MICROBIOLOGY LABORATORY MANUAL



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West Hills College Lemoore

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Microbiology Laboratory Manual

Rosanna Hartline

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Glossary

Detailed Licensing

Licensing

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

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1.1: 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.

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1.2: Media Preparation

Learning Objectives

- Define medium/media.
- Tell that microbes require nutrients to grow in the laboratory.
- Differentiate between broth, slant, deep, and petri plate.
- Calculate the amount of medium powder required to make a specific volume of medium.
- Successfully prepare microbiological media.

Microbiological Media

Just like all other living things, microbes require nutrients (food) to grow and live. Microbiological **media** (singular is "**medium**") is a mixture of water and nutrients necessary to grow microbes. Different types of media also can be used to provide information about the different characteristics microbes have.

Many types of microbiological media can be ordered in powder form from a science supplier. Instructions for preparing the medium are presented on the medium container. Typically, this involves dissolving a certain number of grams of the media powder in one liter of distilled or deionized water ("grams per liter" which is written as g/L). This does not mean that one must always make one liter of medium. The amount can be modified by calculating the amount of microbiological media powder required for the concentration (g/L) listed on the bottle)

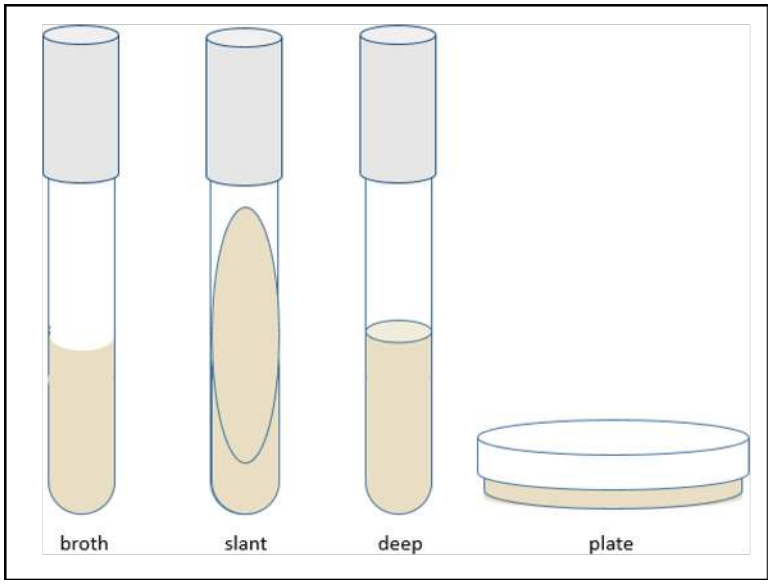


Figure 1: Media can be prepared as a broth (liquid), a slant (agar in a test tube that has been slanted when cooling to create a larger agar surface area), a deep (agar in a test tube typically inoculated using an inoculation needle by stabbing into the agar), and a petri plate (a larger surface area for growing microbes on the surface).

Calculating Amount of Medium

Use the following formula to determine the amount of powdered medium to weigh and dissolve into DI or distilled water:

amount of solution you want to make (mL) x [concentration (g/1000 mL)] = amount of media powder to weigh out (g)

Because 1 L ("one liter") is the same thing as 1000 mL ("one thousand milliliters"), when the instructions on the media powder bottle tell to make a concentration in g/L ("grams per liter"), this is the same thing as g/1000 mL ("grams per thousand milliliters").

Calculation Example

Calculation

- In this example, you want to make 500 mL of medium. Instructions on the bottle say to make a concentration of 40 g/L.
- 40 g/L = 40 g/1000 mL
- Set up the formula:
  - 500 mL x [40 g / 1000 mL]
- Calculate the amount to weigh out:
  - **Do the parentheses first:** 500 mL x [40 g / 1000 mL]
  - **Multiply [note that mL units cancel out]:** 500 mL x 0.04 g/mL
  - **Answer is the amount to weigh out in g ("grams"):** 20 g

How to Make the Medium

1. Measure 500 mL of distilled or deionized water (not tap water) with a graduated cylinder.
2. Weigh 20 g of media powder using a balance.
3. Put a magnetic stir bar into a beaker, flask or bottle (must be larger capacity than 500 mL in for this example - 800 mL or 1000 mL) and add about half of the water.
4. Put the beaker, flask, or bottle onto a stir plate and turn it on so the magnetic stir bar is swirling the water enough to stir it well, but not too much that it is jumping and/or creating lots of bubbles in the water.
5. Gradually add the 20 g of media powder to the stirring water.
6. Add the reminder of the water. By adding the remaining water after the media powder, it will help dissolve any powder floating at the top of the water.
7. Allow to stir until the powder is completely dissolved.
8. If the medium is agar, the solution will need to be heated just to boiling (but not boiling over) starting after step 3. Keep stirring and heating until the solution is clear. Be careful not to over-heat since it is easy for the solution to boil over.
9. If the medium will be distributed into test tubes, measure amount into each test tube and add caps.
10. If the medium is for flasks or bottles, measure the amount into each flask or bottle and add caps or other covers (make sure screw-caps are not screwed on tightly before they are autoclaved).
11. If the medium is for petri plates, make sure the medium is in a container with a cover (make sure screw-caps are not screwed on tightly before they are autoclaved).
12. Autoclave.
13. If test tubes contain agar that will be for slants, prop the rack of test tubes so the agar slants as it cools.
14. If the medium is for petri plates, disinfect a workspace and pour agar into sterile petri plates and allow to cool completely.

