
- 4.2: Fermentation
- 4.3: Starch Hydrolysis
- 4.4: Citrate Test
- 4.5: MR-VP Tests
- 4.6: Coagulase Test
- 4.7: Triple Sugar Iron Medium

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4.1: Required Materials

Cultures Needed

- TSB cultures
 - *E. coli*
 - *B. subtilis*
 - *Proteus vulgaris*
 - *S. marcescens*
 - *E. aerogenes*
 - *S. aureus*
 - *S. epidermidis*

Fermentation Test

- Phenol red glucose w/Durham tube
- Phenol red lactose w/Durham tube
- Phenol red mannitol w/Durham tube

Starch Hydrolysis

- Starch plates

Citrate Test

- Simmons' Citrate

MR-VP Tests

- MR-VP Broth tubes

Coagulase Test

- Coagulase tubes

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4.2: Fermentation

Learning Objectives

- Explain what fermentation is and why it is important for microorganisms.
- Give examples of types of fermentation products, including fermentation products used by humans.
- Tell how fermentation tests can be useful in identification and characterization of bacterial species.
- Describe how the fermentation test works including the functions of phenol red and Durham tubes.
- Tell that fermentation can utilize different carbohydrates resulting in different fermentation reactions.
- Successfully conduct and interpret fermentation tests.

Fermentation is a Metabolic Process

Fermentation is a metabolic process that some microorganisms use to break down glucose and other sugars when O_2 is not available or could not be used by the microorganism. Fermentation is a way bacteria can produce ATP to meet their energy needs (although fermentation produces significantly less ATP than aerobic respiration or anaerobic respiration). Fermentation includes the metabolic pathway **glycolysis** (where a single molecule of glucose is broken down into 2 molecules of pyruvate), as well as additional fermentation reactions that produce a variety of end products (acids, alcohols, gases). The end products are characteristic of individual bacterial species. Fermentation is also possible from non-sugar molecules. Even unusual compounds like aromatics (benzoate), glycerol (sugar-alcohol), and acetylene (hydrocarbons) may be fermented by some bacterial species!

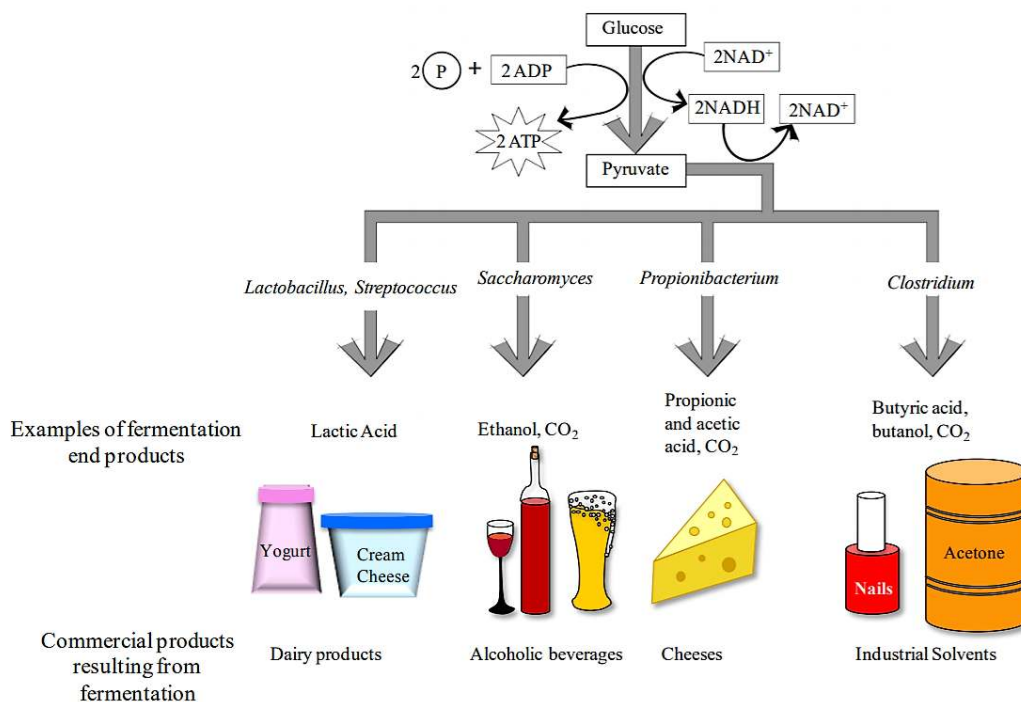


Figure 1: Carbohydrate fermentation. Fermentation is a microbial process utilized to produce a variety of products used by humans including dairy products (yogurt and cream cheese), alcoholic beverages, cheeses, and industrial solvents. Fermentation is a metabolic process that begins with glycolysis to make a small amount of ATP and pyruvate. Pyruvate then undergoes additional fermentation reactions resulting in fermentation byproducts, including those important to humans.

Note that fermentation is mainly a mechanism used by cells to regenerate NAD^+ so that NAD^+ is available for glycolysis to continue when cellular respiration is not occurring. Fermentation also tends to produce waste products that can accumulate in the extracellular environment. By contrast, the waste left over after ATP production by aerobic respiration are limited to CO_2 and H_2O . There can be numerous end products from fermentation, many of which are useful for us humans, but not necessarily the microbes. We use many fermentation products--as diverse as antibiotics, alcohols, and a variety of foods. Microbes such as yeast (e.g. *Saccharomyces cerevisiae*) and bacteria are genetically engineered to produce valuable fermentation products.

Much of the original energy in the substrate remains within the chemical bonds of organic end products such as lactic acid or ethanol. For example, ethanol has so much stored energy it can be used in gasoline solutions to be combusted/burned to release that energy stored in its chemical bonds!

Fermentation of a Variety of Carbohydrates

Bacteria, depending on the species, can ferment different carbohydrates. Although glycolysis (the pathway leading to fermentation) begins with glucose, some bacteria have the enzymes needed for additional chemical reactions to convert other monosaccharides (e.g. fructose and mannose) as well as disaccharides (e.g. lactose and sucrose) so they can enter the glycolysis pathway. These bacteria therefore require the genes in their DNA that code for enzymes capable of converting these sugars into molecules that can enter glycolysis. For example, the enzyme **beta-galactosidase** is necessary to break down lactose. This enzyme is coded in the DNA of microbes that can metabolize lactose so they can produce this enzyme.

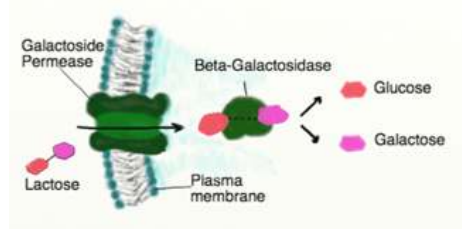


Figure 2: Lactose on the outside of a bacterial cell can be brought into the cell if the cell has the enzyme permease to transport it. Lactose metabolism also requires beta-galactosidase to break down the disaccharide lactose into monosaccharides.

Therefore bacteria can be differentiated both based on their ability to ferment various carbohydrates, as well as the end products that result from the fermentation process. As a result, examining the ability of bacterial species to ferment a variety of carbohydrates is an approach used to characterize bacterial species and is useful for species identification.

Fermentation Test

Some bacteria will produce gases when fermenting a carbohydrate. To detect these gases, a **Durham tube** is used. This is a small inverted glass tube that is placed within the larger glass tube containing the fermentation medium. If gases (typically CO_2) are produced during the fermentation process, a bubble will form at the top of the Durham tube. If you see a bubble in the Durham tube, this means fermentation occurred and gas was produced during fermentation.

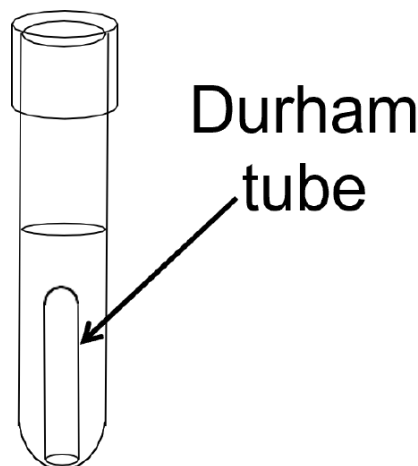


Figure 3: Diagram showing the fermentation test setup with a test tube containing medium and a tiny tube inside of the medium that is upside down to capture gases produced during fermentation. This tiny upside down tube is called a Durham tube. If gas is produced during fermentation, there will be a space in the top of the Durham tube that does not have any medium in it since gas has displaced the medium at the top of the tube forming a bubble.

The medium used to test carbohydrate fermentation is a nutrient broth that contains a fermentable carbohydrate (usually a monosaccharide or a disaccharide), peptone (amino acids) as well as a pH indicator. The pH of the medium is adjusted to approximately 7.5, so it appears orange/red with a **phenol red pH indicator**. These types of carbohydrate fermentation tubes are therefore called **phenol red (sugar) broths**. For example, if the fermentation test is being done to test fermentation of glucose,

glucose is added to phenol red medium and the medium is called **phenol red glucose**. If the fermentation test is being done to test fermentation of lactose, lactose is added to phenol red medium and the medium is called **phenol red lactose**.

If the carbohydrate in the medium is fermented and acidic end products are formed, the color of the medium changes from red-orange to yellow. Occasionally, bacteria will not ferment the carbohydrate, but instead will break down proteins producing ammonia (NH_3) in the growth medium. In this case, the medium will become more alkaline and appear red.

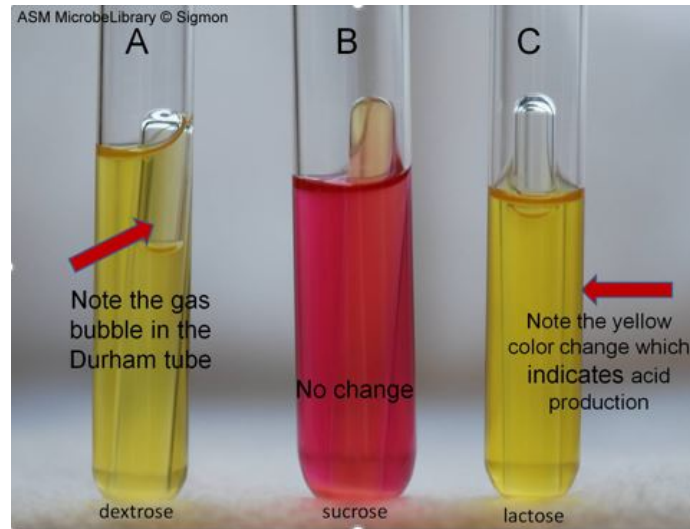


Figure 4: Fermentation reactions produced by *Escherichia coli* in phenol red sugar broths containing dextrose, sucrose, and lactose. Image by Janie Sigmon, York Technical College, Rock Hill, SC.

Results of a fermentation can be interpreted as follows:

- red-orange color indicates no acid was produced
- yellow color indicates acid was produced during fermentation
- a gas bubble trapped in the Durham tube indicates gas was produced during fermentation
- no gas bubble trapped in the Durham tube indicates no gas was produced
- if medium is red-orange **and** no gas bubble is trapped in the Durham tube, no fermentation occurred



? Exercise 22.1



Interpret the result of this fermentation test:

- Did this bacterial species produce gas?
- Did this bacterial species produce acid?
- Did fermentation occur?

Answer

? Exercise 22.2



Interpret the result of this fermentation test:

- Did this bacterial species produce gas?
- Did this bacterial species produce acid?
- Did fermentation occur?

Answer

Laboratory Instructions

Fermentation Test

In this experiment, fermentation of two different carbohydrates will be tested: glucose and lactose.

1. Label three phenol red glucose tubes each with a species names to be tested (*Escherichia coli*, *Bacillus subtilis*, and *Proteus vulgaris*), group name, and medium name. Repeat for three phenol red lactose tubes.
2. Use aseptic technique to transfer a loop of the appropriate cultures to each of the culture broths.
3. Incubate cultures at 37 °C for 48 hours.

Results & Questions

	medium color with glucose (red or yellow)	bubble in Durham tube with glucose (+/-)	acid production with glucose (+/-)	gas production with glucose (+/-)	fermentation of glucose (+/-)
<i>Escherichia coli</i>					
<i>Bacillus subtilis</i>					
<i>Proteus vulgaris</i>					

	medium color with lactose (red or yellow)	bubble in Durham tube with lactose (+/-)	acid production with lactose (+/-)	gas production with lactose (+/-)	fermentation of lactose (+/-)
<i>Escherichia coli</i>					
<i>Bacillus subtilis</i>					
<i>Proteus vulgaris</i>					

1. Complete the tables above based on the results observed from the fermentation tests.
2. Was there a difference in fermentation/fermentation products produced by *E. coli* with glucose versus lactose? Explain your answer.
3. Was there a difference in fermentation/fermentation products produced by *B. subtilis* with glucose versus lactose? Explain your answer.
4. Was there a difference in fermentation/fermentation products produced by *P. vulgaris* with glucose versus lactose? Explain your answer.
5. What is fermentation?
6. Do all bacterial species ferment in the same way and produce the same end products? Explain your answer.
7. Why is fermentation an important process in some bacterial species?
8. Are fermentation tests useful for bacterial species identification and characterization? Explain your answer.
9. What is the purpose of phenol red in the fermentation medium?
10. What is the purpose of the Durham tube in the fermentation tube?

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4.3: Starch Hydrolysis

Learning Objectives

- Explain what the starch hydrolysis test is and how it works.
- Explain how starch hydrolysis relates to the amylase gene and the enzyme amylase.
- State the chemical reaction that occurs when bacteria hydrolyze starch.
- Successfully conduct the starch hydrolysis test.
- Interpret the results of the starch hydrolysis test.
- State why starch hydrolysis is a beneficial characteristic for bacteria.
- Tell that starch hydrolysis is a characteristic that only some bacterial species have and that is therefore useful for species identification and characterization.

Starch Hydrolysis Test

Starch is a *long* carbohydrate molecule made of *hundreds* of glucose molecules bonded together into a very long chain. There is a lot of energy in starch! If bacteria are able to break down starch, this is an advantage because that means they can access the sugars in starch and the energy in those sugars (as well as the carbon).

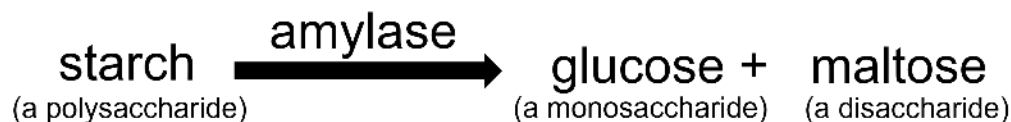


Figure 1: Above is the chemical reaction catalyzed by the enzyme **amylase**. The long polysaccharide starch is broken down (hydrolyzed) by the enzyme amylase. The products are glucose molecules (a monosaccharide) and maltose (a disaccharide). These smaller sugars are small enough that a bacterial cell could transport them inside. Then once inside the bacterial cell, these sugars can be used by the bacteria as a source of energy and carbon.

Some species of bacteria can break down starch and some species of bacteria cannot break down starch. Whether a bacterial species can break down starch or not is based on whether they have a gene (a segment of DNA) that codes for the enzyme **amylase**. Amylase is an enzyme that breaks down starch into smaller sugars.

Starch is too large to pass through the plasma membrane of a cell and must be split into individual glucose molecules. Bacteria that can produce the exoenzyme amylase are able to hydrolyze starch by secreting these enzymes into the environment around them. The enzyme amylase is secreted out of the cells (amylase is considered an **exoenzyme**, meaning it is an enzyme secreted outside of the cells ["exo-" means "outside"]) into the surrounding media, catalyzing the breakdown of starch into smaller sugars. These smaller sugars can then be absorbed by the cells and the cells can use the sugars as a source of energy and carbon.

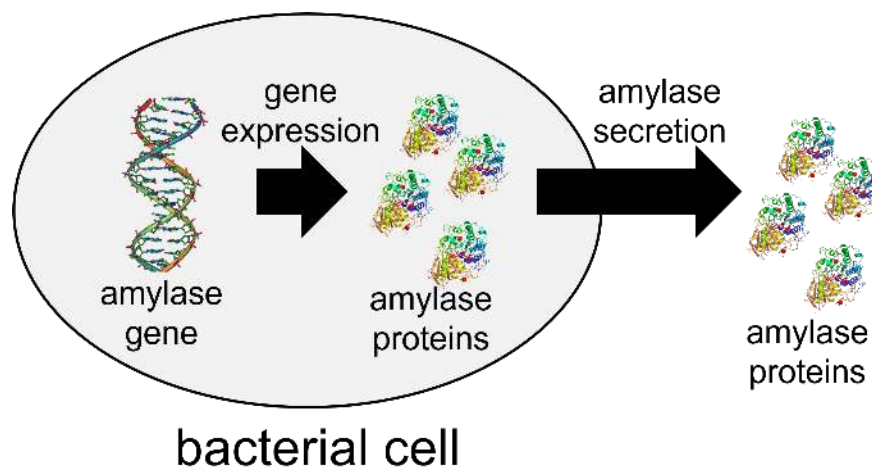


Figure 2: Diagram summarizing how a bacterial cell produces and secretes amylase. The information for building amylase is found in the cell's DNA. The amylase gene is expressed (RNA is created from the information in the gene (the DNA) and from the RNA, ribosomes build the amylase proteins [DNA-->RNA-->protein]). Then amylase is secreted outside of the cell (amylase is an exoenzyme). Amylase can then hydrolyze starch if it is available in the bacterial cell's environment.

Since only some species of bacteria are capable of breaking down starch (aka **starch hydrolysis**) testing if bacteria can break down starch or not is a way we can differentiate one bacterial species from another. Therefore, examining starch hydrolysis of a bacterial species is useful for identifying and characterizing bacterial species. Starch can be added to petri plate media in order to determine if bacteria are capable of breaking down the starch.

Starch inside of a petri plate is clear and cannot be seen without using iodine. Iodine reacts with starch, producing a black or bluish-black color. As starch is hydrolyzed by bacterial amylase and is converted to sugars, there will be less and less starch to react with the iodine. Strong amylase producers may convert all of the starch in the agar to sugars, while weak amylase producers may convert the starch surrounding the growth areas only.

After bacteria are allowed to grow, iodine is added to the petri plate to detect the presence and absence of starch.

- Where starch is on the petri plate will appear blue-black. Iodine reacts with starch to produce blue-black coloration when starch is present.
- Where starch isn't on the petri plate will appear clear or yellowish. Iodine will not produce a blue-black color where starch is absent. These regions therefore appear a clear or yellowish color on the petri plate when starch is not present.

Since the entire petri plate contained starch at the start of the test, clear halos surrounding bacterial growth indicates that the bacterial species was able to hydrolyze the starch resulting in the clear zone without starch (we would say that this bacterial species is **starch hydrolysis positive**). If the growth does not exhibit clear zones surrounding it, the starch is intact throughout the plate (no starch hydrolysis or we say that the bacterial species is **starch hydrolysis negative**).



Figure 3: A starch plate with different bacterial species growing on it after iodine has been added to the petri plate. The black regions are where starch is still present since iodine reacts with starch to create the black color. The clear regions are where starch is no longer present (where starch has been hydrolyzed). The bacterial growth may look light-colored on the plate. It is easy to mistake the bacterial growth from a clear zone that indicates starch hydrolysis. The key here is that **the clear zone will surround the bacterial growth** if starch hydrolysis occurred. Be aware that some species of bacteria hydrolyze starch at such a rapid rate that they may have consumed starch around other bacterial species on the same plate when those other species do not hydrolyze starch.

Laboratory Instructions

Inoculating Starch Plates

1. Obtain a starch agar petri plate.
2. Write on the bottom of the starch agar petri plate to separate the plate into four sections. Label as shown above.
3. Aseptically make a single line streak of the corresponding bacterial species in each of the sections on the petri plate. The "control" region will remain empty (no bacterial streak).
4. Invert the petri plate and incubate at either 25° C or 37° C for 24-28 hours.

Adding Iodine to Visualize Location of Starch

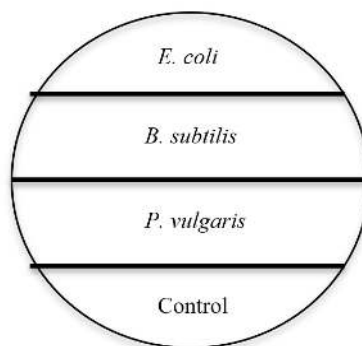


Figure 4: Draw on the bottom of the starch plate the lines and the labels shown above. The bacterial species names have been abbreviated for simplicity. Their full names are *Escherichia coli*, *Bacillus subtilis*, and *Proteus vulgaris*. The control will be used for comparison of how the plate should look when no starch hydrolysis occurs since no bacteria is being streaked in this region.

1. After the starch plate has incubated and bacteria is growing on the surface, placing the agar plate on a white piece of paper or white background since this will really help you to distinguish whether or not clear zones occurred.
2. Cover the agar and growth with iodine.
3. Examine the petri plate. Species that hydrolyze starch will have a region around the bacterial growth that is yellowish or clear **around the growth** (the growth may appear light in color - look for a clear zone surrounding the growth [the growth is raised on the plate]). When starch is not hydrolyzed, the growth may appear lighter in color, but there is no clear zone surrounding the growth (unless the clear zone is extending from a bacterial species that does hydrolyze starch).

Results & Questions

Bacterial Species	Result for Starch Hydrolysis (+ or -)	Does this species produce amylase?	Does this species have the amylase gene?
<i>Escherichia coli</i>			
<i>Bacillus subtilis</i>			
<i>Proteus vulgaris</i>			

1. Complete the table above to indicate the results of this experiment.
2. What does it mean, in the starch hydrolysis test, when there is a clear zone surrounding bacterial growth?
3. What does it mean, in the starch hydrolysis test, when there is no clear zone surrounding bacterial growth?
4. What is hydrolysis?
5. What is starch hydrolysis?
6. What is amylase?
7. Give the chemical reaction catalyzed by amylase.
8. Why is it an advantage for a bacterial species to be able to hydrolyze starch?
9. Do all bacteria hydrolyze starch? Explain your answer.
10. Fill in the blank. Whether a species of bacteria is capable of producing amylase is dependent on _____.
11. The starch hydrolysis test is useful for identifying and characterizing bacteria. Why?

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4.4: Citrate Test

Learning Objectives

- Explain what the citrate test indicates about a bacteria species' genes, enzymes, and metabolism.
- Tell what citrate permease is and what it does for bacterial species that have it.
- Tell that the citrate test is useful for bacterial identification and characterization.
- Successfully conduct a citrate test and interpret the results.

Citrate Test

All living things need carbon to survive. The carbon-containing molecules that bacteria can utilize as a carbon source differs based on the bacterial species and is dependent on their genes. Their genes dictate what enzymes the bacterial species can produce. Since enzymes are necessary for a cell's metabolic reactions, the genes of a bacterial species dictate their abilities to use different carbon sources.

Some bacterial species, but not all bacterial species, can utilize **citrate** as a source of carbon. Organisms that can survive using citrate as the sole source of carbon have a **citrate permease** enzyme that can transport citrate molecules into the cell. The citrate is then made into pyruvate, which can be converted into a variety of different products in the cell.

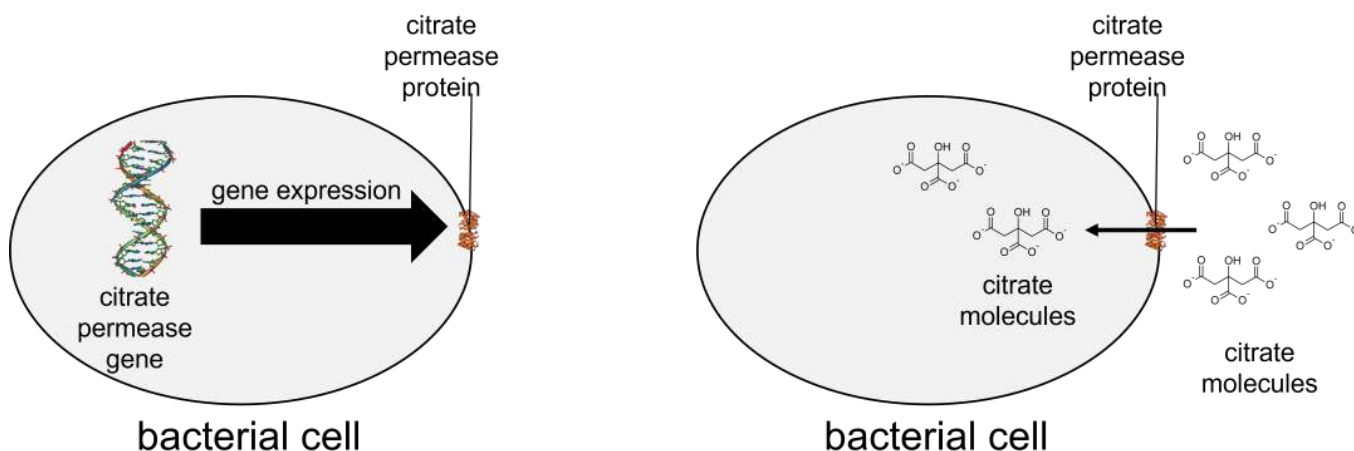


Figure 1: Bacteria with the citrate permease gene can produce the protein citrate permease by gene expression (left). Citrate permease is a membrane protein that enables the transport of citrate into the cell. (Right) When a bacterial cell produces citrate permease, citrate molecules are transported into the bacterial cell. This enables the bacteria to utilize citrate as a source of carbon.

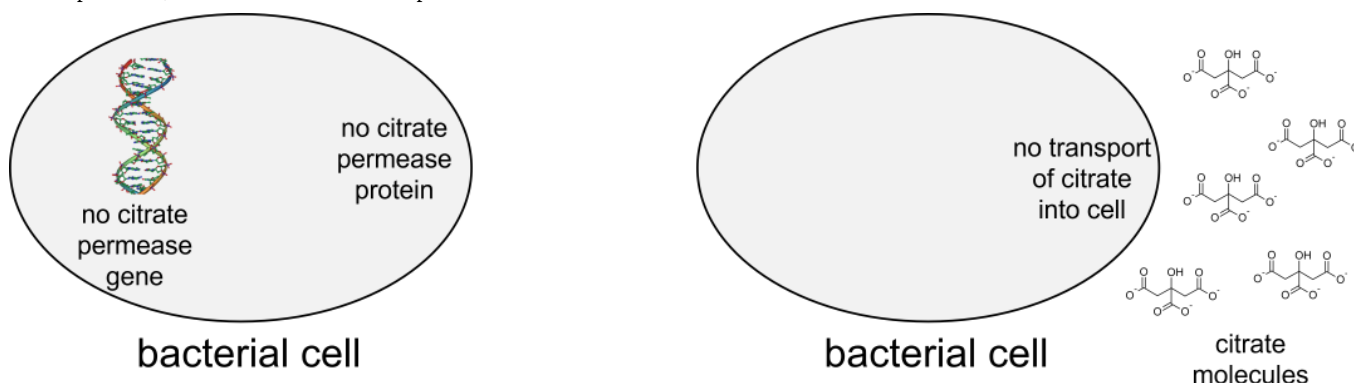


Figure 2: Bacteria without the citrate permease gene cannot produce the protein citrate permease (left). Without the citrate permease protein, citrate is unable to enter the bacterial cell (right). As a result, the bacteria cannot use citrate as a source of carbon.

Simmons' citrate is a chemically defined microbiological medium that contains sodium citrate as the sole carbon source. A pH indicator, bromothymol blue, is also included in Simmons' citrate medium. Bacteria that can grow on this medium (i.e., that can

survive on citrate as the sole source of carbon) produce alkaline byproducts that will change the medium color from green (neutral pH) to blue (alkaline pH).



Figure 3: Results of the citrate test. When the medium stays green (no color change), the test indicates the bacterial species is citrate negative (left). When the medium changes color to blue, the test indicates the bacterial species is citrate positive (right).

Bacterial species that are capable of metabolizing citrate are considered **citrate positive** and will cause Simmons' citrate agar to change from green to blue. Bacterial species that are incapable of metabolizing citrate are considered **citrate negative** and will result in Simmons' citrate agar remaining green (no color change). Since only some bacterial species can metabolize citrate, the citrate test is useful for bacterial identification and characterization.

Laboratory Instructions

Citrate Test

1. Obtain two test tubes with Simmons' citrate agar slants.
2. Use tape to label the test tubes with your group name, bacteria name, and the type of test medium.
3. Using a sterile inoculating loop, aseptically obtain a small amount of bacteria and streak the entire slant of the citrate agar.
Repeat for both species.
4. Incubate the inoculated tube at 37 °C until next lab session.
5. Observe the color of the Simmons's citrate slant.
 - bright blue is citrate positive
 - no color change (agar is still green) is citrate negative

Results & Questions

	citrate (+/-)	bacterial species has citrate permease (+/-)	bacterial species has citrate permease gene (+/-)
<i>Escherichia coli</i>			
<i>Serratia marcescens</i>			

1. Complete the table above with the results observed.
2. How does the citrate test relate to understanding the metabolism of a bacterial species?
3. What does the color change from green to blue mean about the pH of the culture?
4. What does the color change from green to blue mean about the bacterial species in the culture?
5. Can the citrate test be used for bacterial identification and characterization? Explain your answer.

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4.5: MR-VP Tests

Learning Objectives

- Describe the purpose and usefulness of the MR-VP test.
- Compare and contrast the MR test and the VP test.
- Tell how the MR test and VP test are conducted and what the results mean.
- Define fermentation and describe its importance.
- Name the metabolic pathway that occurs in MR positive bacterial species.
- Name the metabolic pathway that occurs in VP positive bacterial species.
- Successfully conduct and interpret the MR-VP test.

Methyl Red–Voges Proskauer (MR-VP) Test

The **MR-VP test** is actually a set of two separate tests that uses one broth medium containing glucose to determine the possible types of glucose fermentation being conducted by a bacterial species. Some bacterial species may ferment glucose using a mixed acid fermentation pathway (detected in the **MR test** or **methyl red test**) while some other bacterial species may ferment glucose via the butanediol fermentation pathway (detected in the **VP test** or **Voges-Proskauer test**).

Since the type of fermentation conducted differs by bacterial species (or strain), testing the type of fermentation pathway is useful for characterizing bacteria and identifying them. Metabolic reactions are dependent on the enzymes that species (or strain) has, which is dependent on the genes that species (or strain) carries in its DNA. Therefore, testing the types of metabolisms species conduct provides insights into differences in their genetics (their DNA).

Fermentation

Fermentation is a type of metabolic pathway some bacterial species conduct to produce ATP (ATP is used as an energy source to keep the cell alive). Fermentation is an anaerobic process (no O₂ is used) and species that conduct fermentation will produce a small amount of ATP that has been derived from the energy in a glucose molecule.

Fermentation begins with **glycolysis**, the metabolic pathway that breaks down glucose into two pyruvate (or pyruvic acid) molecules and will produce a net gain (overall gain) of 2 ATP. Then, in a fermentation pathway (the pathways differ depending on the species), pyruvate is converted into other molecules to regenerate NAD⁺ so the glycolysis pathway can continue (NAD⁺ is a cofactor in a glycolysis reaction that must be available for glycolysis to occur). Additionally, fermentation pathways utilize organic molecules, derived from pyruvate, as final electron acceptors.

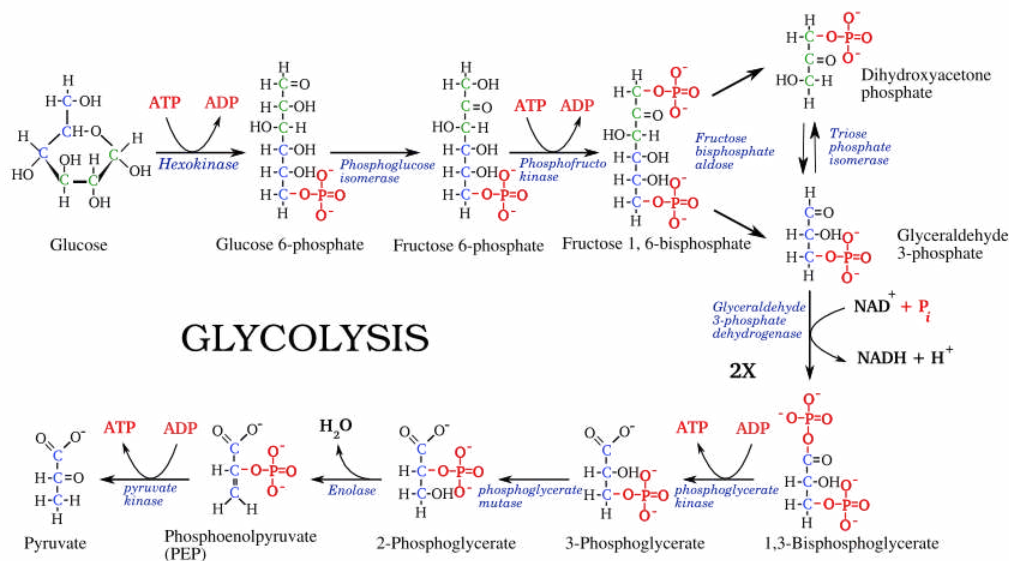


Figure 1: Glycolysis. This chemical pathway begins with a glucose molecule that is converted into two pyruvate molecules. Although two ATP molecules are required at the beginning of this metabolic pathway, in the later reactions, four ATP molecules are produced, leaving a net gain of two ATP molecules for the entire pathway. In species conducting fermentation, pyruvate is used in the fermentation pathway to regenerate NAD⁺ and to produce an organic molecule serving as a final electron acceptor.

Methyl Red (MR) Test Detects Mixed Acid Fermentation

Mixed-acid fermentation pathways are fermentation pathways that some bacterial species conduct to produce a mixture of fermentation products. There is not just one way bacteria conduct mixed acid fermentation, but a number of them. The end-products produced through mixed-acid fermentation is species-dependent and sometimes strain-dependent (depends on the genes that the species has; genes dictate the enzymes produced and therefore the chemical reactions occurring in the species' or strains' metabolism). Below shows one example of glycolysis with a mixed acid fermentation pathway that produces acetoin, formate, lactate, succinate, acetate, and ethanol as byproducts of the process.

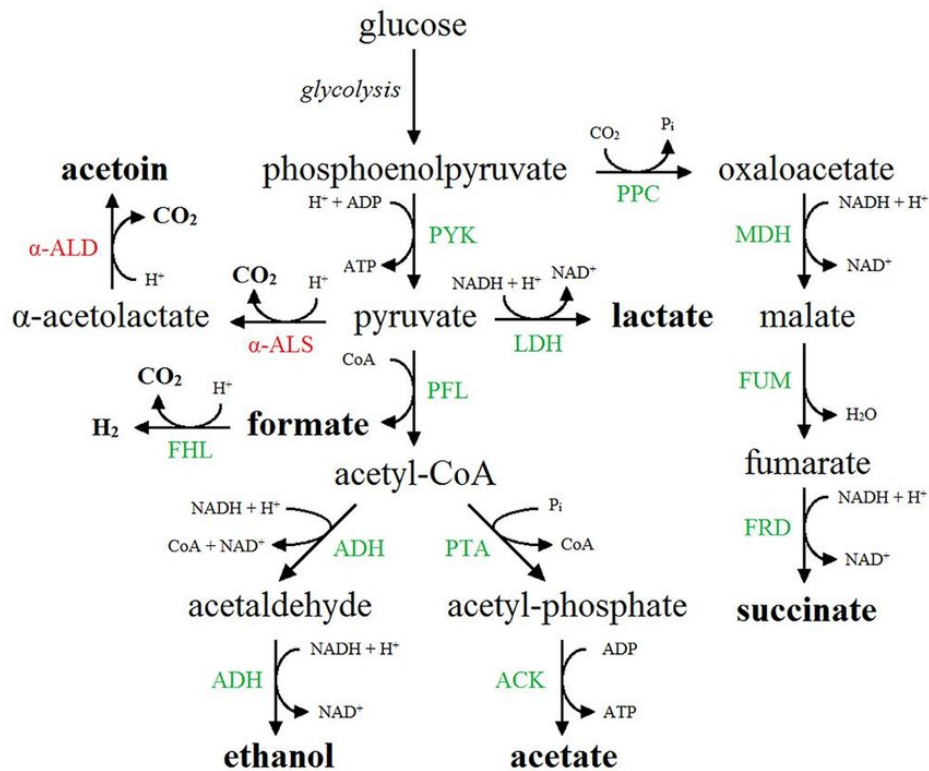


Figure 2: An example of one type of mixed acid fermentation in a strain of *Escherichia coli* that is expressing acetoin synthesis operon (budAB operon). In this example, the fermentation pathway produces acetoin, lactate, formate (which is subsequently broken down to H₂ and CO₂), succinate, acetate, and ethanol. All of these fermentation products, except formate and acetoin pathways, regenerate NAD⁺ to enable the continuation of glycolysis (NAD⁺ is an essential cofactor in glycolysis and required for glycolysis to continue occurring).

The methyl red test (MR) utilizes a liquid medium containing glucose. After this medium is inoculated and incubated to enable bacteria to grow and conduct fermentation, the reagent methyl red is added to the medium. Methyl red is a pH indicator that will detect mixed acid production by changing the medium to a red color (**methyl red positive**). In the absence of mixed acid fermentation, methyl red will not produce a red color (**methyl red negative**).

MR-VP Broth (glucose) → pyruvate → mixed acid fermentation pathway (red color with methyl red)



Figure 3: Results of the methyl red test (MR). (Left) The medium does not turn red in the negative methyl red test indicating that the bacterial species tested does not ferment glucose using the mixed acid fermentation pathway. (Right) The medium turns red in the positive methyl red test indicating that the bacterial species does ferment glucose using the mixed acid fermentation pathway.

Voges-Proskauer (VP) Test Detects Butanediol Fermentation

The Voges-Proskauer test determines if 2,3-butanediol is a product of glucose fermentation by a bacterial species. This fermentation byproduct typically is produced by species of *Klebsiella* and *Enterobacter*.

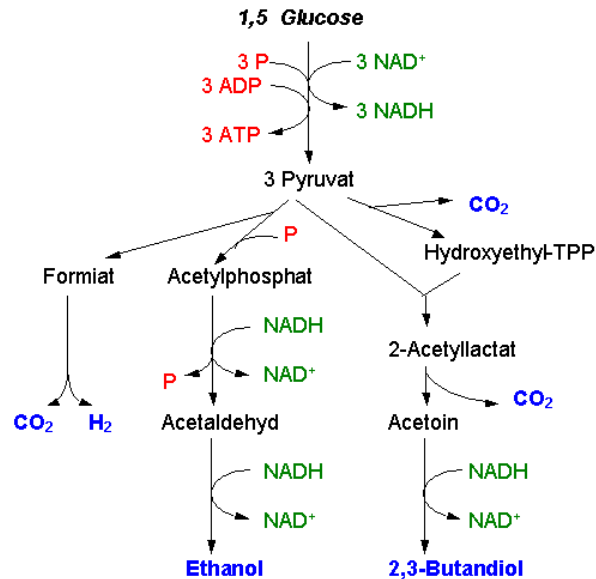


Figure 4: Part of glycolysis is shown with the metabolic reactions occurring in butanediol fermentation (metabolic byproducts are shown in blue).

The Voges-Proskauer test removes some of the MR-VP medium into a separate test tube prior to conducting the methyl red test and adds Barritt's A reagent followed by Barritt's B reagent to the medium. After mixing and a rest period, a liquid layer forms at the top of the test tube indicating the test results. If the top liquid layer is red or red-brown, the bacteria is **Voges-Proskauer positive** indicating it produces 2,3-butanediol as a byproduct of fermentation. If the top liquid layer is yellow, the bacteria is **Voges-Proskauer negative** indicating it does not produce 2,3-butanediol as a byproduct of fermentation.

MR-VP Broth (glucose) → pyruvate → butanediol fermentation pathway (red color in top layers of medium after adding reagents, mixing, and resting)



Figure 5: Results of the Voges-Proskauer test. (Left) When the color at the top of the tube is yellow, this is a negative Voges-Proskauer test indicating that the bacterial species tested does not ferment glucose using the butanediol fermentation pathway. (Right) When the color at the top of the tube is a red-brown color, this is a positive Voges-Proskauer test indicating that the bacterial species tested ferments glucose using the butanediol fermentation pathway.

Laboratory Instructions

1. Obtain a MR-VP broth tube. Use tape to label the tube with your name, your assigned organism(s) and the name of the test media.
2. Aseptically transfer bacteria with a loop to inoculate the MR-VP broth.
3. Incubate the inoculated tube in the class test tube rack until next lab session.
4. After incubation, using a transfer pipette, transfer half of the inoculated MR-VP broth to a clean test tube. Label this tube "VP." Leave the other half of the inoculated broth in the original MR-VP broth test tube. Label this tube "MR."
5. **Methyl red test:**
 1. Add 10 drops of methyl red reagent to "MR" tube.
 2. Examine the color of the medium.
 3. Record the results.
6. **Voges-Proskauer test:**
 1. First add 15 drops of Barritt's A reagent (alpha-naphthol) to the "VP" tube.
 2. Add 5 drops of Barritt's B reagent (40% KOH) to the "VP" tube. **NOTE: A reversal in the order of the reagents may result in a weak-positive or false-negative reaction.**
 3. Hold the test tube from the glass and mix the tube well by flicking it with your fingers.
 4. Let the test tube sit **undisturbed** in a test tube rack for 20 minutes.
 5. Examine the color of the top layer of liquid and record the results.

Results & Questions

bacterial species tested	color of medium for MR test	results from methyl red test (+/-)	species conducts mixed-acid fermentation (+/-)	color of top liquid layer for VP test	results from Voges-Proskauer test (+/-)	species conducts butanediol fermentation (+/-)
<i>Escherichia coli</i>						
<i>Enterobacter aerogenes</i>						

1. Complete the table above to summarize results and interpretations of those results.
2. What is fermentation and why is it important for bacterial species that conduct fermentation?
3. What are the interrelationships between glycolysis and fermentation (there are two main ones)?
4. What is mixed-acid fermentation?
5. What is butanediol fermentation?
6. Explain why the MR-VP test is useful for characterizing and identifying bacterial species.

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4.6: Coagulase Test

Learning Objectives

- Describe what coagulase is and what this enzyme does.
- Tell that bacterial species that produce coagulase are pathogenic.
- Identify that the coagulase test is useful for differentiating between *Staphylococcus* sp.
- Tell how the two types of coagulase tests work and which one is more definitive.
- Successfully conduct and interpret the coagulase test.

Coagulase

The enzyme **coagulase**, produced by *a few* of the *Staphylococcus* species (but not all of them), is a key feature of pathogenic *Staphylococcus* species. The coagulase enzyme produces **coagulation** of blood, allowing the microorganism to "wall" its infection off from the host's protective mechanisms rather effectively. As with other enzymes, whether or not a species can produce an enzyme is dependent upon whether that species has the gene for the enzyme. Therefore, testing for coagulase activity tells us about the genes and DNA of a bacterial species.

This test determines the ability of the bacteria to produce bound coagulase. This exoenzyme will cause the conversion of fibrinogen in blood plasma to fibrin and form a clot. The fibrin covers the surface of the bacteria, making it resistant to phagocytosis.

There are 2 methods to test for coagulase: (1) slide agglutination test and (2) tube agglutination test. The tube agglutination test is more accurate, but the slide agglutination test is faster. If you have an unknown *Staphylococcus* species, you might consider running the slide test, and, if negative, running the tube test. The tube method is the definitive test.

Pathogenic *Staphylococcus* species (e.g. *Staphylococcus aureus*) can be confirmed using the coagulase test. Since there are 2 kinds of coagulase enzymes—bound and free---there are 2 different tests that can be used to identify these enzymes. Both of the enzymes activate fibrinogen in plasma, in different ways. Your instructor will direct you as to which procedure to use, but the tube test is the definitive test: if you get a negative test result for the slide test, run the tube test.

Laboratory Instructions

Tube Agglutination Test

If the slide coagulase test reaction is negative, inoculate a tube of rabbit plasma overnight and check for a clot in the tube. This is considered a positive reaction for free coagulase.

1. Inoculate a tube containing ½ ml of rabbit plasma with the bacterial inoculum.
2. Place at 37° C and check at ½ hour or at next lab period (some strains will give a + reaction in a few hours, other strains take longer) by tipping the slide at an angle.
3. Any degree of coagulation is considered a positive test for the free coagulase enzyme.

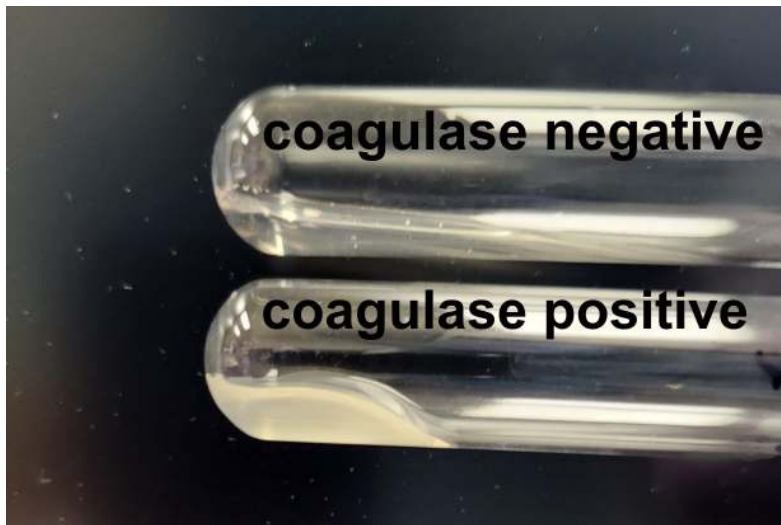


Figure 2: The tube agglutination coagulase test. When the test tube is tilted, if the fluid is not clumped and does not contain clumps (clotting), the result is coagulase negative (top). When the test tube is tilted, the fluid either exhibits a clump or contains clumps in it (clotting), the result is coagulase positive (bottom).

Results & Questions

	coagulase (+/-)	species produces a coagulase enzyme (+/-)	species contains a coagulase gene (+/-)
<i>Staphylococcus aureus</i>			
<i>Staphylococcus epidermidis</i>			

1. Complete the table above to indicate results of the coagulase test.
2. A coagulase positive result indicates what about the bacterial species' pathogenicity?
3. When a bacterial species living inside of a host (such as a human) produces coagulase, what does coagulase do? Be specific.
4. Can testing for coagulase activity be useful for identifying and characterizing bacterial species? Explain your answer.
5. The coagulase test is useful for differentiation bacterial species with the genus _____.

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4.7: Triple Sugar Iron Medium

Triple Sugar Iron is a slant medium with two growth environments: aerobic (on the slant) and anaerobic (in the “butt”). The medium contains three sugars in varying concentrations and a pH indicator that turns yellow at pH measurements below 6.8, and a deeper red at pH measurements above 8.2. Bacteria that ferment typically produce one or more types of acid as a byproduct, therefore, fermentation (both aerobic on the slant and anaerobic in the butt) is noted as a change in the color of the media. The medium also identifies strict aerobes that only grow on the slant surface, and also bacteria that produce H_2S , either as a way to produce ATP anaerobically using sulfur or sulfate as a final electron acceptor, or as a result of the breakdown of proteins that contain high numbers of sulfur-containing amino acids (cysteine or methionine).

The results of this test are reported as appearance of the slant/appearance of the butt, using A to indicate acid reaction (yellow color), K to indicate an alkaline reaction, and NC to indicate no change in the medium. H_2S (detected as a blackening in the media) and the production of gas (CO_2) as a byproduct of fermentation are also reported if observed (see Figure 4).

As an example, and for practice, the interpretation and outcomes for the 4 TSI tests shown are provided in the table below. Note that many other possible reactions may also occur so proper interpretation of this test is important.



Table 1. TSI reactions shown in the cultures in Figure 4, from left to right.

Outcome	Interpretation
Uninoculated control	For color comparison with inoculated samples
K/NC	Aerobic respiration (dark red on the slant) only. Bacteria are strict aerobes.
A/A; gas	Fermentation of all three sugars with CO_2 produced. Bacteria are facultative anaerobes.
K/A; H_2S	Aerobic respiration (dark red on slant), fermentation of glucose (acid only in butt), anaerobic respiration (black in butt). Bacteria are facultative anaerobes.
K/A	Aerobic respiration (dark red on the slant); fermentation of glucose (acid only in butt). Bacteria are facultative anaerobes.

How would you interpret the outcome of the TSI slant, the appearance of which is described below?

Appearance	Outcome and Interpretation
Slant is a dark red color, butt is yellow with noticeable cracking and bubbling.	

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CHAPTER OVERVIEW

5: Lab 5

5.1: Required Materials

5.2: Gelatin Hydrolysis

5.3: Cytochrome c Oxidase

5.4: Catalase Test

5.5: Bacterial Oxygen Requirements

5.6: Nitrate Reduction

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5.1: Required Materials

Cultures Needed

- TSB cultures
 - *E. coli*
 - *P. aeruginosa*
 - *Streptococcus pyogenes*
 - *S. aureus*
 - *Alcaligenes faecalis*

Gelatin Hydrolysis

- Gelatin strips

Cytochrome c Oxidase

- Oxidase reagent
- Filters

Catalase Test

- H₂O₂
- Slides

Bacterial Oxygen Requirements

- Gas Pak and anaerobic chamber

Nitrate Reduction

- Nitrate broth tubes w/Durham tubes
- Zinc dust

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5.2: Gelatin Hydrolysis

Learning Objectives

- Explain what gelatin is and what gelatin hydrolysis is.
- Tell that species that can hydrolyze gelatin have a gelatinase gene and produce a gelatinase enzyme.
- Explain how the gelatin hydrolysis test works.
- Successfully conduct and interpret the gelatin hydrolysis test.
- Tell that gelatin hydrolysis is useful for characterizing and identifying bacterial species.

Gelatin & Gelatin Hydrolysis

Gelatin is a protein derived from collagen, a connective tissue of animals. When chilled on ice, gelatin forms cross-links to itself to create a semi-solid state (gelatin makes Jello! have its unique form and texture). Gelatin provides a rich source of amino acids and peptides for bacteria, but its structure is too large to be transported inside the cell directly. Therefore, it must first be broken down (**gelatin hydrolysis**) by exoenzyme proteins called **gelatinases**. Not all species of bacteria have a gene for a gelatinase enzyme. Only those species of bacteria that do have a gelatinase gene are capable of producing a gelatinase and are capable of breaking down gelatin and using it as a nutrient source. As such, the gelatin hydrolysis test is used to differentiate between bacteria that do and do not hydrolyze gelatin, and therefore is useful for characterizing and identifying bacterial species.

To determine if a bacterial species hydrolyzes gelatin (and therefore has the a gelatinase gene and produces a gelatinase enzyme), the **gelatin hydrolysis test** is used. When bacteria that have this enzyme are inoculated into a nutrient medium containing gelatin, they will produce a gelatinase enzyme and break down the gelatin. After incubation of the bacteria in the medium that hydrolyze gelatin, the medium will no longer form a semi-solid state, even after chilling. Those species that do not hydrolyze gelatin will not have an effect on the gelatin and it will maintain its semi-solid state after chilling.

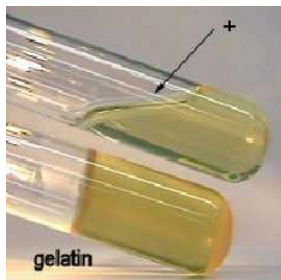


Figure 1: Results from two gelatin hydrolysis tests. The top test tube shows a gelatin medium after chilling that has a more liquid form indicating that gelatin hydrolysis occurred (gelatin hydrolysis positive). The bottom test tube shows a gelatin medium after chilling that has a semi-solid state indicating that gelatin hydrolysis did not occur (gelatin hydrolysis negative).

Laboratory Instructions

1. For each bacterial species being tested, use aseptic technique to collect the bacteria using an inoculation needle and stab the gelatin deep with a needle all the way to the bottom (being careful NOT to get the metal holder into the agar).
2. Incubate at 25° C for a couple days, up to a week. Keep the medium for a week if negative.
3. Examine results by placing the test tubes on ice for 15 minutes or in the fridge for 30 minutes.

Results & Questions

1. Did your specimen hydrolyze gelatin?
2. Why is it that when gelatin is broken down the gelatin medium no longer forms a semi-solid even after chilling?
3. What is gelatin?
4. Why might the ability to hydrolyze gelatin be an advantage for some species of bacteria?
5. Why might gelatin hydrolysis be associated with pathogenic species and not non-pathogenic species?

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5.3: Cytochrome c Oxidase

Learning Objectives

- Describe the role of cytochrome c oxidase in bacterial cells.
- Tell that cytochrome c oxidase is found in only certain species of bacteria and is therefore useful for bacterial species identification and characterization.
- Explain what being "oxidase positive" means about a bacterial species metabolism, electron transport chain, and genetics.
- Explain that being "oxidase negative" does not mean the species is anaerobic, but just that it does not contain the cytochrome c oxidase enzyme and gene.
- Successfully conduct and interpret an oxidase test.

Cytochrome c Oxidase

Aerobic respiration is an O_2 -requiring process that uses energy from nutrient molecules to produce ATP molecules to provide for the cell's energy needs. During aerobic respiration, the electron transport chain transfers high-energy electrons from protein to protein and uses that energy to build up a H^+ gradient that is utilized by ATP synthase to make ATP. At the end of the aerobic electron transport chain, an enzyme transfers the electron from the electron transport chain to O_2 (the electrons at the end of the chain are low energy). In some bacteria capable of aerobic respiration, that enzyme that transfers electrons to O_2 , the final electron acceptor for aerobic respiration, is called **cytochrome c oxidase**.

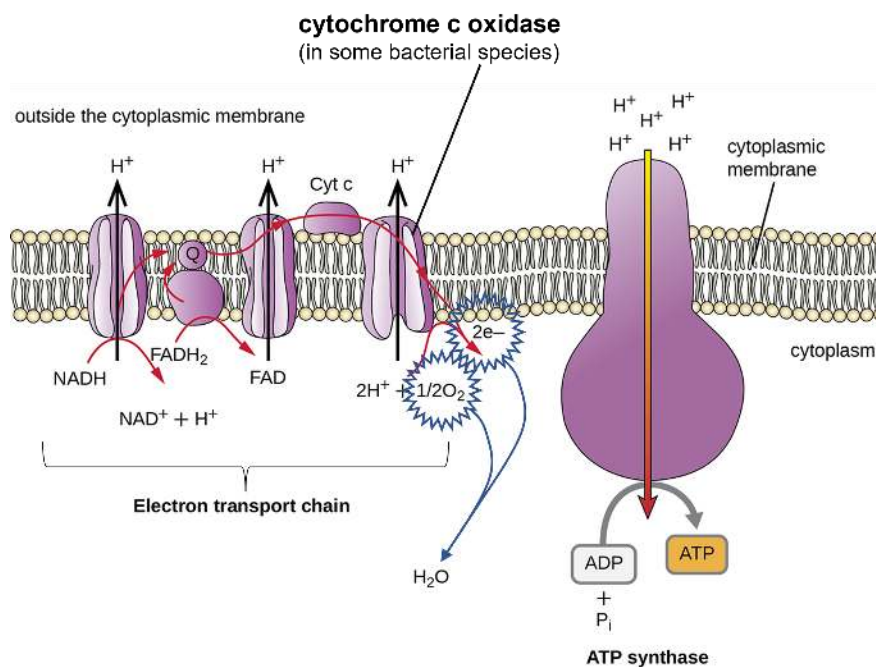


Figure 1: The bacterial electron transport chain is a series of protein complexes, electron carriers, and ion pumps that are used to pump H^+ out of the bacterial cytoplasm into the extracellular space. The last enzyme in the electron transport chain can be (depends on the species) cytochrome c oxidase. The role cytochrome c oxidase plays is to remove electrons from the chain by transferring them to O_2 with H^+ to produce water. In aerobic bacterial species that do not have cytochrome c oxidase, this process still occurs, but using an enzyme that has a different structure and different name than cytochrome c oxidase. The gradient of H^+ produced by the electron transport chain is used by the ATP synthase to make ATP. H^+ flows back down the electrochemical gradient into the bacterial cytoplasm through ATP synthase, providing the energy for ATP production by oxidative phosphorylation. (credit: modification of work by Klaus Hoffmeier)

A test called the **oxidase test** can be used in the laboratory to determine if a bacterial species has cytochrome c oxidase. Those species that have cytochrome c oxidase are called **oxidase positive** and those species that do not have cytochrome c oxidase are called **oxidase negative**. The ability of a bacterial species to produce cytochrome c oxidase is coded in its DNA. If the cytochrome c oxidase gene is present in the bacterial species, it will produce this enzyme. If the gene is absent, that bacterial species cannot

produce cytochrome c oxidase. The oxidase test is therefore useful for characterizing bacterial species and differentiating bacterial species from each other for identification purposes.

Note

Bacterial species that contain cytochrome c oxidase are capable of aerobic respiration. Oxidase positive bacterial species are **all** aerobic.

However, oxidase negative species **are not necessarily anaerobic**. Oxidase negative species may still be aerobic species or facultative anaerobes, but they produce and use a different enzyme, other than cytochrome c oxidase, to transfer electrons from the electron transport chain to O_2 .

The Oxidase Test

The **oxidase test** is a key test to differentiate between the bacterial families *Pseudomonadaceae* (oxidase positive species) and *Enterobacteriaceae* (oxidase negative species), and is useful for speciation and identification of many other bacteria.

The oxidase test utilizes a special reagent called **oxidase reagent**. This reagent is colorless. However, in the presence of cytochrome c oxidase, the oxidase reagent will transfer its electrons to cytochrome c oxidase and exhibit a bluish or purplish color in 30 seconds or less.

- **oxidase positive** bacteria contain cytochrome c oxidase and produce a change in color of the reagent from colorless to bluish or purplish in less than 30 seconds.
- **oxidase negative** bacteria do not contain cytochrome c oxidase and do not change the color of oxidase reagent in less than 30 seconds.

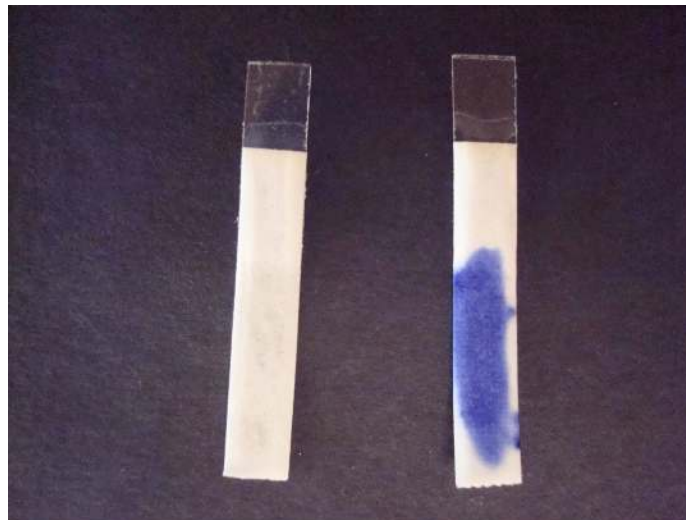


Figure 2: Results from the oxidase test. (Left) An oxidase test strip containing oxidase reagent is still white indicating bacteria is oxidase negative. (Right) The oxidase test strip containing oxidase reagent is purple indicating the bacteria are oxidase positive. The test only indicates oxidase positive if the purple color appears in 30 seconds or less.

Laboratory Instructions

Oxidase Test

1. Obtain an oxidase test strip and half of an empty petri plate.
2. Place the oxidase test strips face up on the half empty petri plate.
3. Lightly moisten the oxidase test strip with a small drop of water. Do not over-moisten the strip!
4. Using a sterile swab or sterile loop, obtain a large amount of bacteria from a petri plate.
5. Rub the bacteria on the swab or sterile loop onto the moistened region of the oxidase test strip.
6. Start a timer for 30 seconds.
7. Watch for purple color to develop on the oxidase test strip within 30 seconds.

- purple or bluish color change in less than 30 seconds is oxidase positive
 - no color change within 30 seconds is oxidase negative (color change after 30 seconds is considered oxidase negative)
8. Repeat for each bacterial species you are testing.

Results & Questions

1. What were the results of the oxidase test for each bacterium?
2. What metabolic pathway is cytochrome c oxidase important in?
3. What would happen to a bacterial cell that lost its ability to produce cytochrome c oxidase?
4. Is a bacterial species that produces cytochrome c oxidase aerobic? Explain your answer.
5. Is a bacterial species that does not produce cytochrome c oxidase anaerobic? Explain your answer.
6. Is the oxidase test useful for bacterial species identification and characterization? Explain your answer.

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5.4: Catalase Test

Learning Objectives

- Describe what catalase is and why it is important for bacterial survival.
- Give the chemical reaction catalyzed by catalase.
- Explain how testing for catalase is useful for characterizing and identifying bacterial species.
- Tell the types of bacterial species that typically produce catalase and those that typically do not produce catalase.
- Successfully conduct a catalase test.
- Interpret results for a catalase test.

Catalase

Catalase is an enzyme produced by some species of bacteria. This enzyme protects bacteria from hydrogen peroxide (H_2O_2) that can damage and kill them. Catalase will convert hydrogen peroxide into liquid water (H_2O) and oxygen gas (O_2). As a result, if catalase is very active due to an abundance of hydrogen peroxide, the rapid production of oxygen gas (O_2) will produce bubbles.

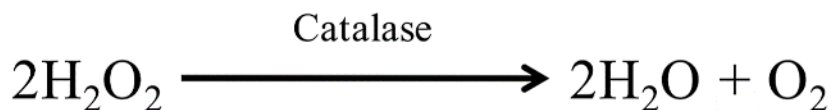


Figure 1: The reaction catalyzed by the enzyme catalase. Two hydrogen peroxide molecules ($2 \text{H}_2\text{O}_2$) is converted into two water molecules ($2 \text{H}_2\text{O}$) and an oxygen molecule (O_2). "Catalase" is written over the reaction arrow to show that this is the enzyme that enables this chemical reaction to occur.

Bacteria that conduct aerobic metabolism (biological reactions that require O_2) produce hydrogen peroxide (H_2O_2) as a toxic byproduct of their metabolism. Toxic hydrogen peroxide can cause intracellular damage, such as damage to DNA, lipids, and proteins. To remove H_2O_2 and other similar compounds, cells produce enzymes to break them down, such as catalase.

Bacteria can only make catalase if they have the gene for catalase in their DNA. When the catalase gene is expressed by the cell (DNA $\rightarrow$ RNA $\rightarrow$ protein), the bacteria produce catalase. Only species that have the catalase gene will make catalase and will test **catalase positive**. Bacterial species that do not have the catalase gene cannot make catalase and will test **catalase negative**. Therefore, testing for the presence or absence of catalase is a way bacterial species can be characterized and another tool for classifying and identifying bacterial species.

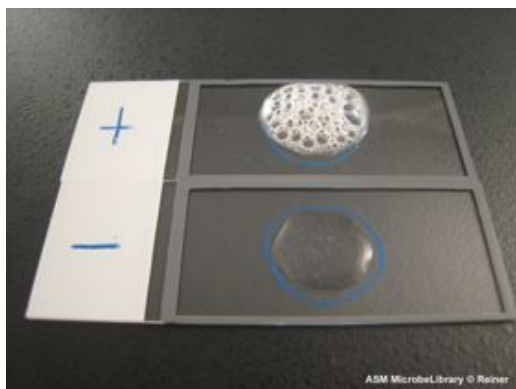
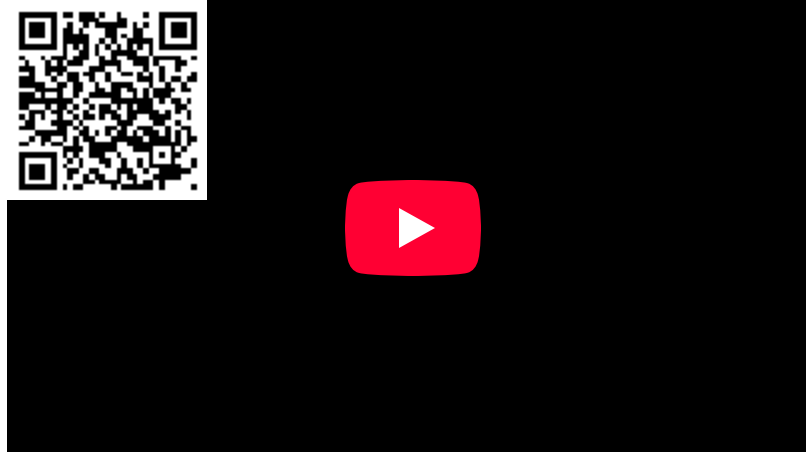


Figure 2: Slide catalase test results. Hydrogen peroxide was added directly to bacteria placed on a microscope slide. A catalase positive reaction is indicated by bubbling (top); a negative reaction is indicated by lack of bubbling (bottom). Image by Karen Reiner, Andrews University, Berrien Springs, MI.

A simple test to determine if bacteria produce catalase is to add hydrogen peroxide to bacteria. This may be done by adding the hydrogen peroxide to bacteria placed on a slide or adding it to bacteria growing on an agar slant. If catalase is present, the hydrogen peroxide will be broken down into water and oxygen gas, resulting in the production of bubbles (catalase positive). This test does

not require any special type of medium, however it should never be performed on organisms that have been grown on blood agar (a medium that contains blood). This is because there is a catalase activity in blood that would produce a false positive result.

Most aerobic bacteria (bacteria that require O_2) and facultatively anaerobic bacteria (bacteria that live and metabolize O_2 or in an environment without O_2) produce catalase. Obligate anaerobe bacterial species (bacteria that must live in an environment without O_2 to survive) lack catalase, which is why they cannot survive in an atmosphere containing oxygen. However, some of them have modified versions of catalase to deal with any possible exposure to oxygen.



Laboratory Instructions

Catalase Test

1. Obtain a glass slide and a bottle of hydrogen peroxide.
2. Using a sterilized inoculating loop, smear a small amount of bacteria onto the dry slide.
3. Place a drop of hydrogen peroxide on top of the bacteria.
4. Look for bubbles immediately:
 - bubbles = catalase positive
 - no bubbles = catalase negative

Results & Questions

1. Was the catalase test positive or negative for each bacterium tested?
2. What is catalase?
3. Why is catalase production an advantage for bacteria to survive?
4. What types of bacteria typically produce catalase?
5. What types of bacteria typically do not produce catalase?
6. Give the chemical reaction catalyzed by the catalase enzyme.
7. When a bacterial species produces the catalase enzyme, what does it tell us about this species genes?
8. When a bacterial species does not produce the catalase enzyme, what does this tell us about this species genes?
9. Why can the catalase test be used to help with bacterial identification?

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5.5: Bacterial Oxygen Requirements

Learning Objectives

- Explain how aerobic and anaerobic respiration differ.
- Define and recognize descriptions of obligate aerobes, obligate anaerobes, facultative anaerobes, microaerophiles, and aerotolerant anaerobes.
- Tell that bacterial species' oxygen requirements are useful for species identification and characterization.
- Describe how thioglycollate agar tubes work and how they can be used to determine bacterial species' oxygen requirements.
- Tell how anaerobic chambers and GasPak systems work to culture obligate anaerobe species.
- Successfully conduct and interpret thioglycollate cultures to determine oxygen requirements of bacteria.
- Successfully grow bacteria in aerobic and anaerobic conditions and interpret the results.

O₂ and Bacterial Metabolism & Growth

Bacteria can differ dramatically in their ability to utilize oxygen (O₂). Under aerobic conditions, if the bacterial species can conduct aerobic respiration, oxygen acts as the final electron acceptor for the electron transport chain located in the plasma membrane of prokaryotes. Bacteria use the electron transport chain to generate a H⁺ gradient that is used by ATP synthase to make ATP. ATP is the energy source for most cellular processes and therefore essentially for keeping cells alive. In the absence of oxygen (O₂), some bacteria can use alternative metabolic pathways including anaerobic respiration and/or fermentation. During anaerobic respiration, other alternative molecules are used as the final electron acceptor for the electron transport chain such as nitrate (NO₃), sulfate (SO₄), and carbonate (CO₃).

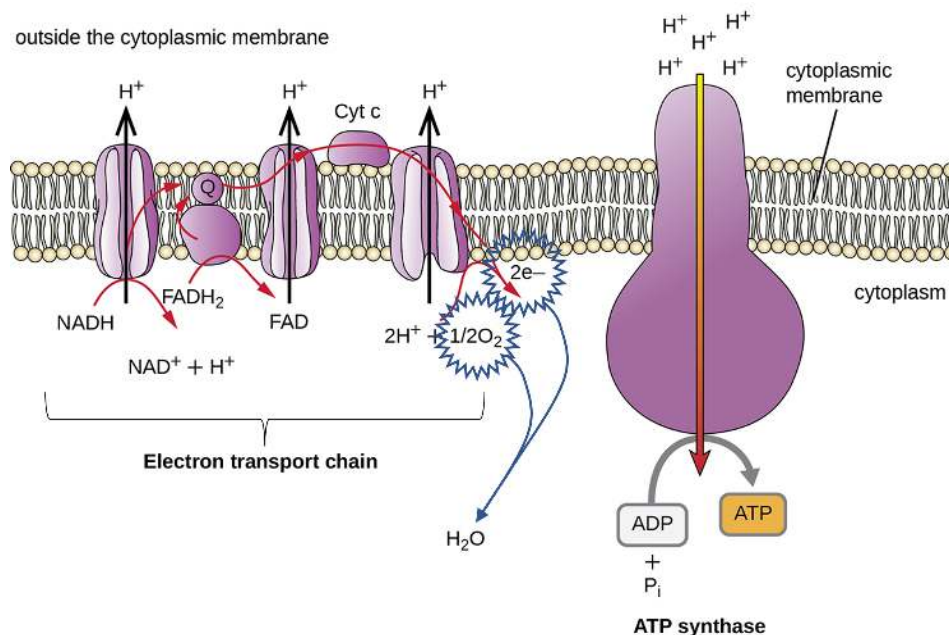


Figure 1: The electron transport chain and oxidative phosphorylation in aerobic respiration. This process is aerobic because oxygen (O₂) is the final electron acceptor and combines with electrons from the electron transport chain to remove them. The electron transport chain and oxidative phosphorylation still occur for bacterial species capable of anaerobic respiration, but instead of using oxygen as the final electron acceptor, they use a different molecule (the molecule used as the final electron acceptor depends on the species but examples include nitrate, NO₃, sulfate, SO₄, and carbonate, CO₃).

The presence or absence of molecular oxygen can be a critical factor in the ability of bacteria to grow in each environment. When bacteria use oxygen in cellular respiration and other chemical reactions, toxic superoxide and peroxides are produced. These highly reactive byproducts damage the cell unless they are quickly neutralized. **Aerobic** bacteria (grow in O₂ environments) produce enzymes such as catalase, peroxidase and/or superoxide dismutase that break down toxic forms of oxygen and their intermediate byproducts. Bacteria called **anaerobes** produce ATP via anaerobic means (anaerobic respiration and/or fermentation). Anaerobes

have no tolerance for oxygen since they cannot produce catalase, peroxidase and/or superoxide dismutase to remove toxic byproducts of O_2 .

Bacterial species are classified by their oxygen requirements as follows:

- **obligate aerobes:** Produce ATP via aerobic respiration. Require around 20% atmospheric oxygen.
- **microaerophiles:** Produce ATP via aerobic respiration or fermentation. Require between 5-15% atmospheric oxygen for growth.
- **aerotolerant anaerobes:** Produce ATP via anaerobic respiration and can conduct fermentation. Oxygen can be present, but they do not utilize it for ATP production or fermentation.
- **facultative anaerobes:** Produce ATP via aerobic respiration, anaerobic respiration, and/or fermentation. These organisms grow equally well in aerobic or anaerobic environments.
- **obligate anaerobes:** Produce ATP via anaerobic respiration or fermentation. These bacteria die in the presence of O_2 because they lack the enzymes needed to break down toxic forms of oxygen and their intermediate byproducts.

Since bacterial species differ in their oxygen requirements, testing this feature is useful for identifying and characterizing bacterial species.

Determining Bacterial Oxygen Requirements with Thioglycollate Medium

Thioglycollate is a medium designed to test the aerotolerance (tolerance to O_2) of bacteria. Along with nutrients, it contains a reducing agent, sodium thioglycollate, which combines with oxygen to produce water. Thioglycollate also contains a small amount of agar which helps reduce oxygen diffusion and helps maintain the stratification of organisms growing in different layers of the broth. Because the thioglycollate can eliminate the oxygen in the bottom of the tube, but not at the surface, varying concentrations of oxygen are found within the tube. On occasion, an indicator is added to the media to indicate the presence or absence of oxygen and shows where the aerobic and anaerobic zones separate. For example, resazurin is pink in the presence of oxygen and colorless when reduced.

One can determine a bacterium's oxygen requirements by cultivating them in a special medium called **thioglycollate agar tubes**. Based on the location and distribution of the bacteria in these tubes, a species can be classified as obligate aerobe, microaerophile, facultative anaerobe, aerotolerant anaerobe, or obligate anaerobe.

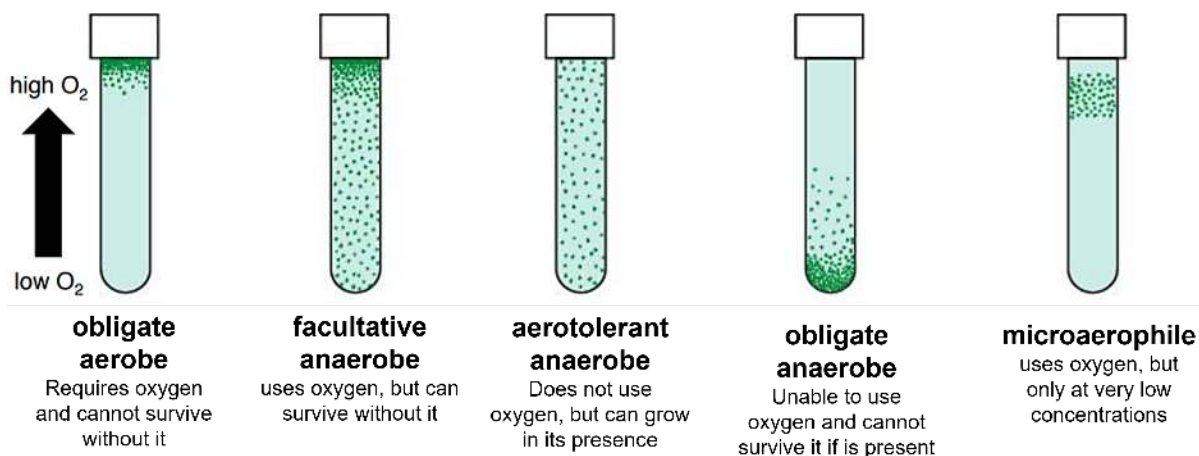


Figure 2: Microbial oxygen requirements can be determined using thioglycollate agar tubes. The green dots in this diagram represent bacterial colonies within the agar or on its surface. The surface of the agar tube is directly exposed to atmospheric oxygen, and will be aerobic. The oxygen content of the thioglycollate medium decreases with depth until the medium becomes anaerobic towards the bottom of the tube.

Growing Bacteria in Strict Anaerobic Conditions

Anaerobic Chambers

The cultivation of anaerobic bacterial species requires an anaerobic chamber. This is a special chamber is a closed environment without O_2 where the microbiologist can work with and cultivate obligate anaerobes without exposing them to oxygen. Anaerobic

chambers contain a hydrogen (H_2) gas mixture that is circulated through a heated palladium catalyst to remove oxygen (O_2) by forming water (H_2O). Anaerobic chambers use a gas mixture of H_2 and nitrogen gas (N_2) (5/95%) or N_2 /carbon dioxide (CO_2)/ H_2 (85/10/5 %) to remove oxygen. An airlock is used to reduce O_2 levels prior to the transfer of samples in and out of the chamber.



Figure 3: A microbiologist using an anaerobic chamber. Gloves reach into the chamber from outside of the chamber to conduct culture transfers and work with anaerobic bacteria. Inside of the anaerobic chamber there is a non- O_2 atmosphere and a catalyst system to remove any O_2 that might enter the chamber when samples and tools are transferred in and out of the chamber.

GasPak Anaerobic Systems

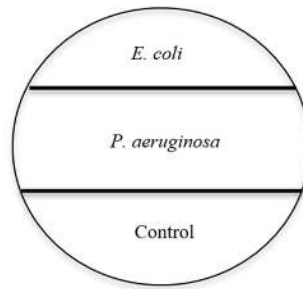
A Gas Pak jar or bag is an alternative way to grow strict anaerobes that must be grown in an atmosphere without oxygen. Plates or tubes are placed in a sealed jar or bag along with a GasPak envelopes that functions as a hydrogen and carbon dioxide generator. The hydrogen combines with the oxygen in the jar to produce water. A palladium catalyst in the chamber or bag catalyzes the formation of water from hydrogen and oxygen, thereby removing the O_2 . To insure anaerobic conditions are effectively produced in the GasPak jar or bag, a strip of paper soaked in methylene blue dye is included in the jar or bag. Methylene blue is colorless in an anaerobic environment and blue in an aerobic environment. These systems are compact, easy to use, and less expensive than an anaerobic chamber.



Figure 4: An anaerobic jar for cultivating obligate anaerobic species. Plates and test tubes with cultures to be cultivated in an anaerobic environment are added to the jar with a gas-generating pouch. The jar is then sealed. The gas generator pouch removes O_2 inside the jar. The jar environment then remains free of O_2 until the jar is opened.

Laboratory Instructions

Growing Bacteria With & Without O₂



1. On the underside of two TSA Petri plates, use a marker to create 3 sections on the Petri plate (see image above) with the sections labeled as *Escherichia coli*, *Pseudomonas aeruginosa*, and Control. Depending on the circumstances, your instructor may also choose to include another section with a *Clostridium* species as well. If that is the case, create an additional section and label it with the *Clostridium* species name. Label one plate as "aerobic" and the other plate as "anaerobic."
2. Aseptically inoculate each section of the petri plates with the corresponding bacterial species. You may use a streak in a straight line or a zig-zag pattern within the labeled section for each. Leave the Control section without any added bacteria.
3. Incubate the aerobic plate as usual at 37 °C. The anaerobic plate will be placed in an anaerobic jar or anaerobic bag and a GasPak pouch. The GasPak will be activated by your instructor to generate an anaerobic environment and then incubated at 37 °C.

Results & Discussion

Growing Bacteria With & Without O₂

1. Examine the aerobic and anaerobic petri plates and record the results.
2. Explain how these results relate to aerobic respiration and anaerobic respiration?
3. True or False. All bacterial species can grow in the presence of oxygen. Explain your answer.
4. True or False. All bacterial species can grow in the absence of oxygen. Explain your answer.
5. How does the GasPak system produce an anaerobic environment?
6. Can testing the oxygen tolerances of bacterial species be useful for species identification and characterization? Explain your answer.

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5.6: Nitrate Reduction

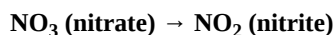
Learning Objectives

- Explain how different bacterial species can conduct nitrate reduction including the possible reactions, possible products, and the name of the enzyme that reduces nitrate.
- Tell the role of nitrate in anaerobic respiration of the species that reduce nitrate.
- Successfully conduct and interpret results of the nitrate reduction test.
- Explain why the nitrate reduction test is useful for characterizing and differentiating species of bacteria.

Nitrate Reduction

Nitrate (NO_3) is a nitrogen-containing molecule that can be reduced by some bacterial species, but not others. Therefore, examining the ability of bacterial species to conduct nitrogen reduction is useful for characterizing and identifying bacterial species.

Reduction of nitrate generally occurs during **anaerobic respiration** in which an organism derives its oxygen from nitrate to serve as the final electron acceptor to remove electrons from the electron transport chain. In some species, nitrate is reduced to nitrite leaving nitrite as evidence of the process:



In other species, nitrate is first reduced to nitrite and then to ammonia:



In still other bacterial species, denitrification occurs where nitrate is reduced completely to N_2 gas and becomes largely unavailable to most living things as a nitrogen source:



Nitrate broth is used to determine if an organism can reduce nitrate. Some bacteria can reduce nitrate (NO_3) to nitrite (NO_2) by producing the enzyme **nitrate reductase**. Other bacteria can reduce nitrate to nitrogen gas by also producing the enzyme nitrite reductase which reduces nitrite to nitrogen gas. Other organisms do not have the ability to reduce nitrate at all.

Nitrate Reduction Test

The nitrate reduction test determines if bacteria conduct nitrate reduction, and therefore if they have the gene for the nitrate reductase enzyme, resulting in the reduction of nitrate (NO_3). To determine if nitrite is produced, nitrite (NO_2) in the medium will form nitrous acid that reacts with the reagent sulfanilic acid (added to the medium), and that reacts with the another reagent naphthylamine (also added to the medium) to form a red color. To determine if denitrification occurs (N_2 is produced), a Durham tube is used to capture the gas. If N_2 or NO_2 are **not** detected, rather than testing for ammonia (NH_3) directly, zinc is used to determine if there is nitrate remaining in the medium. If there **is** nitrate remaining in the medium, this indicates that **no nitrate reduction occurred** and will eliminate the possibility that nitrate was reduced to ammonia. However, if there is no nitrate remaining and N_2 or NO_2 was **not** detected, this indicates that nitrate reduction occurred and ammonia was produced.

Interpretation of the Nitrate Reduction Test

Step 1: Examine Cultures for N_2 Production

There are various ways that a bacterium can utilize nitrate as the final electron acceptor in anaerobic respiration. Some species will completely reduce nitrate to N_2 gas, a process called **denitrification**. If denitrification occurred and therefore N_2 was produced, there will be a pocket of gas (this is N_2 gas) in the top of the Durham tube. The test tube is examined for the presence or absence of N_2 in the Durham tube **before** any reagents are added.



Figure 1: Nitrate can be reduced to nitrogen gas (N_2) by some bacterial species NO_3 (nitrate) $\rightarrow$ NO_2 (nitrite) $\rightarrow$ N_2 (gas). If this occurs, there will be a bubble located inside of the Durham tube.

Step 2: Examine Cultures for NO_2 Production

If there is no nitrogen gas (N_2), it is still possible that nitrate (NO_3) was reduced to nitrite (NO_2). There are still a couple of possible outcomes that need examining to determine whether or not nitrate (NO_3) was reduced:

- **possibility 1:** nitrate (NO_3) reduction to nitrite (NO_2)
- **possibility 2:** nitrate (NO_3) reduction to ammonia (NH_3)
- **possibility 3:** no reduction of nitrate (NO_3)

To determine if nitrate was reduced to nitrite (**possibility 1**), reagents are added to the culture medium. A red color will be produced in the medium only when nitrite (NO_2) is present in the medium, indicating nitrate reduction to nitrite.



Figure 2: Red coloration indicates that nitrate was reduced to produce nitrite NO_3 (nitrate) $\rightarrow$ NO_2 (nitrite).

Step 3: Examine Cultures for NH_3 Production

Even if no N_2 or NO_2 were produced in the culture, it is still possible that nitrate was reduced in the culture. The following two possibilities still exist:

1. **possibility 2:** nitrate (NO_3) reduction to ammonia (NH_3)
2. **possibility 3:** no reduction of nitrate (NO_3)

To differentiate between the above two possibilities, powdered zinc is added. The zinc will not show ammonia production. Instead, the zinc will show if the nitrate is still present in the medium. If the nitrate is still present in the medium, then the nitrate was not reduced and was therefore not reduced to ammonia (nitrate reduction negative).

After zinc is added to the medium, there are two possible outcomes:

- **no pink color forms:** this means that **possibility 2** occurred and nitrate was reduced to ammonia.
- **pink color forms:** this means that **possibility 3** occurred and no nitrate was reduced in the culture. If nitrate (NO_3), is still be present in the tube, then zinc reduces the nitrate to nitrite, which then reacts with the 2 reagents already added to the tube producing a pink color.



Figure 3: If no bubble is present in the Durham tube and the medium did not turn red, it is still possible that nitrate was reduced to ammonia. Zinc is added to determine whether the nitrate was reduced or not. If the nitrate was not reduced by the microbes in the tube, the zinc will cause the medium to turn pink and indicate that the bacteria are nitrate reduction negative. If the medium does not turn pink when zinc is added, this indicates that nitrate was reduced to ammonia by the microbe and is therefore nitrate reduction positive.

REACTION	N ₂ gas	Color after adding reagents	Color after adding zinc
NO ₃ to NO ₂	none	red	(Zn not added)
NO ₃ to N ₂	yes	no color	(Zn not added)
NO ₃ to NH ₃	none	no color	no color
no NO ₃ reduction	none	no color	pink-red

Laboratory Instructions

1. Inoculate the nitrate broths with the assigned bacterial species.
2. Incubate at the optimal temperature, 30° C or 37° C, for these bacterial species.
3. After Incubation: Look for N₂ gas first **before adding reagents** and record results for whether or not N₂ gas was produced.
4. Add 6-8 drops of nitrite reagent A.
5. Add the same number of drops of nitrite reagent B.
6. You should see a reaction within a minute or less. Record results for whether or not nitrite was produced.
7. If you have **not** seen evidence of **either** nitrite (NO₂) or N₂ gas, add a bit of powdered zinc to the culture medium. A bit of zinc is about the amount that sticks to the end of a wood stick.
8. The reduction of unused nitrate (NO₃) by zinc takes a couple of minutes. Record results for whether or not ammonia was produced.

Results & Questions

1. Record the results from the nitrate reduction cultures.
2. Name the 2 major end products of nitrate reduction.
3. What does the gas pocket in the Durham tube indicate about nitrate reduction by that bacterial species? Be specific.
4. What does the red color after adding reagents to the medium mean about nitrate reduction by that bacterial species? Be specific.
5. What does a lack of pink color (no pink) after adding zinc mean about nitrate reduction by that bacterial species? Be specific.
6. What does a pink color after adding zinc mean about nitrate reduction by that bacterial species? Be specific.
7. Species that CAN conduct nitrate reduction may conduct the process differently. Explain this statement.
8. Define denitrification.
9. Is nitrate reduction an aerobic pathway or an anaerobic pathway?

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6.1: Required Materials

Cultures Needed

- TSB Cultures
 - *E. coli*
 - *S. epidermidis*
 - *S. aureus*
 - *P. aeruginosa*
 - *P. vulgaris*
 - *Lactobacillus acidophilus*
 - *Alcaligenes faecalis*

Environmental Effects on Growth: pH

- 4 tubes: TSB pH 4.0
- 4 tubes: TSB pH 6.0
- 4 tubes: TSB pH 8.0
- 4 tubes: TSB pH 10.0

Environmental Effects on Growth: Osmotic Pressure

- 1 TSA plate of each of 2%, 5%, and 10% Sodium ChlorideNaCl" id="MathJax-Element-96-Frame" role="presentation" style="position:relative;" tabindex="0">

Mannitol Salt Agar

- 1 Mannitol Salt Agar plate

EMB Agar

- 3 EMB plates

SIM Deep Tests

- 3 SIM Deep tubes

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SECTION OVERVIEW

6.2: Environmental Effects on Growth- pH

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6.2.1: Learning Objectives

Learning Objectives

After this lab you should be able to:

1. Define pH.
2. Explain the quantitative difference between two different pH values, for example, pH 4 and pH 6.
3. Describe the major effects pH changes can have on a cell.

“Growth of the bacterium *Clostridium botulinum* in canned food may cause botulism—a deadly form of food poisoning. These bacteria exist either as spores or as vegetative cells. The spores, which are comparable to plant seeds, can survive harmlessly in soil and water for many years. When ideal conditions exist for growth, the spores produce vegetative cells, which multiply rapidly and may produce a deadly toxin within 3 to 4 days of growth.

Acidity may be natural, as in most fruits, or added, as in pickled food. Low-acid canned foods are not acidic enough to prevent the growth of these bacteria. Acid foods contain enough acid to block their growth, or destroy them more rapidly when heated. The term “pH” is a measure of acidity; the lower its value, the more acid the food. The acidity level in foods can be increased by adding lemon juice, citric acid, or vinegar.”

National Center for Home Food Preservation: http://nchfp.uga.edu/how/general/ensuring_safe_canned_foods.html

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6.2.2: Introduction

The pH of an environment can affect the growth and survival of the microbes in that environment. pH is a measure of the hydrogen ion concentration or activity, in a solution, expressed as:

$$-\log_{10}[H^+]$$

Every organism has an optimal pH range at which it will grow. If the pH is too high or too low it can affect things like the solubility of molecules, denaturation of proteins/enzymes, membrane transport, proton-motive force/membrane potential, etc. These changes can be devastating to a bacterial population. We have exploited this in the production of foods, for example, pickling is a common method of food preservation and compounds like citric acid are often added to food products to lower the pH and prevent spoilage or toxin formation.

Most bacteria are neutrophils while fungi prefer a more acidic environment from pH 4-6. Archaea are found in all ranges. Nonetheless, all microbes keep their internal environment near neutral and some have developed mechanisms to allow them to survive in the more extreme ranges.

In microbiology we also use a variety of biochemical tests based on pH changes in the media as diagnostic tests. Many bacteria produce acids that are released into the environment as metabolic products of fermentation. These acids can have a big effect on the pH of a closed system like a test tube or agar plate. You will utilize some of these biochemical tests in the future to help you identify bacteria and key characteristics of them.

The pH scale is a tool to measure the relative acidity of solutions, food, natural environments like lakes (water), and as stated, biochemical reactions. The scale is logarithmic reflecting the definition of pH and actually making the scale easy to use. pH 0, which is the most acidic level, has a hydrogen ion concentration of 100 moles/L; pH 14, the most alkaline, has a hydrogen ion concentration of 10⁻¹⁴ moles/L. Pure water is neutral and therefore has a pH of 7 (10⁻⁷ moles/L). Each pH level represents then a tenfold decrease or increase in hydrogen ion concentration. So, pH6 is ten times more acidic than pH 7, and ten times more alkaline than pH 5.

There are 3 main groups of microbes classified by the pH range they can survive in:

Acidophiles: pH 0-5.5

Neutrophiles: pH 5.5-8.0

Alkaliphiles: pH 8.0-11.5

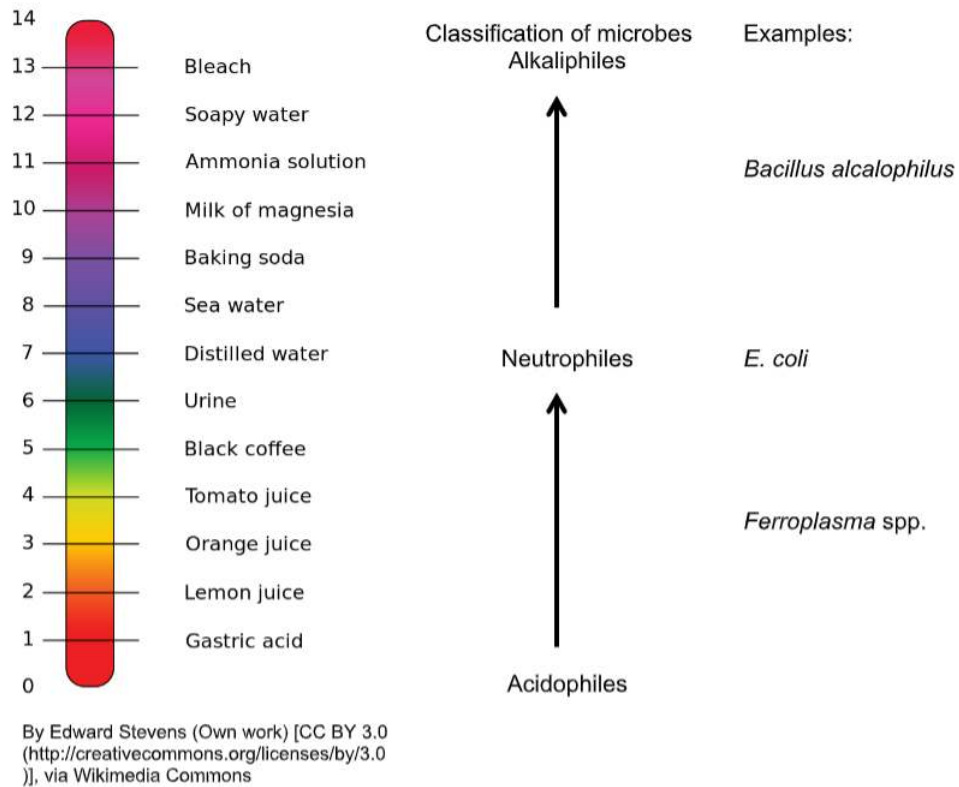


Figure 6.2.2.1: Classification of microbes

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6.2.3: Pre-lab Questions

1. Quantify the difference between:
 - a. pH 2 and pH 3
 - b. pH 6 and pH 8
 - c. pH 5 and pH 9
2. Explain pH in your own words:
3. Explain why we used a selective media, Sabouraud Dextrose Agar (SDA) earlier in the course to inhibit bacteria and select for fungi.

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6.2.4: Materials and Procedures

Materials

- Organisms used from the Student Stock Cultures(TSB):
 - Proteus vulgaris*
 - Escherichia coli*
 - Alcaligenes faecalis*
 - Lactobacillus acidophilus*
- 4 tubes: TSB pH 4.0
- 4 tubes: TSB pH 6.0
- 4 tubes: TSB pH 8.0
- 4 tubes: TSB pH 10.0

Procedures

Each Group:

- Use a sterile transfer pipette to inoculate each of the 4 organisms into each pH tube:
 - add 1 drop of broth culture to each pH broth tube.
 - Staphylococcus saprophyticus* → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0
 - Escherichia coli* → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0
 - Alcaligenes viscolactis* → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0
 - Lactococcus lactis* → TSB pH 4.0, pH 6.0, pH 8.0, pH 10.0
- Place the tubes in buckets and incubate in your classroom incubator.

Results

Enter your ratings for each bacterium/pH and give the pH classification for bacterium.

Organism	pH 4	pH 6	pH 8	pH 10	Optimum pH	pH Classification

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6.2.5: Post-lab Questions

1. A buffer is a chemical often added to growth media to prevent shifts in the pH of the media. A buffering system will absorb or release H^+ ions to prevent the media from becoming too acidic or alkaline, respectively. Why would it be important to add a buffer to growth media?
2. Based on your results, is there overlap in the pH ranges of the organisms? Why might this be?
3. The pH of seawater is around 8.3. What factors or media adjustments would you have to make when trying to culture marine organisms?

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6.2.6: Conclusion and Resources

Conclusion

Later in course you will use different media to help characterize and identify bacteria. Several tests use fermentation of sugars to differentiate bacteria.

1. How does fermentation of sugars relate to pH?
2. How might you use pH changes to help you identify different bacteria?

Resources

Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2014). *Prescott's Microbiology* (9th ed.) (pp. 141-149). New York, New York: McGraw-Hill.

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SECTION OVERVIEW

6.3: Environmental Effects on Growth- Osmotic Pressure

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6.3.1: Learning Objectives

Learning Objectives

After this lab you should be able to:

1. Define osmosis, hypertonic, hypotonic, and isotonic.
2. Analyze scenarios with regard to net water movement in and out of a cell and discuss the effect of osmotic pressure on microorganisms.
3. Classify organisms based on their salinity preferences.

Gather the family/friends/roommates around and try this at home:

Place a piece of lettuce in a bowl of super salty water. Let it sit for a half hour or so. Prediction? Observations? Next, place the same piece of lettuce in a bowl of plain tap water. Let it sit again—this may take a bit longer. Prediction? Observations?

Try to explain what happened, or (should you be out of lettuce) what you think would happen.

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6.3.2: Introduction

Like other cells, the osmotic environment surrounding microorganisms can affect them. **Osmosis** is the net movement of water molecules across a semi-permeable membrane, from an area of low solute (high water activity) concentration to higher solute concentration (lower water activity). Water molecules want to balance the solute concentration on either side of the membrane to a more **isotonic** state. Osmotic pressure is the physical force to draw water to one side of the membrane or other. In a **hypertonic** solution (more solutes outside of the cell, higher osmotic pressure) there will be net movement of water out of the cell (in order to create equal osmotic pressure on both sides of the membrane). **Plasmolysis**, cell shrinkage, may occur. In a **hypotonic** solution the opposite will happen. There are more solutes inside the cell relative to the solution and the net movement of water will be into the cell, which may result in **lysis** of the cell.

It's important to remember that osmosis is relative to the two side of the membrane. When considering a microorganism and its environment, one should consider the inside of the cell relative to the environment. Is the environment hypertonic (higher solutes) or hypotonic (lower solutes) relative to the cell? This will determine the net movement of water.

Net movement of water

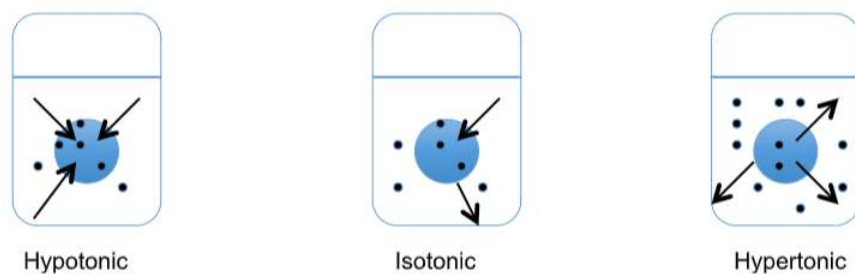


Figure 6.3.2.1: Net movement of water

The bacterial cell wall helps those (with a cell wall) maintain their osmotic pressure in a fairly stable environment so that neither plasmolysis nor lysis will occur. Most bacteria live in a narrow range of salinity, usually less than 3% **NaCl**. **Halophiles** will grow in higher than 3% **NaCl**. **Extreme halophiles** require a very high salt concentration (up to 30%) and can be found in places like the Dead Sea, Great Salt Lake, etc. These organisms have proteins, enzymes, membranes, etc., that function in the high osmotic environment. In addition, some bacteria have adapted mechanisms to survive in a wider range of osmotic environments and are **osmotolerant**.

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6.3.3: Pre-lab Questions

1. What is the primary effect of osmotic pressure on a cell?
2. Look up the salinity and pH of seawater. What challenges would you have, and what would you do to overcome them, with culturing marine bacteria in the lab?

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6.3.4: Materials and Procedures

Materials

- Organisms-
 - Staphylococcus epidermidis*
 - Escherichia coli*
 - Lactobacillus acidophilus*
- 1 plate of each Sodium Chloride (**NaCl**) concentration:
- TSA with 2.0% **NaCl**
- TSA with 5.0% **NaCl**
- TSA with 10.0%**NaCl**

Procedures

Inoculate each plate with the following organisms: *Staphylococcus saprophyticus*, *Escherichia coli*, *Lactococcus lactis*

- Use a single streak on $\frac{1}{3}$ of the plate for each organism; make sure to label each streak line with the correct organism:

Staphylococcus saprophyticus, *E. coli*, *Lactococcus lactis* → TSA with 2.0% **NaCl**

Staphylococcus saprophyticus, *E. coli*, *Lactococcus lactis* → TSA with 5.0% **NaCl**

Staphylococcus saprophyticus, *E. coli*, *Lactococcus lactis* → TSA with 10.0% **NaCl**

- Invert and incubate.

Results

- After incubation assess the growth of each bacteria on each plate.
- Fill out the chart below:

Enter your ratings for each bacterium/salinity and give the salinity classification for bacterium.

Organism	2.0% NaCl	5.0% NaCl	10.0% NaCl	Optimum salinity	Salinity Classification

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6.3.5: Post-lab Questions

1. How do hypotonic and hypertonic environments generally affect most bacteria?
2. What difference between bacterial cells and animal cells helps to protect bacteria against osmotic changes in their environment?
3. How would you characterize marine bacteria in relationship to osmotic pressure?
4. Define water activity.

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6.3.6: Conclusion and Resources

Conclusion

Reflect on your results and discuss how osmotic pressure might relate to food preservation.

Resources

Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2014). *Prescott's Microbiology* (9th ed.) (pp. 141-149). New York, New York: McGraw

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6.4: Mannitol Salt Agar

Learning Objectives

- Define selective medium and differential medium.
- Explain how mannitol salts agar is selective.
- Explain how mannitol salts agar is differential.
- Successfully utilize mannitol salts agar to characterize/identify bacterial species and interpret results.

Mannitol Salts Agar

Mannitol salts agar is a microbiological medium that is both selective and differential. Selective means the medium will only allow certain microorganisms to grow. Differential means the medium will show some characteristic of the microorganisms growing on it and can be used to differentiate between different species.

Mannitol salts agar is selective since it has a high salt concentration and will only allow **halophilic** (salt-loving species) or **halotolerant** (salt-tolerant) species to grow on it. This medium contains 7.5% NaCl (salt), whereas typical media contains about 0.5% NaCl.

- species that grow on mannitol salts agar: **mannitol utilization and salt resistance positive**
- species that do not grow on mannitol salts agar: **mannitol utilization/salt resistance negative**

Mannitol salts agar is differential since it will detect acid production as a result of fermentation of mannitol (a sugar in the medium) in species that can ferment mannitol to produce acid. The medium contains a pH indicator, phenol red, that is red-orange when the pH is neutral (around pH 7). If a species ferments mannitol and produces acid, the pH of the medium decreases and phenol red will become yellow.

- medium remains red-orange after growth and growth observed: **mannitol fermentation negative**
- medium is yellow after growth: **mannitol fermentation positive**

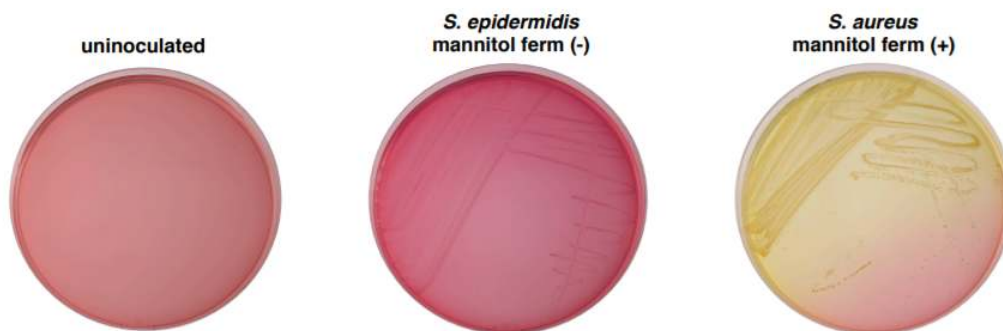


Figure 1: Mannitol salts agar. (Left) The uninoculated medium has a light red to red-orange color. (Middle) The medium with *Staphylococcus epidermidis* growing on it shows the appearance with a mannitol fermentation negative species and appears red to red-orange. (Right). The medium with *Staphylococcus aureus* growing on it shows the appearance with a mannitol fermentation positive species and appears yellow in the region of the medium where the bacteria is growing.

Use of mannitol salts agar is useful for differentiation of species of *Staphylococcus* and *Micrococcus*. Mannitol fermentation by pathogenic staphylococci, such as *Staphylococcus aureus*, is indicated by the media changing to yellow, whereas *Staphylococcus epidermidis*, a non-pathogenic species of staphylococci, does not produce a yellow color.

Laboratory Instructions

1. Obtain a mannitol salts agar petri plate and label with your name/group name or number and "mannitol salts agar."
2. Draw a straight line on the underside of the petri plate to separate the plate into two halves. Label one side *Staphylococcus aureus* and the other side *Staphylococcus epidermidis*.

3. Aseptically inoculate each half of the plate with the corresponding bacterial species with a streak that is a straight line in the center of each half of the petri plate.
4. Invert the petri plate and incubate for 24 hours.
5. Observe, record, and interpret results.

Results & Questions

bacterial species	growth (+/-)	salt resistance and mannitol utilization (+/-)	medium color surrounding growth	mannitol fermentation (+/-)
<i>Staphylococcus aureus</i>				
<i>Staphylococcus epidermidis</i>				

1. Fill in the table above with results and interpretations.
2. Define selective medium.
3. Explain how mannitol salts agar is a selective medium.
4. Define differential medium.
5. Explain how mannitol salts agar is a differential medium.
6. Explain how phenol red, a component of mannitol salts agar medium, detects if a species conducts mannitol fermentation.

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6.5: EMB Agar

Learning Objectives

- Identify that EMB medium is both selective and differential.
- Explain how EMB is a selective medium and how this works.
- Explain how EMB is differential and how this works.
- Define coliform.
- Successfully utilize EMB medium and interpret its results.

Eosin-Methylene Blue (EMB) Medium

Eosin-methylene blue (EMB) medium is a **selective medium** and a **differential medium** used for isolation of Gram-negative rods in a variety of specimen types, including being used frequently in clinical laboratories. EMB is a selective medium because it inhibits Gram-positive bacterial growth. The two dyes in this medium, eosin and methylene blue, act as selective agents to inhibit Gram-positive bacteria, but allow for the growth of Gram-negative bacteria.

EMB enables differentiation of bacterial species that are **coliforms** due to its dyes and its lactose content. EMB contains two types of sugars: lactose and sucrose. It is the lactose that is the key to this medium's ability to differentiate coliform bacteria. Lactose-fermenting bacteria (*Escherichia coli* and other coliforms) produce acid using lactose. When acids are produced by bacteria, the combination of the dyes (which serve as pH indicators in this medium) produces color variations in the colonies because of the acidity. Strong acidity produces a deep purple colony with a green metallic sheen, whereas less acidity may produce a brown-pink coloration of colony. Nonlactose fermenters appear as translucent or pink.

Definition

coliform: A classification of bacterial species that is typically associated with animal digestive tracts and fecal contamination in the environment. These bacterial species are Gram-negative bacilli that do not produce endospores and have the β -galactosidase gene, and can therefore break down lactose. Coliform bacteria break down lactose to produce acid and gas.

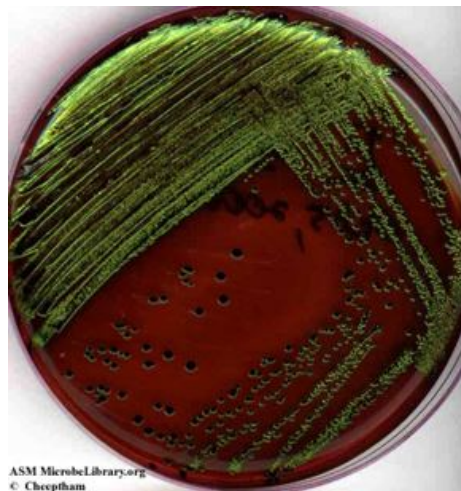


Figure 1: Eosin-methylene blue (EMB) agar plate inoculated with *Escherichia coli* (a Gram-negative coliform bacterium) showing good growth of dark blue-black colonies with metallic green sheen indicating vigorous fermentation of lactose and acid production which precipitates the green metallic pigment. Image by Naowarat Cheeptham, Thompson Rivers University, Kamloops, BC, Canada.

Laboratory Instructions

1. To observe the effects of EMB medium as selective and differential, aseptically conduct **quadrant streak plates** on three different EMB plates with:
 - *Escherichia coli* - a Gram-negative coliform species

- *Pseudomonas aeruginosa* - a Gram-negative non-coliform species
 - *Staphylococcus aureus* - a Gram-positive species
2. Invert and incubate the plates for 24-48 hours.
 3. Observe, record, and interpret the results.

Results & Questions

bacterial species	growth (+/-)	species is likely Gram (+/-)	colony color(s)	lactose utilization produces acid (+/-)	species is a coliform (+/-)
<i>Escherichia coli</i>					
<i>Pseudomonas aeruginosa</i>					
<i>Staphylococcus aureus</i>					

1. Record the results and interpretations from the EMB plates in the table above.
2. What types of bacterial species is EMB used to identify?
3. How is EMB a selective medium?
4. How is EMB a differential medium?
5. Define coliform.
6. Explain the roles of the dyes in EMB (there are two).
7. Explain the role of lactose in EMB.

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6.6: SIM Deep Tests

Learning Objectives

- Explain what a SIM deep is and tell the tests that can be conducted in a SIM deep.
- List possible metabolic reasons why a species of bacteria could be producing hydrogen sulfide.
- Tell what the indole test examines and what enzyme it tests for.
- Explain what the motility is and that indicates whether or not bacterial species produce flagella for motility.
- Successfully conduct SIM tests and interpret results of these tests.

SIM Medium

SIM (sulfur reduction, indole, motility) medium is an example **combination medium**, meaning that one can determine several bacterial activities/characteristics through the use of one medium. SIM medium tests for sulfur reduction, indole production and motility. SIM is an example of a The form of medium used for this test is an **agar deep**. SIM Medium contains the following: pancreatic digest of casein, peptic digest of animal tissue, ferrous ammonium sulfate $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)$, sodium thiosulfate $\text{Na}_2\text{S}_2\text{O}_3$, agar (3.5 g/L) and distilled or deionized water.

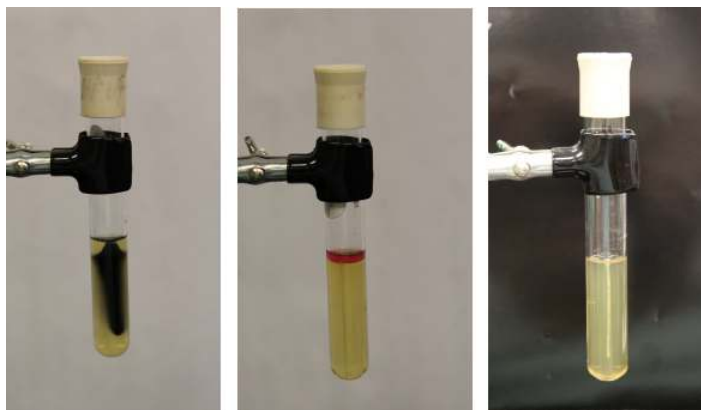


Figure 1: SIM medium is for deep cultures and an inoculation needle is used to inoculate it. After incubation, a black color indicates that the bacteria has produced hydrogen sulfide (H_2S). (Left) This culture turned black and therefore this bacterial species is positive for hydrogen sulfide production. (Right and middle) These cultures did not turn black and is therefore are negative for hydrogen sulfide production. If bacteria are motile, the growth will fan away from the stab. (Right) In this image the bacteria are localized to the stab and therefore no motility occurred and is therefore negative for motility. (Middle) After incubation of a SIM deep, Kovac's reagent may be added to the top of the SIM medium. When Kovac's reagent turns red, this indicates that the bacterial species produced indole and is therefore an indole positive species.

SIM - Sulfur Reduction

Sulfur can be reduced producing hydrogen sulfide (H_2S) by bacteria in two unrelated ways:

1. One process occurs during putrefaction. When proteins putrefy, the resulting foul "rotten egg" smell is due to the production of hydrogen sulfide gas (H_2S). Hydrogen sulfide is a byproduct of the conversion of the amino acid cysteine to pyruvate by the enzyme **cysteine desulfurase**.
2. The second mode of H_2S generation involves anaerobic respiration. In some prokaryotes, thiosulfate ($\text{S}_2\text{O}_3^{2-}$) is the terminal electron acceptor in an anaerobic respiration. When thiosulfate is reduced (picks up electrons) the result is H_2S gas. In either case, invisible H_2S gas is produced.

Because hydrogen sulfide gas is colorless (though not odorless!), SIM medium uses an indicator reaction. Iron (in the form of ferrous ammonium sulfate) in the medium combines with H_2S gas to form iron sulfide, FeS , a black precipitate. Any black color in the medium indicates the bacterial species is **positive for sulfur reduction**. If there is no black color in the medium, the bacterial species is **negative for sulfur reduction**.

Unfortunately, this test does not distinguish between the hydrogen sulfide produced as a result of putrefaction and hydrogen sulfide produced at the end of an anaerobic respiration.

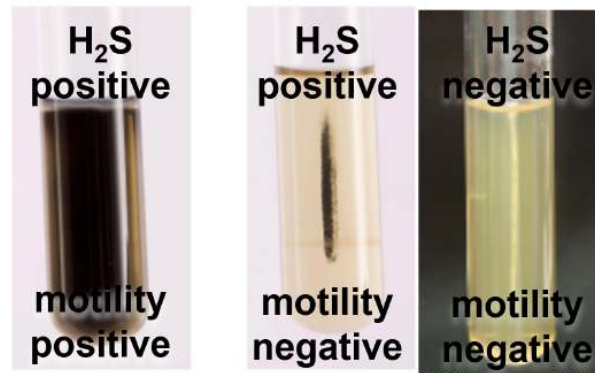


Figure 2: Results of SIM deep cultures (no Kovac's reagent). (Left) The medium is black throughout indicating hydrogen sulfide production and motility. (Middle) The medium is black along the stab line only indicating hydrogen sulfide production and no motility. (Right) The medium is not black and there is no growth away from the stab line indicating this bacterial species is hydrogen sulfide negative and motility negative.

SIM - Indole

Tryptophan is an amino acid found in most proteins. Some bacteria produce **tryptophanase**, an enzyme that breaks tryptophan down into **indole**, ammonia and pyruvate (see below). Not all bacterial species produce tryptophanase. Whether a bacterial species produces tryptophanase is dependent on its genes. Testing for the activity of tryptophanase using the indole test is an effective way to differentiate one bacterial species from another and to characterize bacteria.

The pyruvate and ammonia (NH_3) are converted into other molecules, but the indole accumulates, and thus can be detected in the media.

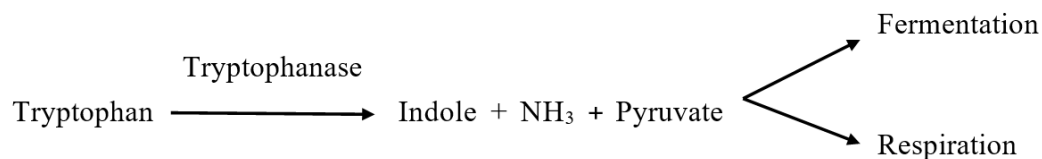


Figure 3: Chemical reaction catalyzed by tryptophanase. Bacteria that have the tryptophan gene can break down tryptophan to produce indole. Indole is not used by the bacteria and therefore will accumulate in the medium. Kovac's reagent can be used to detect the accumulated indole and therefore is an effective way of determining if the bacterial species produces the enzyme tryptophanase. Pyruvate is utilized by bacteria in either the cellular respiration metabolic pathway or in a fermentation pathway.

The presence of indole therefore indicates that an organism produces the enzyme tryptophanase. Indole can be detected using a chemical known as **Kovac's reagent**. Indole forms a red ring with the addition of Kovac's reagent indicating the bacterial species is **indole positive** and that it produces tryptophanase. When a bacterial species is **indole negative** (indicating no tryptophanase activity), the Kovac's reagent will produce a dark yellow ring.

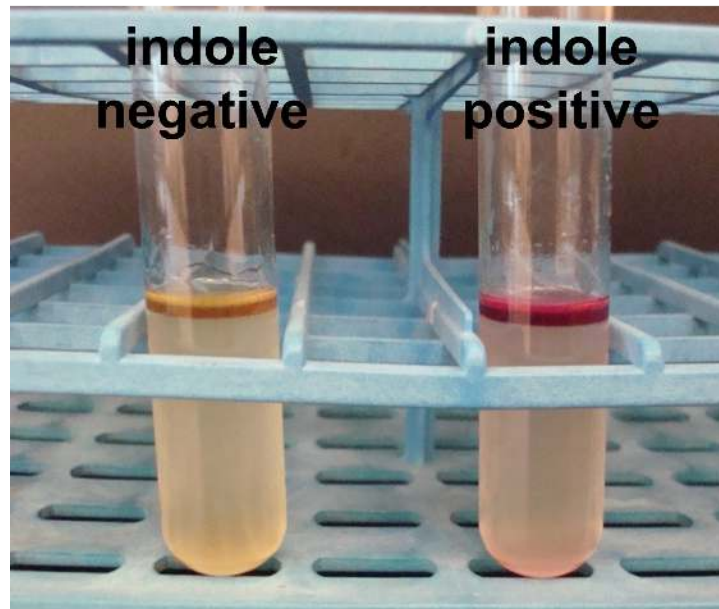


Figure 4: Results from indole test. Kovac's reagent is on top of the SIM agar deep inside of these test tubes. The reagent appears yellow when the bacterial species is indole negative (left) and the reagent appears red when the bacterial species is indole positive (right).



Figure 5: Reaction that occurs between indole and Kovac's reagent. A reaction between indole and Kovac's reagent produces a dye that is pink-red when indole is present.

SIM - Motility

Motility is the ability of a microbe to “swim” using flagella. The reduced agar content of this medium, 3.5 g/L compared to 12-15 g/L in most solid media, creates a semi liquid environment allowing motile cells to spread from their original placement. The **stab technique** deposits cells in a straight line down the center of the deep using an **inoculation needle** rather than an inoculation loop. If growth is observed beyond the stab line into the periphery of the tube, the test is positive for motility. Avoid confusing growth produced by the lateral movement of the needle during an imperfect stab inoculation with actual motility. Rotating the tube for a side view will help you determine if growth is confined to the original inoculation line or has truly spread into the periphery of the tube.

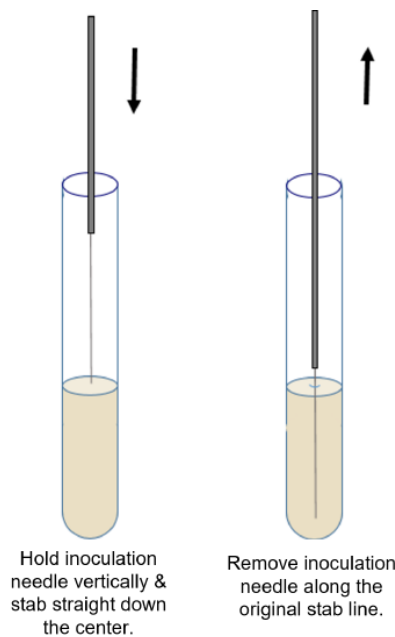


Figure 6: How to inoculate a deep with an inoculation needle. Hold the inoculation needle vertically and stab straight down the center of the deep. Then remove the inoculation needle along the original stab line.

Motility is indicated by the ability of the organism to ‘fan’ away from the streak. Or, the entire tube may appear cloudy when compared to an un-inoculated control. If the organism is non-motile, the growth will only appear along the stab line.

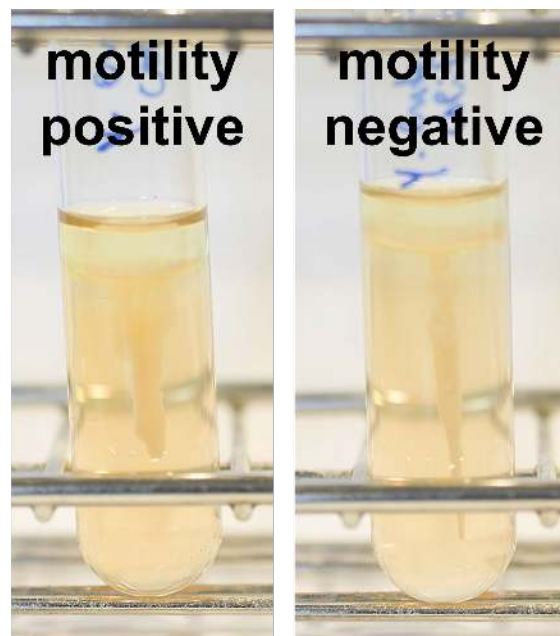


Figure 7: SIM deep results shown with Kovac's reagent present on top of the medium. (Left) Bacterial growth has moved away from the stab and therefore is **motility positive**. The Kovac's reagent is not red and therefore is indole negative. The medium did not produce any black coloration and is therefore hydrogen sulfide reduction negative. (Right) Bacterial growth stayed close to the initial stab and there is **motility negative**. The Kovac's reagent is not red and therefore is indole negative. The medium did not produce any black coloration and is therefore hydrogen sulfide reduction negative.

Laboratory Instructions

1. Obtain a deep of SIM medium.
2. Label the test tube with your group name, the medium name, and the bacterial species name.
3. Using an inoculating needle, stab the medium about 2/3 of the way down and out the same pathway as quickly as possible with the bacterial species provided by your instructor.

4. Repeat steps 1-3 if testing multiple bacterial species.
5. Incubate the tube for at least 48 hours.
6. After the incubation period, examine your tube.

Indole Test

1. After the SIM deep has incubated for at least 48 hours, examine results for motility and hydrogen sulfide production and record results.
2. Add 10 drops of Kovac's reagent to the top of the SIM medium tube.
 - if Kovac's reagent produces a dark yellow ring indicates the species is indole negative
 - if Kovac's reagent produces a red ring indicates the species is indole positive

Results & Questions

bacterial species	medium color	hydrogen sulfide production (+/-)	Kovac's reagent color	indole (+/-)	location of growth (along stab / fanned out)	motility (+/-)
<i>Escherichia coli</i>						
<i>Proteus vulgaris</i>						
<i>Staphylococcus aureus</i>						

1. Complete the table above with results from the SIM deep tests.
2. A bacterial species that is positive for hydrogen sulfide production could be producing H₂S in one of two ways. What are these?
3. Is hydrogen sulfide black? Explain your answer.
4. A bacterial species that is positive for indole produces what enzyme? What does this enzyme do?
5. What is the role of Kovac's reagent in the indole test?
6. A bacterial species that is positive motility has what type of bacterial cell structure found only in some cells?
7. Is using a SIM deep useful for bacterial species identification and characterization? Explain your answer.

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7.3: Temperature

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7.1: Required Materials

TSB Cultures

- *S. marcescens*
- *B. subtilis* (veg)
- *B. subtilis* (endo)

UV

- 5 TSA plates
- UV lamps

Temperature

- Water bath at 80 C
- 5 TSB tubes
- 1 TSA plate

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SECTION OVERVIEW

7.2: Environmental Effects on Growth - UV Radiation and Temperature

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7.2.1: Desiccation

Desiccation, or drying, generally has a **static** effect on microorganisms. Lack of water inhibits the action of microbial enzymes. Dehydrated and freeze-dried foods, for example, do not require refrigeration because the absence of water inhibits microbial growth.

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7.2.2: Introduction

A. INTRODUCTION TO THE CONTROL OF MICROORGANISMS

The next two labs deal with the inhibition, destruction, and removal of microorganisms. Control of microorganisms is essential in order to prevent the transmission of diseases and infection, stop decomposition and spoilage, and prevent unwanted microbial contamination.

Microorganisms are controlled by means of physical agents and chemical agents. Physical agents include such methods of control as high or low temperature, desiccation, osmotic pressure, radiation, and filtration. Control by chemical agents refers to the use of disinfectants, antiseptics, antibiotics, and chemotherapeutic antimicrobial chemicals.

Basic terms used in discussing the control of microorganisms include:

1. Sterilization

Sterilization is the process of destroying all living organisms and viruses. A sterile object is one free of all life forms, including bacterial endospores, as well as viruses.

2. Disinfection

Disinfection is the elimination of microorganisms, but not necessarily endospores, from inanimate objects or surfaces.

3. Decontamination

Decontamination is the treatment of an object or inanimate surface to make it safe to handle.

4. Disinfectant

A disinfectant is an agent used to disinfect inanimate objects but generally is too toxic to use on human tissues.

5. Antiseptic

An antiseptic is an agent that kills or inhibits growth of microbes but is safe to use on human tissue.

6. Sanitizer

A sanitizer is an agent that reduces, but may not eliminate, microbial numbers to a safe level.

7. Antibiotic

An antibiotic is a metabolic product produced by one microorganism that inhibits or kills other microorganisms.

8. Chemotherapeutic antimicrobial chemical

Chemotherapeutic antimicrobial chemicals are synthetic chemicals that can be used therapeutically.

9. Cidal

An agent that is cidal in action will kill microorganisms and viruses.

10. Static

An agent that is static in action will inhibit the growth of microorganisms.

These two labs will demonstrate the control of microorganisms with physical agents, disinfectants and antiseptics, and antimicrobial chemotherapeutic agents. Keep in mind that when evaluating or choosing a method of controlling microorganisms, you must consider the following factors which may influence antimicrobial activity:

1. the concentration and kind of a chemical agent used;
2. the intensity and nature of a physical agent used;
3. the length of exposure to the agent;
4. the temperature at which the agent is used;
5. the number of microorganisms present;
6. the organism itself; and
7. the nature of the material bearing the microorganism.

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7.2.3: Temperature

Microorganisms have a minimum, an optimum, and a maximum temperature for growth. Temperatures **below the minimum** usually have a **static** action on microorganisms. They inhibit microbial growth by slowing down metabolism but do not necessarily kill the organism. Temperatures **above the maximum** usually have a **cidal** action, since they denature microbial enzymes and other proteins. Temperature is a very common and effective way of controlling microorganisms.

1. High Temperature

Vegetative microorganisms can generally be killed at temperatures from 50°C to 70°C with moist heat. Bacterial **endospores**, however, are very resistant to heat and extended exposure to much higher temperature is necessary for their destruction. High temperature may be applied as either moist heat or dry heat. The lowest temperature at which all microorganisms in a liquid suspension will be killed in 10 minutes is referred to as the **thermal death point**.

a. Moist heat

Moist heat is generally more effective than dry heat for killing microorganisms because of its ability to **penetrate** microbial cells. Moist heat kills microorganisms by **denaturing their proteins** (causes proteins and enzymes to lose their three-dimensional functional shape). It also may **melt lipids** in cytoplasmic membranes.

1. Autoclaving

Autoclaving employs **steam under pressure**. Water normally boils at 100°C; however, when put under pressure, water boils at a higher temperature. During autoclaving, the materials to be sterilized are placed under **15 pounds per square inch of pressure** in a pressure-cooker type of apparatus. When placed under 15 pounds of pressure, the boiling point of water is raised to **121°C**, a temperature sufficient to kill bacterial endospores.

The time the material is left in the autoclave varies with the nature and amount of material being sterilized. Given sufficient time (generally 15-45 minutes), autoclaving is **cidal** for both vegetative organisms and endospores, and is the most common method of sterilization for materials not damaged by heat.

2. Boiling water

Boiling water (100°C) will generally kill vegetative cells after about 10 minutes of exposure. However, certain viruses, such as the hepatitis viruses, may survive exposure to boiling water for up to 30 minutes, and endospores of certain *Clostridium* and *Bacillus* species may survive even hours of boiling.

b. Dry heat

Dry heat kills microorganisms through a process of **protein oxidation** rather than protein coagulation. Examples of dry heat include:

1. Hot air sterilization

Microbiological ovens employ very high dry temperatures: 171°C for 1 hour; 160°C for 2 hours or longer; or 121°C for 16 hours or longer depending on the volume. They are generally used only for sterilizing glassware, metal instruments, and other inert materials like oils and powders that are not damaged by excessive temperature.

2. Incineration

Incinerators are used to destroy disposable or expendable materials by burning. We also sterilize our inoculating loops by incineration.

c. Pasteurization

Pasteurization is the mild heating of milk and other materials to kill **particular spoilage organisms or pathogens**. It does not, however, kill all organisms. Milk is usually pasteurized by heating to 71°C for at least 15 seconds in the flash method or 63-66°C for 30 minutes in the holding method.

2. Low Temperature

Low temperature **inhibits** microbial growth by **slowing down microbial metabolism**. Examples include refrigeration and freezing. Refrigeration at 5°C slows the growth of microorganisms and keeps food fresh for a few days. Freezing at -10°C stops microbial growth, but generally does not kill microorganisms, and keeps food fresh for several months.

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7.2.4: Radiation

1. Ultraviolet Radiation

The ultraviolet portion of the light spectrum includes all radiations with wavelengths from 100 nm to 400 nm. It has low wavelength and low energy. The microbicidal activity of ultraviolet (UV) light depends on **the length of exposure**: the longer the exposure the greater the cidal activity. It also depends on the **wavelength of UV used**. The most cidal wavelengths of UV light lie in the **260 nm - 270 nm range** where it is absorbed by nucleic acid.

In terms of its mode of action, UV light is absorbed by microbial DNA and causes adjacent thymine bases on the same DNA strand to covalently bond together, forming what are called **thymine-thymine dimers** (see Fig. 7.2.4.1). As the DNA replicates, nucleotides do not complementary base pair with the thymine dimers and this terminates the replication of that DNA strand. However, **most of the damage from UV radiation actually comes from the cell trying to repair the damage to the DNA by a process called SOS repair**. In very heavily damaged DNA containing large numbers of thymine dimers, a process called SOS repair is activated as kind of a last ditch effort to repair the DNA. In this process, a gene product of the SOS system binds to DNA polymerase allowing it to synthesize new DNA across the damaged DNA. However, this **altered DNA polymerase loses its proofreading ability** resulting in the synthesis of DNA that itself now contains many misincorporated bases. In other words, **UV radiation causes mutation** and can lead to faulty protein synthesis. **With sufficient mutation, bacterial metabolism is blocked and the organism dies**. Agents such as UV radiation that cause high rates of mutation are called **mutagens**.

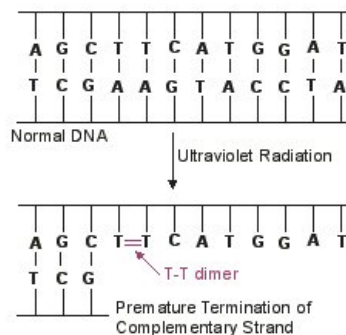


Figure 7.2.4.1: **Mutation as a Result of Exposure to Ultraviolet Radiation.** (Copyright; Gary E. Kaiser, Ph.D. The Community College of Baltimore County, Catonsville Campus [CC-BY-3.0](https://creativecommons.org/licenses/by/3.0/))

The effect of this improper base pairing may be reversed to some extent by exposing the bacteria to strong visible light immediately after exposure to the UV light. The visible light activates an enzyme that breaks the bond that joins the thymine bases, thus enabling correct complementary base pairing to again take place. This process is called **photoreactivation**.

UV lights are frequently used to reduce the microbial populations in hospital operating rooms and sinks, aseptic filling rooms of pharmaceutical companies, in microbiological hoods, and in the processing equipment used by the food and dairy industries.

An important consideration when using UV radiation is that it has **very poor penetrating power**. Only microorganisms on the **surface** of a material that are exposed directly to the radiation are susceptible to destruction and **bacterial endospores are more resistant to ultraviolet radiation**. UV radiation can also damage the eyes, cause burns, and cause mutation in cells of the skin.

2. Ionizing Radiation

Ionizing radiation, such as **X-rays and gamma rays**, has much more energy and penetrating power than ultraviolet radiation. It ionizes water and other molecules to form radicals (molecular fragments with unpaired electrons) that can **disrupt DNA molecules and proteins**. It is often used to sterilize pharmaceuticals and disposable medical supplies such as syringes, surgical gloves, catheters, sutures, and petri plates. It can also be used to retard spoilage in seafoods, meats, poultry, and fruits.

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7.2.5: Filtration

Microbiological membrane filters provide a useful way of sterilizing materials such as vaccines, antibiotic solutions, animal sera, enzyme solutions, vitamin solutions, and other solutions that may be damaged or denatured by high temperatures or chemical agents. The filters contain pores small enough to prevent the passage of microbes but large enough to allow the organism-free fluid to pass through. The liquid is then collected in a sterile flask (**Fig. 7.2.5.1**). Filters with a pore diameter from 25 nm to 0.45 μm are usually used in this procedure. Filters can also be used to remove microorganisms from water and air for microbiological testing (see Appendix E).

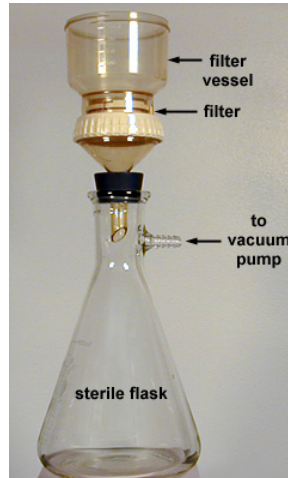


Figure 7.2.5.1: **Micropore Filtration Apparatus.** (Copyright; Gary E. Kaiser, Ph.D. The Community College of Baltimore County, Catonsville Campus [CC-BY-3.0](#))

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SECTION OVERVIEW

7.2.6: Procedure and Results

Video Reviews of Techniques

- [Aseptic Technique Tips](#)
- [Aseptic Technique: Inoculation of broth tubes, slant tubes, and stab tubes](#)

7.2.6.1: Procedure for Osmotic Pressure

7.2.6.2: Results for Osmotic Pressure

7.2.6.3: Procedure for Ultraviolet Radiation

7.2.6.4: Results for Ultraviolet Radiation

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7.2.6.1: Procedure for Osmotic Pressure

MEDIA

- 2 plates of Trypticase Soy agar,
- 2 plates of 5% glucose agar,
- 2 plates of 10% glucose agar,
- 2 plates of 25% glucose agar,
- 2 plates of 5% NaCl agar,
- 2 plates of 10% NaCl agar, and
- 2 plates of 15% NaCl agar.

ORGANISMS

- Trypticase Soy broth cultures of *Escherichia coli* and *Staphylococcus aureus*;
- a spore suspension of the mold *Aspergillus niger*.

A. OSMOTIC PRESSURE PROCEDURE (to be done by tables)

1. Divide one plate of each of the following media **in half**. Using your **inoculating loop**, streak one half of each plate with *E. coli* and the other half with *S. aureus* (see Fig. 6). Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

- Trypticase Soy agar (control)
- Trypticase Soy agar with 5% glucose
- Trypticase Soy agar with 10% glucose
- Trypticase Soy agar with 25% glucose
- Trypticase Soy agar with 5% NaCl
- Trypticase Soy agar with 10% NaCl
- Trypticase Soy agar with 15% NaCl

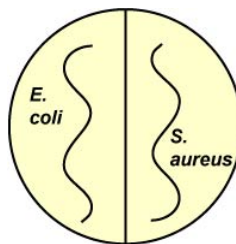


Figure 7.2.6.1.1: Inoculation of Salt and Sugar Plates with *Escherichia coli* and *Staphylococcus aureus*. (Copyright; Gary E. Kaiser, Ph.D. The Community College of Baltimore County, Catonsville Campus [CC-BY-3.0](https://creativecommons.org/licenses/by/3.0/))

2. Using a **sterile swab**, streak one plate of each of the following media with a spore suspension of the mold *A. niger* (see Fig. 7). Incubate **the plates upside down at room temperature** for 1 week.

- Trypticase Soy agar (control)
- Trypticase Soy agar with 5% glucose
- Trypticase Soy agar with 10% glucose
- Trypticase Soy agar with 25% glucose
- Trypticase Soy agar with 5% NaCl
- Trypticase Soy agar with 10% NaCl
- Trypticase Soy agar with 15% NaCl

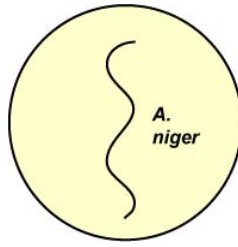


Figure 7.2.6.1.2: **Inoculation of Salt and Sugar Plates with *Aspergillus niger*.** (Copyright; Gary E. Kaiser, Ph.D. The Community College of Baltimore County, Catonsville Campus [CC-BY-3.0](#))

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7.2.6.2: Results for Osmotic Pressure

Observe the 2 sets of plates from the osmotic pressure experiment and record the results below.

Plate	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Aspergillus niger</i>
Control	+++	+++	+++
5% NaCl			
10% NaCl			
15% NaCl			
5% glucose			
10% glucose			
25% glucose			

+ = Scant growth
 ++ = Moderate growth
 +++ = Abundant growth
 - = No growth

<i>Escherichia coli</i> and <i>Staphylococcus aureus</i>	<i>Aspergillus niger</i>
Control	Control
5% NaCl	5% NaCl
10% NaCl	10% NaCl
15% NaCl	15% NaCl
5% glucose	5% glucose
10% glucose	10% glucose
25% glucose	25% glucose

Conclusions:

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7.2.6.3: Procedure for Ultraviolet Radiation

MEDIA

5 plates of Trypticase Soy agar

ORGANISM

Trypticase Soy broth culture of *Serratia marcescens*

ULTRAVIOLET RADIATION PROCEDURE (to be done by tables)

1. Using **sterile swabs**, streak all 5 Trypticase Soy agar plates with *S. marcescens* as follows:
 - a. Dip the swab into the culture.
 - b. **Remove all of the excess liquid by pressing the swab against the side of the tube.**
 - c. Streak the plate so as to **cover the entire agar surface with organisms.**
2. Expose **3 of the plates to UV light** as follows:
 - a. **Remove the lid** of each plate and place a piece of cardboard with the letter "V" cut out of it over the top of the agar.
 - b. Expose the first plate to UV light for **1 second**, the second plate for **3 seconds**, and the third plate for **5 seconds**.
 - c. Replace the lids and incubate the plates **upside down at room temperature** until the next lab period.
3. **Leaving the lid on**, lay the cardboard with the letter "V" cut out over the fourth plate and expose to UV light for **30 seconds**. Incubate the plates **upside down at room temperature** with the other plates.
4. Use the fifth plate as a **non-irradiated control** and incubate the plates **upside down at room temperature** with the other plates.

Caution

Do not look directly at the UV light as it may harm the eyes.

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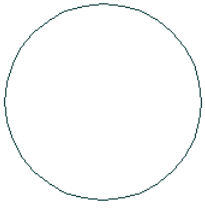
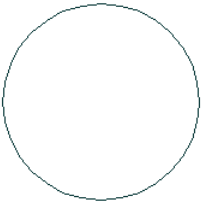
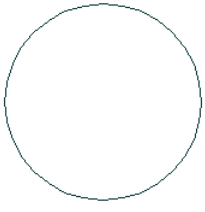
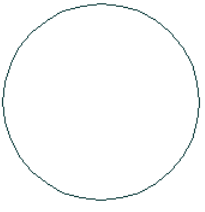
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7.2.6.4: Results for Ultraviolet Radiation

1. Make drawings of the 5 plates from the ultraviolet light experiment.

		
Non-irradiated control	1 second UV exposure; lid off	
		
3 second UV exposure; lid off	5 second UV exposure; lid off	30 second UV exposure; lid on

Conclusions:

2. Observe the plates exposed to UV light for any **non-pigmented** colonies. Aseptically **pick-off** one of these nonpigmented colonies and streak it in a plate of Trypticase Soy agar. Incubate **at room temperature** until the next lab period.

3. After incubation, observe the plate you streaked with the nonpigmented colony.

Does the organism still lack chromogenicity?

What would account for this?

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7.3: Temperature

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CHAPTER OVERVIEW

8: Lab 8

8.1: Required Materials

8.2: Food Microbiology

8.3: Microbiology of Milk and Food

8.3.1: Introduction

8.3.2: Procedures

8.3.3: Results

8.3.4: Review Questions

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8.1: Required Materials

Food and Milk

- 10 9 ml tubes of sterile diH₂O
 - 2 1 g samples of different food products
 - 2 microcentrifuge tubes of various milk product
 - 10 TSA plates
-

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8.2: Food Microbiology

Learning Outcomes

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

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

Introduction

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

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

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

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

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

Serial Dilutions

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

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

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

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

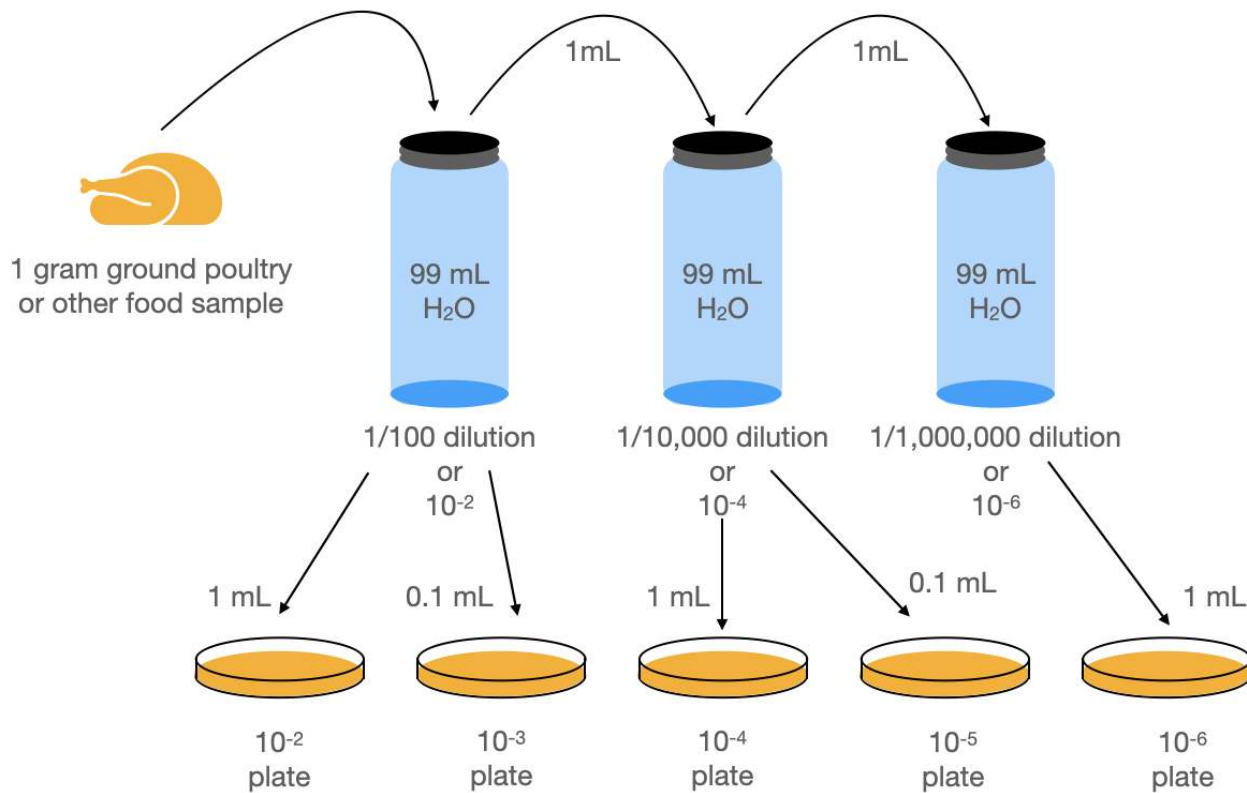
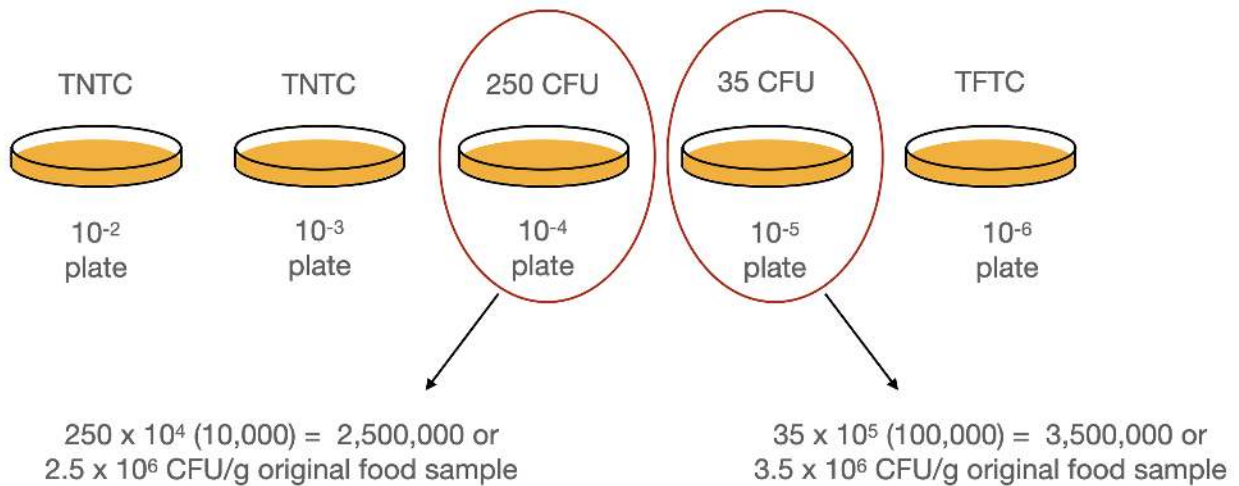


Figure 8.2.1: Schematic diagram of how to set up serial dilutions and plates to determine CFU/gram of each type of food

Referring to Figure 8.2.1 - you can see how 1 gram of food is diluted into 99 mL of water. Water weighs 1g/mL, so that's the same as 99 grams of water or a 1/100 (1 g food/100 g total) dilution. If you take 1 mL of that dilution and prepare a TSA plate with that sample, the number of colonies (CFU) on that plate will be 1/100th the number of CFU per original gram of food. The same calculation can be made for the 10⁻⁴ and 10⁻⁶ dilutions. Although we are not preparing 1/1000 (10⁻³) or 1/100,000 (10⁻⁵) dilutions, we can easily prepare plates representing these dilutions by plating only 0.1 mL (one-tenth of a mL) of the 10⁻² and 10⁻⁴ dilutions respectively.

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

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



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

Figure 8.2.2: Example data set. To calculate the number of CFU in the original sample, you would take 250 CFU and multiply it by ten thousand, or 35 and multiply it by a million. Together, this gives you a final answer of around 2-4 million CFU per gram of food.

The “Who” Question

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

Eosin-Methylene Blue Agar

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

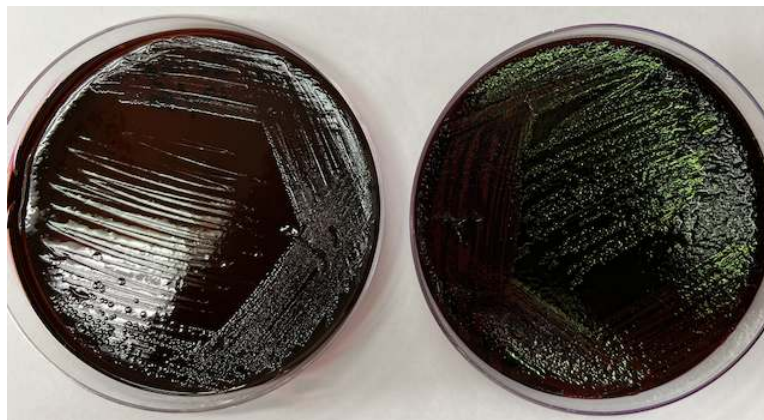


Figure 8.2.3: Typical growth on EMB. Left - a non-lactose fermentor. Right - a vigorous lactose fermenter such as *E. coli* produces a characteristic green sheen.

Hektoen Enteric Agar

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

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



Figure 8.2.4: Left - *Salmonella enterica* produces hydrogen sulfide and turns black on Hektoen. Right - *Shigella sonnei* is not supposed to ferment lactose...but in our lab it always does producing acid and turning the medium orange.

Materials

Per Student Group:

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

Experiment

Day 1

For steps 1-10, refer to Figure 8.2.1.

Prepare Dilution blanks

1. Collect three 99 mL water blanks and label them 10-2, 10-4, and 10-6.

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

Prepare Plates

1. Collect 5 empty Petri dishes, and label them with your names, dates, and the dilution they represent (10-2, 10-3, 10-4, 10-5, 10-6).
2. Ensure that each dilution blank is well-mixed. Using correct aseptic technique, pipette
 - o 1.0 mL of the 10-2 dilution into the 10-2 Petri plate
 - o 0.1 mL of the 10-2 dilution into the 10-3 Petri plate
 - o 1.0 mL of the 10-4 dilution into the 10-4 Petri plate
 - o 0.1 mL of the 10-4 dilution into the 10-5 Petri plate
 - o 1.0 mL of the 10-6 dilution into the 10-6 Petri plate
3. Retrieve molten TSA deeps one at a time from the dry bath. Pour one deep into each plate, swirling gently (make a Figure 8.2.8 with the plate) to mix the dilutions with the agar. Do not swirl so hard that the agar touches the plate lid.
4. Allow the agar to cool and solidify on the lab bench.
5. Stack the five plates and secure them with a rubber band. Incubate at 37 °C for 48 hours or at 30 °C for 5 days.

6. Prepare Hektoen and EMB Plates

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

Day 2

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

Data

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

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

Your data

Food Sample _____

Table 1: Student Data From Standard Plate Counts

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

EMB/Hektoen Plate Results:

Table 2: Student Data from Hektoen and EMB Plates

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

Class Data:

Record as CFU/gram of the original food sample

Table 3: Class Data from Standard Plate Counts

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

Questions

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

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SECTION OVERVIEW

8.3: Microbiology of Milk and Food

8.3.1: Introduction

8.3.2: Procedures

8.3.3: Results

8.3.4: Review Questions

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8.3.1: Introduction

Learning Objectives

- Learn how to use serial dilutions.
- Learn how to do a standard plate count.
- Understand the importance of standard microbiological tests for food safety

Pathogens can be introduced into foods at any stage: during growth/production at the farm, during processing (grinding, chopping, milling, etc.), during handling and packaging, and when the food is prepared in the kitchen. In many cases, small numbers of pathogenic bacteria are not dangerous, but improper storage and/or cooking conditions can allow these bacteria to multiply to dangerous levels.

Fecal contamination of water (and through water, contamination of food materials) is another one of the ways in which pathogens can be introduced. **Coliform bacteria** are Gram-negative non-spore forming bacteria that are capable of fermenting lactose to produce acid and gas. A subset of these bacteria are the **fecal coliforms**, which are found at high levels in human and animal intestines. Fecal coliform bacteria such as *E. coli*, are often used as **indicator species**, as they are not commonly found growing in nature in the absence of fecal contamination. The presence of *E. coli* suggests feces are present, indicating that serious pathogens, such as *Salmonella* species and *Campylobacter* species, could also be present.

In this lab, you will examine bacteria found in milk, chicken, and in other assorted food materials.

Milk

Milk contains carbohydrates, minerals, fats, vitamins, and proteins, and is therefore susceptible to breakdown by a wide variety of microorganisms. Several different kinds of bacteria may be present in milk, most commonly the genera *Lactobacillus*, *Micrococcus*, and *Streptococcus*. As discussed in Lab 7, regular pasteurization is the process used to reduce microbial loads to acceptable levels in foods like milk and fruit juices. Milk that has undergone a regular pasteurization procedure can still contain bacteria. If this milk is stored at 4°C (refrigerator temperature) the bacteria are prevented from multiplying, but if the milk is left out at room temperature, the bacteria will reproduce and the milk will spoil.

If done aseptically, **UHT (ultra high temperature) pasteurization** can sterilize foods. This is why UHT milk can be left unopened at room temperature for long periods of time.

Microorganisms of concern in milk include *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Salmonella* species (most common: *S. enteritidis* and *S. typhimurium*), and *Yersinia enterocolitica*.

Chicken

Raw poultry products are frequently contaminated by pathogens (e.g. *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens*, *Campylobacter jejuni*, etc.). Salmonellosis is one of the most common serious foodborne infection caused by contaminated poultry. The prevalence of *Salmonella* in raw fresh and frozen poultry approaches 80% in some countries.

Fruits and Vegetables

Fruits and vegetables may become contaminated with potential pathogens from the soil or water they come in contact with while growing. They may also pick up harmful bacteria during processing, storage and preparation. While the use of potable (uncontaminated, drinkable) water to wash and freshen harvestable fruits and vegetables is useful both commercially and in the home, residual water may support extensive microbial growth during further storage.

Standard Plate Count and Serial Dilution Techniques

One of the most common methods of determining the amount of bacteria in a food product is a **standard plate count**. In this method, serial dilutions of the food are plated on general purpose and/or differential/selective growth media. Bacterial colonies are

then counted, and the number of **CFUs** (colony forming units) in the original undiluted sample is calculated. (CFUs are as a measure of the number of bacteria to take into account that one colony might be the product of more than a single bacterium).

Serial dilution is a technique that is used to produce very dilute solutions without the necessity of measuring very small quantities of liquids. It is a series of stepwise dilutions, in which one first dilutes a solution, then dilutes the dilution, then dilutes the dilution and so forth. The dilution factor at each step is usually constant, resulting in a geometric progression of concentration. An example of a serial dilution is seen below. In this example, each dilution is a 10-fold dilution (transferring 1 ml into 9 ml of H₂O results in a 1/10 dilution; i.e., 1 ml in a total volume of 10 ml).

Serial dilutions are often used in standard plate counts because the number of bacteria in a sample (water, food, or a medical sample such as a urine or a fecal sample) is unknown. The sample is diluted to obtain a number of CFUs that supplies statistically significant results, yet is still easily countable. The general recommendation for a countable plate is between 30 – 300 CFUs/plate.

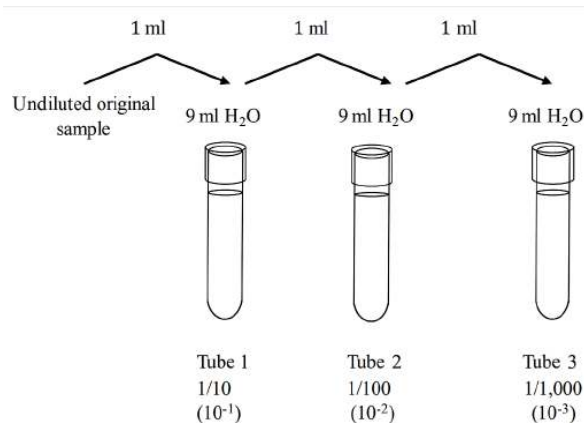


Figure 9.1.1: *Serial Dilution*

After dilutions are prepared, a set amount of liquid (typically between 0.1- 1 ml) is spread out over the surface of an agar plate, and then incubated to allow for bacterial growth. CFU counts from these diluted plates are used to calculate the number of bacterial cells/ml in your original (undiluted) sample. If you plate a full milliliter (ml) of your dilution, you would simply multiply the number of CFU's counted by the dilution factor of the plate you counted.

- Example: I count 55 CFU's on a plate diluted 1:1000 (10⁻³) in which I added a full ml of my dilution. My calculation is $55 \times 1000 = 55,000$ (5.5×10^4) cells/ml.

However, it is important to note that we do not always plate a full ml (it can be difficult to get that much liquid to be absorbed into an agar plate). Therefore an additional calculation is often necessary to be able to express your results as cells/ml. If you plate 0.1 ml of sample, you will need to multiply the number of CFU's by 10 to determine # CFU's/ml. If you plate 0.5 ml of a sample, you will need to multiply by 2 to determine the number of CFU's/ml.

- Here's another example: you set up serial dilutions for a milk sample and plate out 0.5 ml of your dilutions. The countable plate that came from your 1:100 (10⁻²) dilution has 114 colonies.

Your calculation:

1. Multiply: #CFU's x dilution factor = $114 \times 100 = 11400$ cells/0.5 ml.
2. Multiply by 2 to express your result as cells/ml $11400 \times 2 = 22800$ cells/ml (or 2.28×10^4) If dilutions are done properly, we should expect to see a geometric progression in the number of cells in each dilution as shown below.

	Dilution	Number of bacteria/ml in each tube after dilution	Number of CFU's (1 ml plated)	Number of CFU's (0.1 ml plated)
Undiluted sample		1,000,000/ml = 10^6 /ml	1,000,000 (10^6)	100,000 (10^5)
Tube 1	$1/10 = 10^{-1}$	100,000/ml = 10^5 /ml	100,000 (10^5)	10,000 (10^4)
Tube 2	$1/100 = 10^{-2}$	10,000/ml = 10^4 /ml	10,000 (10^4)	1,000 (10^3)
Tube 3	$1/1000 = 10^{-3}$	1,000/ml = 10^3 /ml	1,000 (10^3)	100 (10^2)

Of course your results would not turn out as perfectly as those shown above. Slight inaccuracies in pipetting would cause variations in the final results. So in the real world, the results of multiple dilutions are averaged together when determining the final number of bacteria/ml in a sample.

For a review of scientific notation and additional practice problems to help you understand serial dilution, see Appendix II. For additional information about the use of pipettors for measuring small volumes, see Appendix III.

Key Terms

CFU, indicator bacteria, serial dilution, coliform bacteria, fecal coliform bacteria, standard plate count, UHT pasteurization

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8.3.2: Procedures

Note

You will be using sterile spreaders when you inoculate plates in Procedures A and B of this lab. Each table will have a tinfoil packet of sterile spreaders. Please be careful when you open the packet (unfold the narrow end first) so you do not contaminate the spreaders. There will be a beaker of vesphene specifically for these spreaders at each table. Place the spreaders in this beaker. DO NOT throw the spreaders in the garbage—they will be autoclaved and reused for later lab sections. DO NOT use spreaders from the disinfectant beakers in your experiment.

A. The Microbiology of Milk

In this exercise, you will compare the amount of bacteria found in regular pasteurized milk with the amount found in UHT pasteurized milk. You will also examine the effects of leaving the two different types of milk at room temperature.

Per pair of students: 2 TSA plates

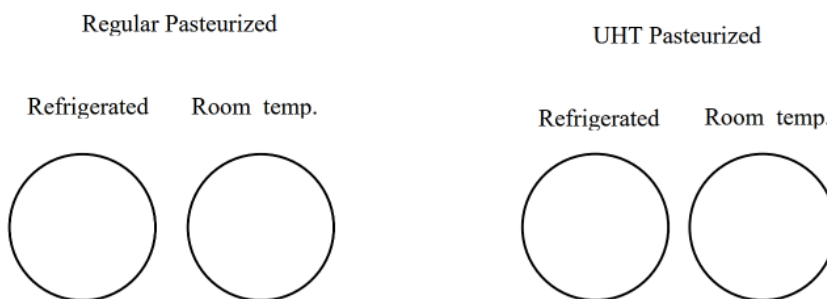
Per pair of students:

Either: 2 tubes of pasteurized milk: one refrigerated, one left out at room temperature for 1 week, **or** 2 tubes of UHT pasteurized milk: one refrigerated, one left out at room temperature for 1 week

1. Each pair of students will choose either the regular pasteurized milk or the UHT pasteurized milk.
2. Using the 20-200 μ l pipettor (also called the P200), transfer 200 μ l of the refrigerated milk on a TSA plate. (Appendix III provides instructions on how to use micropipettors.)
3. Use a sterile spreader to spread the 200 μ l of the milk across the plate.
4. Using a **different** sterile spreader, repeat Steps 2-3 using the room temperature milk.
5. Incubate the plates until the next lab.

Type of milk chosen: _____

Per Table, you should have the following plates:



What type of bacteria will grow on the TSA plates? _____

Predict which type of milk will have the most bacteria. Explain the rationale for your prediction.

B. The Microbiology of Poultry

In this exercise you will determine the number of bacteria found on chicken wings using serial dilutions followed by a standard plate count.

There will be 1 resealable plastic bag containing 9 ml of sterile water and a chicken wing at the instructor's table.

Per table: 3 TSA plates ,4 tubes, each containing 9 ml of sterile H₂O,1 tube of chicken wing wash fluid (given to you by your instructor)

1. The instructor will rinse the chicken wing in the water, massaging the chicken wing to remove any bacteria that are adhering to the skin. Using a 200 - 1000 μl pipettor (also called the P1000), the instructor will transfer 1 ml of the wash fluid into 99 ml of H_2O , and will mix thoroughly. This will be a dilution of $1/100$ (10^{-2}). The instructor will transfer 2-3 ml of this $1/100$ dilution into 4 sterile tubes (1/table).

2. Label one of the tubes of sterile water 10^{-3} ($1/1,000$)—this will be Tube 1. Label one of the tubes of sterile water 10^{-4} ($1/10,000$)—this will be Tube 2. Label the next tube of sterile water 10^{-5} ($1/100,000$)—this will be Tube 3. Label the last tube of sterile water 10^{-6} ($1/1,000,000$)—this will be Tube 4.

3. Using the P1000, remove 1 ml of the $1/100$ wash fluid from the tube given to you by the instructor, and transfer it to Tube 1. Mix the contents of the tube by carefully by flicking the tube. Using the same pipettor, transfer 1 ml from Tube 1 into Tube 2; transfer 1 ml from Tube 2 to Tube 3, and transfer 1 ml from Tube 3 to Tube 4. After each transfer, flick to mix thoroughly. The figure below shows you how to make the transfers.

Instructions for plating

Using the P200, transfer 100 μl from Tube 2, Tube 3 and Tube 4 to 3 different TSA plates. Make sure to label your plates. The figure below indicates how the transfers should be done.

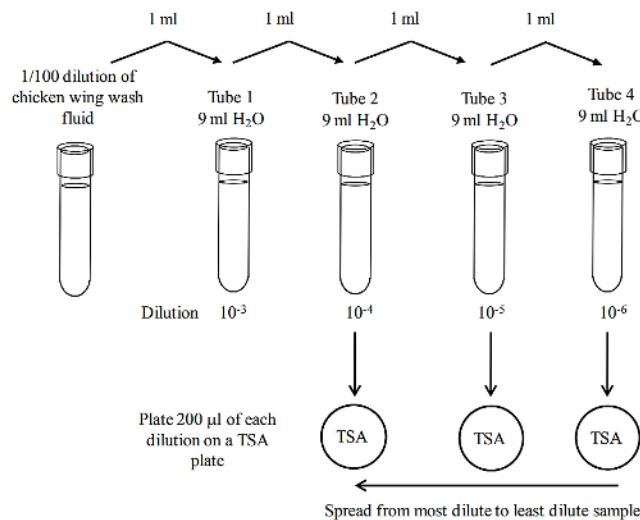


Figure 9.2.1: Serial dilutions and transfers to plates

You will be using 1 sterile spreader for all of your plates, so you must be careful to work from the most dilute sample to the least dilute sample. Make sure that all of the fluid has been absorbed into the plate before you invert the plate for incubation.

C. Microbiology of Food

Per table: 1 TSA plate

Per table: Choose 1 of the following four food materials: ground beef, mushroom, bread or lettuce.

Chosen food material: _____

1. Flame sterilize forceps. (Note: follow the recommendations for forcep sterilization described in Lab 9.)
2. Pick up a portion of your chosen food product, gently rub it over the surface of the TSA plate, then discard the food.
3. Incubate the plate until the next lab. Of the four food materials, which do you think will contain the most bacteria?

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8.3.3: Results

A. The Microbiology of Milk

Record the results of your experiment in the table below. Record growth as 0, +, ++, or +++. Describe any notable growth characteristics.

TSA Plates

	Amount of Growth	Growth Characteristics
Regular pasteurized (refrigerated)		
Regular pasteurized (room temperature)		
UHT pasteurized (refrigerated)		
UHT pasteurized (room temperature)		

Which type of milk had the largest number of bacteria? _____

Was your earlier prediction correct? _____

B. The Microbiology of Poultry

Count the CFUs on your plates. Record your numbers in the table below. If there are too many CFUs to count, just write “TMTTC” (too many to count). Remember—a countable plate that is considered statistically significant should have between 30 -300 CFUs.

Record your plate counts below. Make note of any notable growth characteristics (e.g., fungal-like growth, colony colors, etc.) Note: not all of your plates may be in the countable plate range (they may have many more or many fewer colonies). You need only record the numbers for the countable plates.

Based on the results for each countable dilution, determine the total number of bacteria in the original sample. Then average these numbers to obtain a more significant result.

Dilution plated	Amount spread on plate	Growth characteristics	Number of CFUs/plate	Number of bacteria/ml in original sample (No. CFUs x Dilution factor x 10)
Tube 2 = 10^{-4} 0.1 ml				
Tube 3 = 10^{-5} 0.1 ml				
Tube 4 = 10^{-6} 0.1 ml				

Average number of bacteria/ml in original sample:

If you also did this experiment using EMB plates, would you expect more colonies on the TSA or the EMB plates? Explain your answer.

What type of bacteria could you identify by using the EMB plates, and why would these bacteria be of concern for human health?

Class Data

For the results of the experiment to be more statistically significant, data from the entire class will be collected and written in the following table.

	Number of bacteria/ml in original sample
Table 1	
Table 2	
Table 3	
Table 4	
Average	

C. Microbiology of Food

Observe the plates of all of the food materials. Rate the food material based on the relative number of bacterial colonies seen on each plate:

least bacteria _____ < _____ < _____ < _____ most
bacteria

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8.3.4: Review Questions

1. You are testing ground beef to determine how many bacteria are present per gram of meat. You place 1 gram of meat in sterile water, mix vigorously, and then do serial dilutions as shown below. You inoculate a plate with 0.1 ml of the sample from the last tube. The next day you count 122 colonies. How many bacteria/ml were in the undiluted sample?

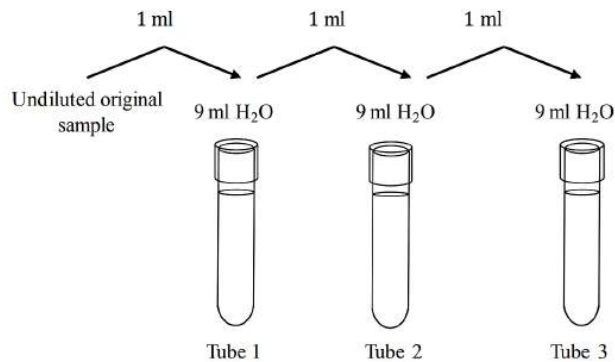


Figure 9.4.1: Serial Dilutions for Question 1

2. When doing serial dilutions, why is it necessary to plate more than one dilution?
3. If you observe *Escherichia coli* in a food sample, what could this indicate?
4. You left a carton of orange juice on your counter for 3 days. When you taste it, it is very bubbly (as though it was carbonated), and it tastes more bitter than usual. (Note: acids taste bitter). What do you think could have happened? (Note: you can't just say that bacteria grew—you must explain how the growth of the bacteria resulted in the changes in the orange juice.)
5. Based on what you now know about the presence of bacteria on chicken and beef, why is it a good idea to use separate chopping boards for meat and for vegetables?
6. You are testing unpasteurized milk for the presence of bacterial contamination. Starting from the undiluted milk, you do serial dilutions as shown below, and plate 1.0 ml of each dilution on agar. If the undiluted milk contains 5×10^6 bacteria/ml, how many colonies would you expect to see on each plate?

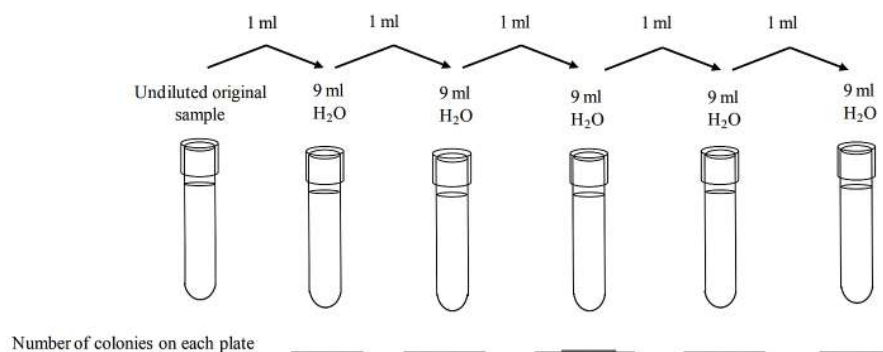


Figure 9.4.2: Serial Dilutions for Question 6

7. Do you think standard plate counts are very accurate? Why or why not?

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CHAPTER OVERVIEW

9: Lab 9

[9.1: Required Materials](#)

[9.2: Control of Microbial Growth](#)

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9.1: Required Materials

TSB cultures

- *E. coli*
- *S. aureus*

5 TSB plates

Various soaps and disinfectants

sterile diH₂O

Sterile cotton-tipped applicators

Sterile droppers

Sterile paper disks

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9.2: Control of Microbial Growth

Learning Objectives

- Define the following terms: fomites, sterilization, asepsis, sepsis, commercial sterilization, disinfection, sanitization, antisepsis, degerming
- Give at least five examples of the categories of physical controls for microbes and what they involve.
- Give at least three examples of categories of chemical controls for microbes and what they involve.
- Successfully conduct an experiment comparing soap, disinfectant, and untreated surfaces (fomites) and the microbial load present.
- Graph and analyze results from control of microbial growth experiment.
- Interpret t-test results and state conclusions based on the interpretation.

Clean Enough?

How clean is clean? People wash their cars and vacuum the carpets, but most would not want to eat from these surfaces. Similarly, we might eat with silverware cleaned in a dishwasher, but we could not use the same dishwasher to clean surgical instruments. As these examples illustrate, “clean” is a relative term. Car washing, vacuuming, and dishwashing all reduce the microbial load on the items treated, thus making them “cleaner.” But whether they are “clean enough” depends on their intended use. Because people do not normally eat from cars or carpets, these items do not require the same level of cleanliness that silverware does. Likewise, because silverware is not used for invasive surgery, these utensils do not require the same level of cleanliness as surgical equipment, which requires sterilization to prevent infection.

Location	Average number CFUs per 6.5 × 6.5 cm area
Door latch	256
Door lock	14
Door lock control	182
Door handle	29
Window control	4
Cruise control button	69
Steering wheel	239
Interior steering wheel	390
Radio volume knob	99
Gear shifter	115
Center console	506



Figure 1: Most environments, including cars, are not sterile. A study¹ analyzed 11 locations within 18 different cars to determine the number of microbial colony-forming units (CFUs) present. The center console harbored by far the most microbes (506 CFUs), possibly because that is where drinks are placed (and often spilled). Frequently touched sites also had high concentrations. (credit “photo”: modification of work by Jeff Wilcox)

Why not play it safe and sterilize everything? Sterilizing everything we come in contact with is impractical, as well as potentially dangerous. As this chapter will demonstrate, sterilization protocols often require time- and labor-intensive treatments that may degrade the quality of the item being treated or have toxic effects on users. Therefore, the user must consider the item’s intended application when choosing a cleaning method to ensure that it is “clean enough.”

Fomites

To prevent the spread of human disease, it is necessary to control the growth and abundance of microbes in or on various items frequently used by humans. Inanimate items, such as doorknobs, toys, or towels, which may harbor microbes and aid in disease transmission, are called **fomites**. Two factors heavily influence the level of cleanliness required for a particular fomite and, hence, the protocol chosen to achieve this level. The first factor is the application for which the item will be used. For example, invasive applications that require insertion into the human body require a much higher level of cleanliness than applications that do not. The

second factor is the level of resistance to antimicrobial treatment by potential pathogens. For example, foods preserved by canning often become contaminated with the bacterium *Clostridium botulinum*, which produces the neurotoxin that causes botulism. Because *C. botulinum* can produce endospores that can survive harsh conditions, extreme temperatures and pressures must be used to eliminate the endospores. Other organisms may not require such extreme measures and can be controlled by a procedure such as washing clothes in a laundry machine.

Control of Microbial Growth: Sterilization

The most extreme protocols for microbial control aim to achieve **sterilization**: the complete removal or killing of all vegetative cells, endospores, and viruses from the targeted item or environment. Sterilization protocols are generally reserved for laboratory, medical, manufacturing, and food industry settings, where it may be imperative for certain items to be completely free of potentially infectious agents. Sterilization can be accomplished through either physical means, such as exposure to high heat, pressure, or filtration through an appropriate filter, or by chemical means. Chemicals that can be used to achieve sterilization are called **sterilants**. Sterilants effectively kill all microbes and viruses, and, with appropriate exposure time, can also kill endospores.

For many clinical purposes, **aseptic technique** is necessary to prevent contamination of sterile surfaces. Aseptic technique involves a combination of protocols that collectively maintain sterility, or **asepsis**, thus preventing contamination of the patient with microbes and infectious agents. Failure to practice aseptic technique during many types of clinical procedures may introduce microbes to the patient's body and put the patient at risk for **sepsis**, a systemic inflammatory response to an infection that results in high fever, increased heart and respiratory rates, shock, and, possibly, death. Medical procedures that carry risk of contamination must be performed in a **sterile field**, a designated area that is kept free of all vegetative microbes, endospores, and viruses. Sterile fields are created according to protocols requiring the use of sterilized materials, such as packaging and drapings, and strict procedures for washing and application of sterilants. Other protocols are followed to maintain the sterile field while the medical procedure is being performed.

One food sterilization protocol, **commercial sterilization**, uses heat at a temperature low enough to preserve food quality but high enough to destroy common pathogens responsible for food poisoning, such as *C. botulinum*. Because *C. botulinum* and its endospores are commonly found in soil, they may easily contaminate crops during harvesting, and these endospores can later germinate within the anaerobic environment once foods are canned. Metal cans of food contaminated with *C. botulinum* will bulge due to the microbe's production of gases; contaminated jars of food typically bulge at the metal lid. To eliminate the risk for *C. botulinum* contamination, commercial food-canning protocols are designed with a large margin of error. They assume an impossibly large population of endospores (10^{12} per can) and aim to reduce this population to 1 endospore per can to ensure the safety of canned foods. For example, low- and medium-acid foods are heated to 121 °C for a minimum of 2.52 minutes, which is the time it would take to reduce a population of 10^{12} endospores per can down to 1 endospore at this temperature. Even so, commercial sterilization does not eliminate the presence of all microbes; rather, it targets those pathogens that cause spoilage and foodborne diseases, while allowing many nonpathogenic organisms to survive. Therefore, "sterilization" is somewhat of a misnomer in this context, and commercial sterilization may be more accurately described as "quasi-sterilization."

Control of Microbial Growth: Non-Sterilizing Approaches

Sterilization protocols require procedures that are not practical, or necessary, in many settings. Various other methods are used in clinical and nonclinical settings to reduce the microbial load on items. Although the terms for these methods are often used interchangeably, there are important distinctions.

The type of protocol required to achieve the desired level of cleanliness depends on the particular item to be cleaned. For example, those used clinically are categorized as critical, semicritical, and noncritical. Critical items must be sterile because they will be used inside the body, often penetrating sterile tissues or the bloodstream; examples of **critical items** include surgical instruments, catheters, and intravenous fluids. Gastrointestinal endoscopes and various types of equipment for respiratory therapies are examples of **semicritical items**; they may contact mucous membranes or nonintact skin but do not penetrate tissues. Semicritical items do not typically need to be sterilized but do require a high level of disinfection. Items that may contact but not penetrate intact skin are **noncritical items**; examples are bed linens, furniture, crutches, stethoscopes, and blood pressure cuffs. These articles need to be clean but not highly disinfected.

Figure 1 summarizes common protocols, definitions, applications, and agents used to control microbial growth.

Common Protocols for Control of Microbial Growth			
Protocol	Definition	Common Application	Common Agents
For Use on Fomites			
Disinfection	Reduces or destroys microbial load of an inanimate item through application of heat or antimicrobial chemicals	Cleaning surfaces like laboratory benches, clinical surfaces, and bathrooms	Chlorine bleach, phenols (e.g., Lysol), glutaraldehyde
Sanitization	Reduces microbial load of an inanimate item to safe public health levels through application of heat or antimicrobial chemicals	Commercial dishwashing of eating utensils, cleaning public restrooms	Detergents containing phosphates (e.g., Finish), industrial-strength cleaners containing quaternary ammonium compounds
Sterilization	Completely eliminates all vegetative cells, endospores, and viruses from an inanimate item	Preparation of surgical equipment and of needles used for injection	Pressurized steam (autoclave), chemicals, radiation
For Use on Living Tissue			
Antisepsis	Reduces microbial load on skin or tissue through application of an antimicrobial chemical	Cleaning skin broken due to injury; cleaning skin before surgery	Boric acid, isopropyl alcohol, hydrogen peroxide, iodine (betadine)
Degerming	Reduces microbial load on skin or tissue through gentle to firm scrubbing and the use of mild chemicals	Handwashing	Soap, alcohol swab

Figure 2: Summary of different approaches for reducing microbes. For use on fomites includes disinfection, sanitization, and sterilization. For use on living tissue involves antisepsis (for broken tissues and Preparation for surgery) and degerming (handwashing). The only method that completely eliminates all microbes is sterilization. All other approaches are for reducing microbial load.

Disinfection

The process of **disinfection** inactivates most microbes on the surface of a fomite by using antimicrobial chemicals or heat. Because some microbes remain, the disinfected item is not considered sterile. Ideally, **disinfectants** should be fast acting, stable, easy to prepare, inexpensive, and easy to use. An example of a natural disinfectant is vinegar; its acidity kills most microbes. Chemical disinfectants, such as chlorine bleach or products containing chlorine, are used to clean nonliving surfaces such as laboratory benches, clinical surfaces, and bathroom sinks. Typical disinfection does not lead to sterilization because endospores tend to survive even when all vegetative cells have been killed.

Antisepsis

Unlike disinfectants, **antiseptics** are antimicrobial chemicals safe for use on living skin or tissues. Examples of antiseptics include hydrogen peroxide and isopropyl alcohol. The process of applying an antiseptic is called **antisepsis**. In addition to the characteristics of a good disinfectant, antiseptics must also be selectively effective against microorganisms and able to penetrate tissue deeply without causing tissue damage.

Degerming

The act of handwashing is an example of **degerming**, in which microbial numbers are significantly reduced by gently scrubbing living tissue, most commonly skin, with a mild chemical (e.g., soap) to avoid the transmission of pathogenic microbes. Wiping the skin with an alcohol swab at an injection site is another example of degerming. These degerming methods remove most (but not all) microbes from the skin's surface.

Sanitization

The term **sanitization** refers to the cleansing of fomites to remove enough microbes to achieve levels deemed safe for public health. For example, commercial dishwashers used in the food service industry typically use very hot water and air for washing and drying; the high temperatures kill most microbes, sanitizing the dishes. Surfaces in hospital rooms are commonly sanitized using a chemical disinfectant to prevent disease transmission between patients.

Physical Approaches for Controlling Microbial Growth

Physical Methods of Control			
Method	Conditions	Mode of Action	Example Uses
Heat			
Boiling	100 °C at sea level	Denatures proteins and alters membranes	Cooking, personal use, preparing certain laboratory media
Dry-heat oven	170 °C for 2 hours	Denatures proteins and alters membranes, dehydration, desiccation	Sterilization of heat-stable medical and laboratory equipment and glassware
Incineration	Exposure to flame	Destroy by burning	Flaming loop, microincinerator
Autoclave	Typical settings: 121 °C for 15 minutes at 15 pounds per square inch (psi)	Denatures proteins and alters membranes	Sterilization of microbiological media, heat-stable medical and laboratory equipment, and other heat-stable items
Pasteurization	Can vary. One type is 72 °C for 15 seconds (HTST)	Denatures proteins and alters membranes	Prevents spoilage of milk, apple juice, honey, and other ingestible liquids
Cold			
Refrigeration	0 °C to 7 °C	Inhibits metabolism (slows or arrests cell division)	Preservation of food or laboratory materials (solutions, cultures)
Freezing	Below -2 °C	Stops metabolism, may kill microbes	Long-term storage of food, laboratory cultures, or medical specimens
Pressure			
High-pressure processing	100–800 MPa	Denatures proteins and can cause cell lysis	Preservation of food
Hyperbaric oxygen therapy	Air pressure three times higher than normal	Inhibits metabolism and growth of anaerobic microbes	Treatment of certain infections (e.g., gas gangrene)
Desiccation			
Simple desiccation	Drying	Inhibits metabolism	Dried fruits, jerky
Reduce water activity	Addition of salt or water	Inhibits metabolism and can cause lysis	Salted meats and fish, honey, jams and jellies
Lyophilization	Rapid freezing under vacuum	Inhibits metabolism	Preservation of food, laboratory cultures, or reagents
Radiation			
Ionizing radiation	Exposure to X-rays or gamma rays	Alters molecular structures, introduces double-strand breaks into DNA	Sterilization of spices and heat-sensitive laboratory and medical items; used for food sterilization in Europe but not widely accepted in US
Nonionizing radiation	Exposure to ultraviolet light	Introduces thymine dimers, leading to mutations	Disinfection of surfaces in laboratories and rooms in health-care environment, and disinfection of water and air

Physical Methods of Control (continued)			
Method	Conditions	Mode of Action	Example Uses
Sonication			
Sonication	Exposure to ultrasonic waves	Cavitation (formation of empty space) disrupts cells, lysing them	Laboratory research to lyse cells; cleaning jewelry, lenses, and equipment
Filtration			
HEPA filtration	Use of high-efficiency particulate air (HEPA) filter with 0.3 μm pore size	Physically removes microbes from air	Laboratory biological safety cabinets, operating rooms, isolation units, heating and air conditioning systems, vacuum cleaners
Membrane filtration	Use of membrane filter with 0.2- μm or smaller pore size	Physically removes microbes from liquid solutions	Removal of bacteria from heat-sensitive solutions like vitamins, antibiotics, and media with heat-sensitive components

Figure 2: Summary of the physical methods for controlling microbes. These approaches include the following categories: heat, cold, pressure, desiccation, radiation, sonication, and filtration. There are multiple ways for controlling microbes within some of these categories. For example, controlling microbes with cold could involve refrigeration or it could involve freezing.

Chemical Approaches for Controlling Microbial Growth

Table 1: Chemical disinfectants can be used to control microbes. These types of chemicals fall into different chemical categories: phenolics, metals, halogens, alcohols, surfactants, bisbiguanides, alkylating agents, peroxygens, supercritical gases, chemical food preservatives, and natural food preservatives.

Chemical Disinfectants		
Chemical	Mode of Action	Example Uses
Phenolics		
Cresols o-Phenylphenol Hexachlorophene Triclosan	Denature proteins and disrupt membranes	Disinfectant in Lysol Prevent contamination of crops (citrus) Antibacterial soap pHisoHex for handwashing in hospitals
Metals		
Mercury Silver Copper Nickel Zinc	Bind to proteins and inhibit enzyme activity	Topical antiseptic Treatment of wounds and burns Prevention of eye infections in newborns Antibacterial in catheters and bandages Mouthwash Algicide for pools and fish tanks Containers for long-term water storage
Halogens		
Iodine Chlorine Fluorine	Oxidation and destabilization of cellular macromolecules	Topical antiseptic Hand scrub for medical personnel Water disinfectant Water treatment plants Household bleach Food processing Prevention of dental carries
Alcohols		
Ethanol Isopropanol	Denature proteins and disrupt membranes	Disinfectant Antiseptic
Surfactants		
Quaternary ammonium salts	Lowers surface tension of water to help with washing away of microbes, and disruption of cell membranes	Soaps and detergent Disinfectant Antiseptic Mouthwash
Bisbiguanides		
Chlorhexidine Alexidine	Disruption of cell membranes	Oral rinse Hand scrub for medical personnel
Alkylating Agents		
Formaldehyde Glutaraldehyde o-Phthalaldehyde Ethylene oxide β-Propionolactone	Inactivation of enzymes and nucleic acid	Disinfectant Tissue specimen storage Embalming Sterilization of medical equipment Vaccine component for sterility
Peroxygens		
Hydrogen peroxide Peracetic acid Benzoyl peroxide	Oxidation and destabilization of cellular macromolecules	Antiseptic Disinfectant

Chemical Disinfectants		
Chemical	Mode of Action	Example Uses
Carbamide peroxide		Acne medication
Ozone gas		Toothpaste ingredient
Supercritical Gases		
Carbon dioxide	Penetrates cells, forms carbonic acid, lowers intracellular pH	Food preservation Disinfection of medical devices Disinfection of transplant tissues
Chemical Food Preservatives		
Sorbic acid Benzoic acid Propionic acid Potassium sorbate Sodium benzoate Calcium propionate Sulfur dioxide Nitrites	Decrease pH and inhibit enzymatic function	Preservation of food products
Natural Food Preservatives		
Nisin Natamycin	Inhibition of cell wall synthesis (Nisin)	Preservation of dairy products, meats, and beverages

Laboratory Instructions

Purpose

The goal of this experiment is to examine how various cleaners perform in sterilizing surfaces and directly affecting bacterial growth.

1. The surface as it is, untreated
2. The surface that has been cleaned with a soap
3. The surface that has been cleaned with a disinfectant
4. Soap is placed directly in bacterial culture on plate
5. Disinfectant is placed in direct contact with bacterial culture on plate

Each person will choose a surface. Work as a group so that the surfaces you choose will be diverse and different. Surfaces may be anything that it will be safe to use soap and disinfectant on. Examples include:

- Door handles
- Pens/pencils
- Cell phones (being mindful of not getting sensitive parts wet when cleaning)
- The floor
- A shoe
- A lab bench
- Lab equipment
- A sink in the lab or the bathroom
- Other parts of the bathroom
- A computer mouse (being mindful of not getting sensitive parts wet when cleaning)
- Keys
- Anything else you can think of

Instructions

1. Choose a surface you would like to examine for microbes.
2. Label three petri plates with:
 - a. Your name
 - b. Your group number
 - c. The sample being swabbed
 - d. Label one of each as:
 - i. uncleaned
 - ii. soap cleaned
 - iii. disinfectant cleaned
3. Dip a sterile cotton swab into dH₂O and then swab the surface of interest and streak across the Petri plate labeled "uncleaned."
4. Clean a **separate spot** of that same surface with soap and wipe dry. Dip a sterile cotton swab into dH₂O and then swab the surface you cleaned with soap and streak across the Petri plate labeled "soap cleaned."
5. Clean a **separate spot** of that surface with disinfectant and wipe dry. Dip a sterile cotton swab into dH₂O and then swab the surface you cleaned with disinfectant and streak the Petri plate labeled "disinfectant cleaned."
6. Invert all plates and place in the class bin (these will be put in the incubator until next class).
7. Choose one additional plate and draw 6 separate sections on the bottom plate. Choose 6 soaps and or disinfectants to test and label each section accordingly.
8. Inoculate with one of the available bacterial cultures.
9. Aseptically place one sterile paper disk in each of the 6 sections of the plate. Add 2 drops of your chosen soaps/disinfectants to each disk. Once finished, place plate in incubator.

Results & Questions

1. The surface I am examining in this experiment is...
2. I predict the following will have the **most** bacterial growth (*circle one*): uncleaned, soap cleaned, disinfectant cleaned
3. I predict the following will have the **least** bacterial growth (*circle one*): uncleaned, soap cleaned, disinfectant cleaned
4. Predict how your selected soap/disinfectants will compare to each other in terms of affecting microbial growth.
4. Record your individual colony numbers for each test.
5. Record class results (average colony number).
6. Discuss your results.
 - Which treatment produced the most microbial growth?
 - Which treatment produced the least microbial growth

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CHAPTER OVERVIEW

10: Lab 10

[10.1: Required Materials](#)

[10.2: Bacterial Susceptibility to Antibiotics \(Kirby-Bauer Test\)](#)

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10.1: Required Materials

TSB Cultures

- *S. aureus*
- *P. aeruginosa*
- *E. coli*
- *S. epidermidis*
- *A. faecalis*
- *B. subtilis*

2 TSB plates

Antibiotic disks

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10.2: Bacterial Susceptibility to Antibiotics (Kirby-Bauer Test)

Learning Objectives

- Define "antibiotic" and tell where these chemicals come from in nature (for non-synthetic or non-semisynthetic).
- Differentiate between broad spectrum antibiotics and narrow spectrum antibiotics.
- Tell that use of broad spectrum antibiotics contributes to increased antibiotic resistance in bacteria.
- Tell the purpose of the Kirby-Bauer test.
- Explain how the Kirby-Bauer test works including how diffusion of the antibiotics is important in the creation zones of inhibition.
- Define "zone of inhibition."
- Successfully conduct a Kirby-Bauer test (modified) and interpret the results.
- Explain why the Kirby-Bauer test is important for reducing antibiotic resistance.
- Describe why antibiotic resistance is a threat for successful treatment of bacterial infections.

Introduction to Antibiotics

Antibiotics are chemicals produced by some bacteria and fungi that, in small quantities, can inhibit the growth of bacteria. Much of the success we have achieved in treating infections since World War II is due to the discovery of antibiotics. Utilizing the information gleaned from studying these natural chemicals, scientists have artificially synthesized other useful antimicrobial chemicals in the laboratory. Sometimes these antimicrobials are completely synthesized in the lab (synthetic) and sometimes they are partly produced in nature and partly synthesized in the lab (semisynthetic).

Many microbes can produce antibiotics, but four genera produce most of the antibiotics used for treating human and animal infections. *Bacillus* and *Streptomyces* are bacteria. *Penicillium* and *Cephalosporium* are fungi. It is a constant challenge to develop new antibiotics to replace those antibiotics for which microbes have developed resistance.

The range of bacteria killed by an antibiotic determines its "spectrum of activity". Antibiotics that are only effective against Gram-positive or only effective against Gram-negative bacteria have a narrow spectrum of activity. Antibiotics that are effective against many different types of bacteria are called **broad spectrum antibiotics**. Broad spectrum antibiotics are probably contributing to the escalating drug resistance we are seeing in microorganisms. Broad spectrum antibiotics often wipe out a person's normal microbiome as well as the pathogen they are intended to kill, resulting in superinfections from organisms such as *Candida albicans* and *Clostridium difficile* that grow out of control when they do not have to compete with microbes in the normal microbiomes.

Empiric therapy takes place when an antimicrobial agent is given to the patient without performing a culture or other diagnostic test to determine the specific cause of the disease. Empiric therapy is prescribed in instances where the causative pathogen is likely and where diagnostic tests will not change the treatment. The selection of which drug to use is based solely on experience, observation and relevant clinical information including current resistance patterns in suspected pathogens. These antibiotics are typically broad-spectrum, in that they treat a wide variety of possible microorganisms. Examples of this include antibiotics prescribed for strep throat, pneumonia, urinary tract infections, and suspected bacterial meningitis in newborns aged 0 to 6 months.

Physicians are beginning to target infections with narrow spectrum antibiotics, or synergistically treat infections with small doses of multiple antibiotics to try to prevent antibiotic resistance.

The laboratory can aid the physician in selecting which antimicrobial agent is likely to kill the pathogen that is causing an infection in a patient. There are several methods that are used by clinical microbiologists in this determination, including the **Kirby-Bauer Test**.

Kirby-Bauer Test

The results of the Kirby-Bauer Test provide an accurate prediction of which antibiotics are likely to be effective against the pathogen. Because the Kirby-Bauer test is relatively simple to perform and is inexpensive, it has been extensively used in medical practice. The results are reported as **S (sensitive)**, **I (intermediate)**, and **R (resistant)** to an antibiotic. A sensitive result indicates the bacteria will die when it is exposed to the antibiotic. An intermediate result indicates the antibiotic must be used in combination with another antibiotic to clear the infection. A resistant result indicates the antibiotic does not kill the bacteria.

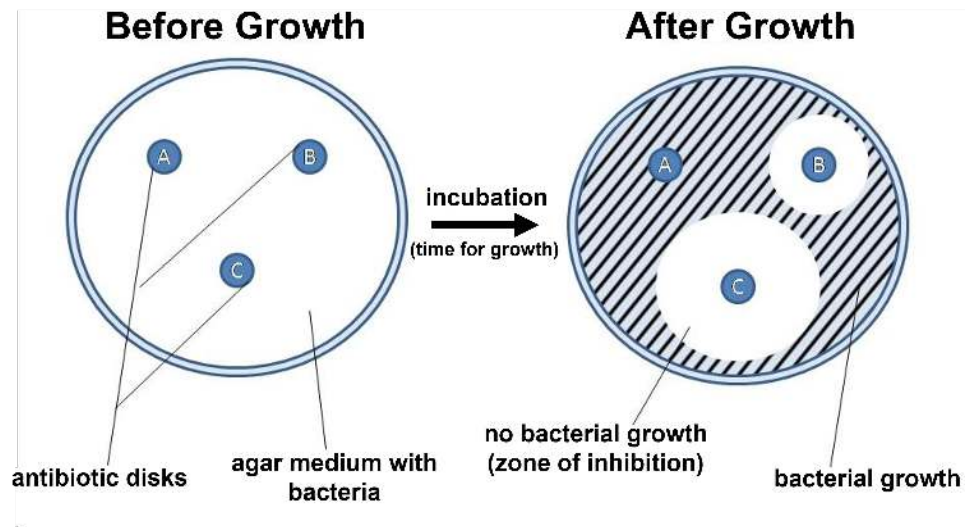


Figure 1: A summary diagram showing how the Kirby-Bauer approach works. A petri plate with nutrient agar is spread with the bacteria being tested. Antibiotic disks are placed on the agar. After incubation, zones on inhibition will occur surrounding the antibiotic disks based on the bacteria's sensitivity to the antibiotics tested. The zone of inhibition is a region where no bacterial growth occurred since it was inhibited/killed by the concentration of antibiotic present on the petri plate at that location (based on the diffusion of the antibiotic away from the antibiotic disk).

Petri plates with nutrient agar are covered with the bacteria that is being tested for its antibiotic sensitivity. After spreading the bacteria over the entire surface of the petri plates, small disks containing different antibiotics are placed at a distance from each other (or alone) on the petri plate. Each antibiotic will diffuse outward from the antibiotic disk creating a concentration gradient of the antibiotic in a circle surrounding the disk. Closest to the antibiotic disk will be the highest concentration of antibiotic. Further from the antibiotic disk will be the lowest concentration of antibiotic. Based on how sensitive the bacterial species/strain is to each antibiotic, bacterial will begin to grow at one of the antibiotic concentrations. Bacteria that can grow at higher concentrations of an antibiotic (closer to the disk) will have more resistance to that antibiotic. Bacteria that can only grow at lower concentrations of an antibiotic (further from the disk) will be more susceptible to that antibiotic. As a result, the size of the **zone of inhibition** (the region of the petri plate surrounding the antibiotic disk that does not have bacterial growth) will determine if the bacterial species/strain is sensitive, intermediate, or resistance to that antibiotic.

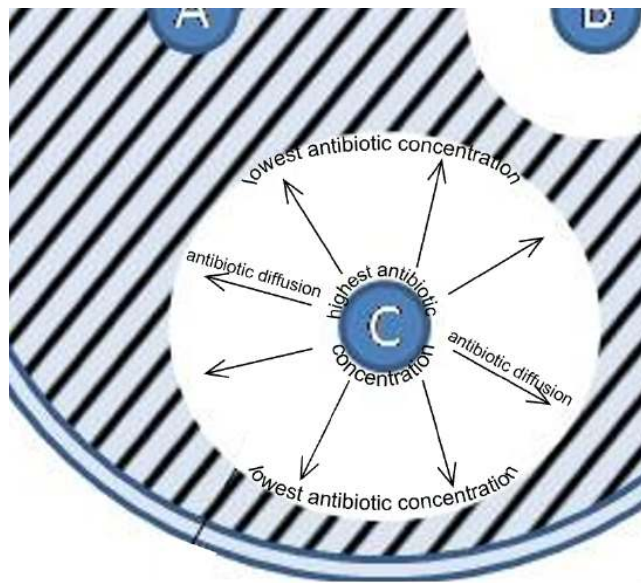


Figure 2: A closer look at antibiotic diffusion and how this produces zones of inhibition. Closest to the antibiotic disk is the highest antibiotic concentration and the antibiotic diffuses outward to produce a concentration gradient of antibiotic. The further from the antibiotic disk, the lower the antibiotic concentration. This approach enables a quick way of seeing a spectrum of antibiotic concentrations and how this impacts inhibition of the bacterial species/strain being tested.

The Kirby-Bauer test, known as agar disk diffusion, must be strictly regulated for the results to be interpreted correctly. Such characteristics as the stability of the antibiotic, the rate of diffusion of the antibiotic, the bacteria being tested, the pH of the culture medium, the depth of the culture medium, the inoculum density, the incubation time, the incubation temperature, and the concentration of the antibiotic can affect the results. Nevertheless, when the Kirby-Bauer test is performed under standardized conditions (on Mueller-Hinton agar inoculated with a pure culture of microbes that is the correct turbidity to match McFarland 0.5 standard, etc.) and the results are interpreted according to the Interpretative Zone Standards published by the National Committee for Clinical Laboratory Standards. In this laboratory, this is a simplified version of the Kirby-Bauer Test in lab that is not standardized, but will allow you to learn the general principles involved in this procedure.

Laboratory Instructions

Kirby-Bauer Test for Antibiotic Sensitivity (Modified for Simplicity)

1. Label 3 TSA plates with your group name and "Kirby-Bauer."
2. Dip a sterile cotton swab into a *Staphylococcus aureus* TSB culture and completely coat the surface of the TSA plate with *S. aureus*. Cotton swabs should go into an antimicrobial solution and be left there for at least 10 minutes before disposal.
3. Repeat step 2 for all the TSA plates.
4. Allow the plates to dry for 5 – 10 minutes.
5. Gently place one penicillin G disk (disk is labeled as P) in the center of one of the petri plates. DO NOT press the disk into the agar and do not move the disk once placed on the agar.
6. Repeat step 5. for an erythromycin disk (disk is labeled as E) on a different petri plate.
7. On the third petri plate, place a streptomycin disk (disk is labeled as S) centrally on one side of the petri plate and a tetracycline disk (labeled as T or TE) centrally on the other side of the same petri plate making sure they are spread out from each other.
8. DO NOT INVERT THE PLATES! Place the petri plates in the incubator.
9. After given time for growth (24-48 hours), measure the zones of inhibition in mm and record results in the results table.

10. Identify which of the size ranges that each zone of inhibition falls into in the interpretation table. This will determine if this bacterial strain is susceptible, intermediate, or resistant to each antibiotic.



Figure 3: Example of how to measure the diameter of a zone of inhibition in mm. The example shown here has a zone of inhibition with a diameter of 32 mm.

Results & Questions

antibiotic disk abbreviation	antibiotic name	antibiotic disk concentration	strain is resistant for this size of zone of inhibition (mm)	strain is intermediate for this size of zone of inhibition (mm)	strain is susceptible for this size of zone of inhibition (mm)
E	erythromycin	15 µg	13 or less	14 – 22	23 or more
P	penicillin G	10 units	28 or less		29 or more
S	streptomycin	10 µg	6 or less	7 – 9	10 or more
T (TE)	tetracycline	30 µg	14 or less	15 – 18	19 or more

antibiotic disk	diameter of the zone of inhibition (mm)	this <i>S. aureus</i> strain is... (resistant, intermediate or susceptible)... to this antibiotic
E (erythromycin)		
P (penicillin G)		
S (streptomycin)		
T (TE) (tetracycline)		

1. Complete the table above using measurements of the diameter of the zones of inhibition and the interpretation table above to determine if *S. aureus* is resistant, intermediate, or susceptible to each antibiotic.
2. If you had a patient with an infection of this strain of *S. aureus*, which antibiotic(s) might be a good choice for treatment? Explain your answer.
3. Define "zone of inhibition."
4. Explain how the size of the zone of inhibition relates to the concentration of the antibiotic.
5. Explain how the size of the zone of inhibition relates to the susceptibility or resistance to an antibiotic.
6. What is "antibiotic resistance?"
7. Why is using this Kirby-Bauer approach helpful to prevent bacterial strains from becoming resistant to antibiotics?
8. **True or False.** It is easy for scientists to develop new antibiotics.
9. What are the treatment options for a bacterial infection where the bacterial species is resistant to all types of antibiotics?
10. Why is it important to take steps to prevent bacterial strains from becoming antibiotic resistant?

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CHAPTER OVERVIEW

11: Lab 11

[11.1: Required Materials](#)

[11.2: Plaque Assay \(Day 1\)](#)

[11.3: Worksheet](#)

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11.1: Required Materials

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11.2: Plaque Assay (Day 1)

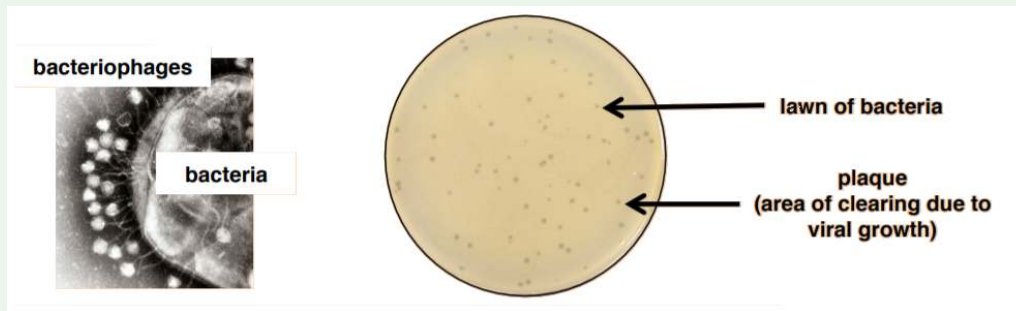
How do we count viruses?

Unlike bacteria, many of which can be grown on an artificial nutrient medium, viruses require a living host cell for replication. Infected host cells (eukaryotic or prokaryotic) can be cultured and grown, and then the growth medium can be harvested as a source of virus. In this lab, we will be quantifying bacteriophages (viruses that attack bacteria) using a plaque assay. This means that we must grow both bacteria and virus.

As the virus replicates and grows, it will lyse bacteria resulting in an area of clearing that can be observed on a bacterial lawn. These clearings are called plaques. A plaque assay, therefore, is a method by which the number of plaques observed can be used to estimate the amount of virus present in the original culture. The technique required to do so is called the pour-plate technique because you will mix the virus with the bacteria with melted agar and then pour the mixture onto a plate.

The unit of viral titer (concentration) is plaque-forming units per milliliter (pfu/ml).

As we do not know the starting concentration of virus, we will need to do a serial dilution (similar to the standard plate count). After incubation, the number of plaques formed will be used to calculate the original viral (phage) titer.



Plaque Assay:

Complete the Following Lab with Your Partner:

1. Obtain 14 microcentrifuge tubes and label either #1-7 or E. coli #1-7.
2. Obtain 7 nutrient agar plates. Label them A-G and place them in the 37°C incubator to warm up until needed.
3. Aseptically add 990 µL of sterile saline to tubes #1.
4. Aseptically add 900 µL of sterile saline to tubes #2-7.
5. Finger vortex the E. coli broth. Then, aseptically add 300 µL to all seven E. coli microcentrifuge tubes.
6. Vortex the T4 bacteriophage suspension and then aseptically transfer 10 µL to tube #1. Close the lid and vortex. This is your 10⁻² dilution.

Note

Enterobacteria phage T4 is a bacteriophage that infects *Escherichia coli* bacteria.

7. Aseptically transfer 100 µL from tube #1 to tube #2, close the lid and vortex. This is your 10⁻³ dilution.
8. Aseptically transfer 100 µL from tube #2 to tube #3, close the lid and vortex. This is your 10⁻⁴ dilution.
9. Aseptically transfer 100 µL from tube #3 to tube #4, close the lid and vortex. This is your 10⁻⁵ dilution.
10. Aseptically transfer 100 µL from tube #4 to tube #5, close the lid and vortex. This is your 10⁻⁶ dilution.
11. Aseptically transfer 100 µL from tube #5 to tube #6, close the lid and vortex. This is your 10⁻⁷ dilution.

12. Aseptically transfer 100 μ L from tube #6 to tube #7, close the lid and vortex. This is your 10⁻⁸ dilution.
13. Aseptically transfer 100 μ L of SALINE into tube E. coli #1, close the lid and vortex. This will become your negative control.
14. Aseptically transfer 100 μ L of dilution tube #2 to E. coli #2, close the lid and vortex.
15. Aseptically transfer 100 μ L of dilution tube #3 to E. coli #3, close the lid and vortex.
16. Aseptically transfer 100 μ L of dilution tube #4 to E. coli #4, close the lid and vortex.
17. Aseptically transfer 100 μ L of dilution tube #5 to E. coli #5, close the lid and vortex.
18. Aseptically transfer 100 μ L of dilution tube #6 to E. coli #6, close the lid and vortex.
19. Aseptically transfer 100 μ L of dilution tube #7 to E. coli #7, close the lid and vortex.
20. Let all seven tubes stand for 15 minutes. We are allowing time for the virus to infect the bacteria.
21. Remove one soft agar tube (containing 2.5 ml of agar) from the hot water bath and add the entire contents of E. coli #1 to it. Using a sterile plastic transfer pipet, mix the contents by pipetting up and down twice. (DO NOT USE YOUR MICROPIPETTER FOR THIS OR YOU WILL LOSE POINTS!!!)
22. Immediately pour the mixture onto plate A. Then, gently tilt the plate back and forth until the soft agar mixture is spread evenly across the solid medium.

 Note

Remember that you need to take the soft agar plates and nutrient agar plates out one at a time. Otherwise, the agar will solidify before you have a chance to pour.

23. Allow the agar to solidify completely with the lid on at your table (15-20 minutes). Then, invert and place in the 37°C incubator to grow overnight.

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11.3: Worksheet

Name: _____

Section: _____

Student ID#: _____

Work in groups on these problems. You should try to answer the questions without referring to your textbook. If you get stuck, try asking another group for help.

Plague Assay

Record your results below. A countable plate has between 30-300 plaques. If there are more than 300 plaques record TMTC (too many to count), if there are fewer than 30 plaques record TFTC (too few to count) (1).

	A	B	C	D	E	F	G
Plaques Counted							
Sample Volume	2.5 mL.	2.5 mL.	2.5 mL.	2.5 mL.	2.5 mL.	2.5 mL.	2.5 mL.
$\frac{PFU}{mL}$							
$\frac{PFU}{mL \text{ of starting tube}}$							

1. Why is it important in this assay to make sure that there are enough bacteria present to produce a lawn? (1)
2. What would happen if you had not waited the 15 minutes that the protocol requires? (1)
3. Why was the water bath set to 50°C? (1)
4. What is the difference between a CFU and a PFU? (1)

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CHAPTER OVERVIEW

12: Lab 12

[12.1: Required Materials](#)

[12.2: DNA Restriction and Electrophoresis](#)

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12.1: Required Materials

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12.2: DNA Restriction and Electrophoresis

Learning Objectives

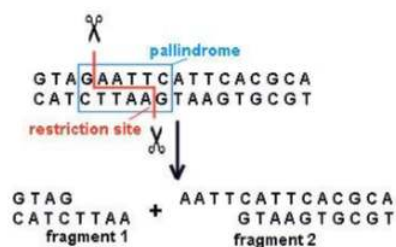
- Learn how to cut DNA into fragments with enzymes.
- Load and separate DNA fragments by electrophoresis
- Analyze electrophoresis gels

DNA RESTRICTION ANALYSIS

In this experiment, DNA from the bacteriophage Lambda (48,502 base pairs in length) is cut with a variety of restriction enzymes and the resulting fragments are separated using gel electrophoresis. Three samples of Lambda (phage) DNA are incubated at 37° C, each with one of the 3 restriction endonuclease enzymes: Pst1, EcoRI, and HindIII. A fourth sample will be the negative control in that it will be incubated without any endonuclease. Each of the 3 enzymes recognizes a different sequence of bases on DNA called a **palindrome**, and cuts within it at a specific site called a "restriction site."

The DNA samples are then loaded into wells of an agarose gel and electrophoresed, along with **loading dyes** (see procedure below). An electrical field applied across the gel causes the DNA fragments in the samples to move from their origin (a sample well) through the gel matrix toward the positive electrode. Small DNA fragments migrate faster than larger ones, so restriction fragments of differing sizes separate into distinct bands during electrophoresis. The loading dyes are of 2 different sizes, corresponding to very small DNA fragments and very large DNA fragments. They can be seen as the electrophoresis progresses, and they form a 'bracket' in between which the DNA fragments are moving.

Otherwise, one cannot tell how far the DNA fragments have moved through the agar. The characteristic number and pattern of bands produced by each restriction enzyme are made visible by staining with a compound that binds to the DNA molecule---**methylene blue**.



MATERIAL NEEDED

- restriction buffer
- loading dye
- ice
- 37° C water bath
- Lambda phage DNA
- TBE buffer mix
- restriction enzymes: Pst1, EcoR1, Hind111
- agarose to be poured (in 60° C water bath)
- reaction tubes
- 4 microtubes: clear, violet, green, orange
- staining tray

PROCEDURES

Use of the micropipetter

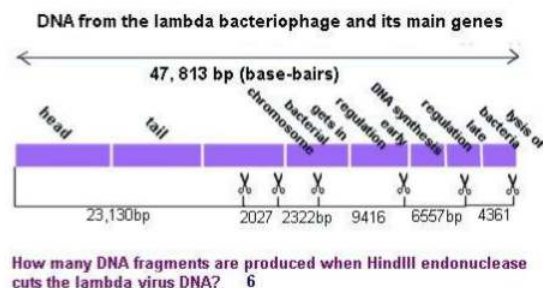
1. Be sure that the amount set on the pipetter is correct.
2. Place new tip firmly on micropipetter.
3. Depress plunger to **first stop**, and hold this position. This step eliminates any air in tip.
4. Dip tip into solution to be pipetted, and draw fluid into tip by releasing plunger. Always touch pipette tip to side of tube to dislodge any small amount stuck to tip. You now have a sample inside of your pipette tip.
5. To expel sample, touch pipette tip to inside wall of tube into which you want to empty sample. This creates a capillary effect which will help draw fluid out of tip.

- Slowly depress plunger to first stop and then depress to second stop to blow out last bit of fluid in tip. **DO NOT release plunger before removing tip from fluid in tube. Otherwise, it will suck fluid back into tip.**
- When taking a sample, **always** check for air at the tip. If it is present, put the sample back and begin again.

Digest DNA with restriction endonucleases (keep all enzymes on ice)

- Label four 1.5ml tubes, in which you will perform restriction digestion: "P" for **PstI** enzyme, "E" for **EcoRI** enzyme, "H" for **HindIII** enzyme, and "L" for **Lambda** DNA uncut.
- Using table below, add reagents to each tube in this order: DNA, restriction buffer, water, and enzymes last (ask for them). Use a fresh pipette tip for each different reagent. The amounts are given in microliter (millionths of a liter).
- Pool and mix reagents by tapping the tube bottom on counter a couple of times (or use a microcentrifuge).
- Incubate all reaction tubes for 30 minutes at 37° C.
- The tubes will go into the freezer until the next period when the electrophoresis will be done.

color	tube code	enzyme	DNA	buffer	PstI	EcoRI	HindIII
clear	L	uncut DNA	4	6	---	---	---
violet	P	PstI	4	5	1	---	---
green	E	EcoRI	4	5	---	1	---
orange	H	HindIII	4	5	---	---	1



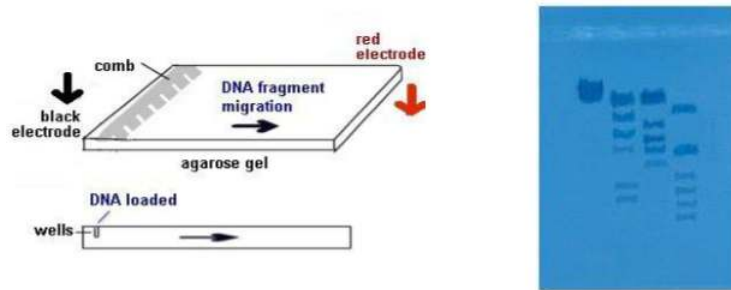
Casting agarose gel

- Set gel-casting tray into the tray apparatus, screw tight, and insert well-forming comb into space marked with red line. There is a leveling bubble which can be used to level the tray (by turning knobs at bottom).
- Place tray **FLAT** where agarose can be poured and allowed to set **UNDISTURBED**.
- Carefully pour 40 ml of agarose solution (liquified in 60° C water bath) into casting tray. Use a toothpick to move any bubbles to edges (this must be done **BEFORE** gel hardens).
- Gel will solidify within 20 minute. Do **NOT** move tray while agarose is solidifying.
- Gently remove comb, pulling it straight up and taking care not to rip wells.
- When solidified, remove the gel tray from the gel-casting tray and place on platform of electrophoresis box, so that comb is at negative (BLACK) cathode end. The - charged DNA fragments will migrate towards the + anode end.
- Fill box with TBE buffer, to level that just covers entire surface of gel by about 2 mm.
- Make certain that sample wells left by comb are completely submerged by buffer.
- The gel is now ready to load with DNA.

Loading gel with DNA

- Remove the 4 tubes from the fridge and pulse spin the tubes in a centrifuge or tap the tubes firmly down on the table top so that all contents go down to the bottom of the tube.
- Add 2 µl loading dye to each reaction tube and tap contents of tube on table top.
- Use pipette to load contents of each reaction tube into a separate well in gel (total of 4 wells). **Set your micropipetter on 12 µl** (that should be the total contents in the tube).
- You will need to remember the order of your tubes since there is **NO** way that the gel can be marked. Use a clean pipette tip for each different tube.
 - Steady pipette over well using 2 hands.
 - Expel any air first from pipette tip.

- Dip pipette tip through buffer, positioned over the well, and slowly expel the mixture (do not punch thru bottom of gel).
5. The loading dye contains sucrose which is heavier than the DNA. It weighs the mixture down so it will sink into the bottom of the well.



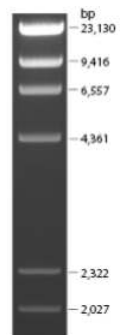
Electrophoresis

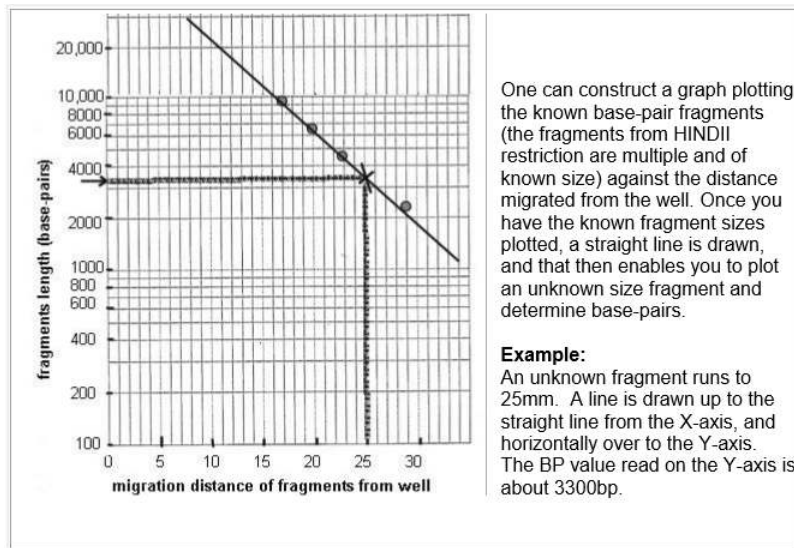
1. The electrophoresis chamber top is placed on the chamber, the electrodes connected to power supply--anode to anode (red-red) and cathode to cathode (black-black).
2. Power supply is turned on and voltage set---120 V. The higher the voltage, the faster the electrophoresis time. In a few minutes, you should begin to notice the loading dye moving through the gel toward the + pole (anode). We will let it run for about 1 hour.
3. The **loading dye** will resolve into 2 bands of color. The faster-moving, purplish band is the dye **bromophenol blue**, the slower-moving, aqua band is **xylene cyanol**. Bromophenol blue migrates through the gel at the same rate as a DNA fragment approximately 300 base pairs long. Xylene cyanol migrates at the a rate equivalent to approximately 2,000 base pairs long.
4. After 1 hour, the bromophenol band should be nearing the end of the gel. Turn off the power supply, disconnect the leads, and remove the top of chamber.
5. Carefully remove the casting tray and keep it horizontal until you are ready to put into the plastic container. Slide gel easily into staining tray labeled with your group name.
6. Add enough methylene blue DNA stain to cover the gel and place cover on it. They will sit in the dye overnight.
7. The gels will be washed a couple of times in distilled water (standing for 10-20 min), and will refrigerate until next lab period when we will look at them.
8. The gels can then be placed on gel support film, which binds the gel and dehydrates it, if your instructor so chooses.

INTERPRETATION

Examine your stained gel on a light box, overhead projector, or a UV box. Which restriction enzyme produced the most restriction sites on the lambda DNA?

The HindIII digest of lambda DNA yields at least 6 fragments suitable for use as molecular weight standards for gel electrophoresis. This is called a ladder. After the sample is ran, the unknown fragments can be compared with the ladder fragments to determine the approximate size of the unknown DNA bands by how they match up to the known bands of the ladder.





QUESTIONS

1. What is the source of the DNA being used in this exercise?
2. The speed of the DNA fragment migration correlates directly with the _____ of the fragment.
3. Why use 3 different restriction enzymes to cut DNA?
4. How can you account for differences in band separation and intensity between your gel and the ideal gel?
5. Small restriction fragments of nearly the same length will appear as a single band on this gel, even though it may be run to the very end. Why?

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