Practice Calculations

? Exercise 2.1

In order to make 400 mL of medium with a concentration of 15 g/L, how much medium powder would you weigh in grams?

Answer

? Exercise 2.2

In order to make 650 mL of medium with a concentration of 20 g/L, how much medium powder would you weigh in grams?

Answer

? Exercise 2.3

In order to make 200 mL of medium with a concentration of 30 g/L, how much medium powder would you weigh in grams?

Answer

? Exercise 2.4

In order to make 300 mL of medium with a concentration of 45 g/L, how much medium powder would you weigh in grams?

Answer

? Exercise 2.5

In order to make 150 mL of medium with a concentration of 35 g/L, how much medium powder would you weigh in grams?

Answer

How to Make Petri Plates



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1.3: The Myth of Spontaneous Generation

Learning Objectives

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

The Myth of Spontaneous Generation

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

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

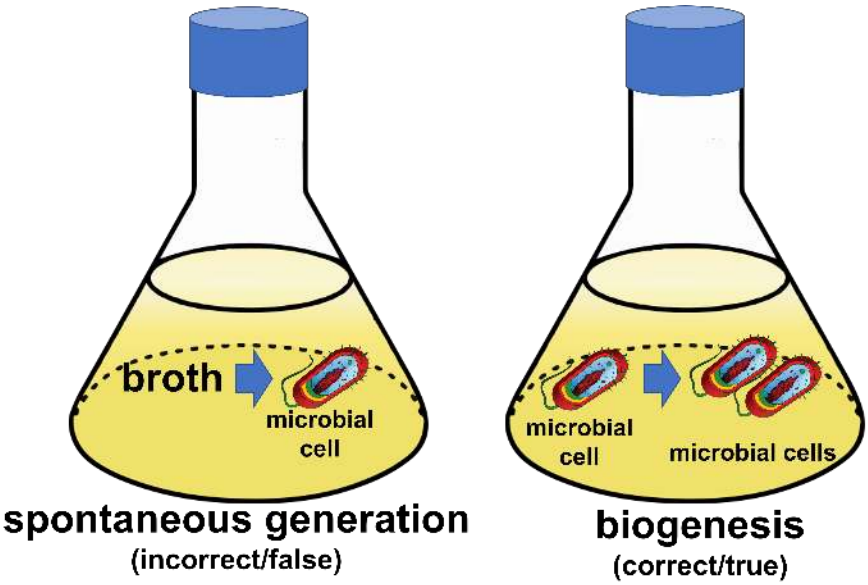


Figure 1: (Left) Spontaneous generation is an incorrect/false theory where scientists of the past believed that a liquid broth could create microbial cells from the broth itself (life coming from non-living matter). (Right) In reality, cells come from other cells (cell theory) or life comes from life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

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

Historical Experiments Disproving Spontaneous Generation of Microorganisms

Spallanzani's Experiment Disproving Spontaneous Generation Theory of Microorganisms

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

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

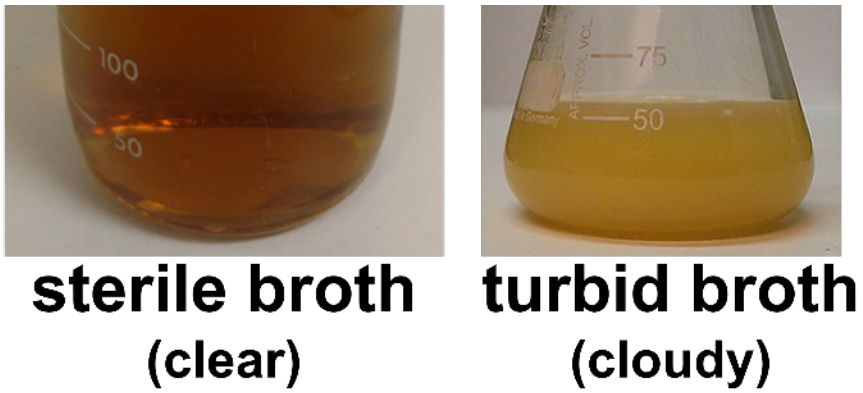


Figure 2: Comparison of sterile microbiology medium (left) that is a clear liquid and a turbid medium (right) that is a cloudy liquid. The sterile medium lacks live microorganisms. The turbid broth contains a dense population of microorganisms.

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

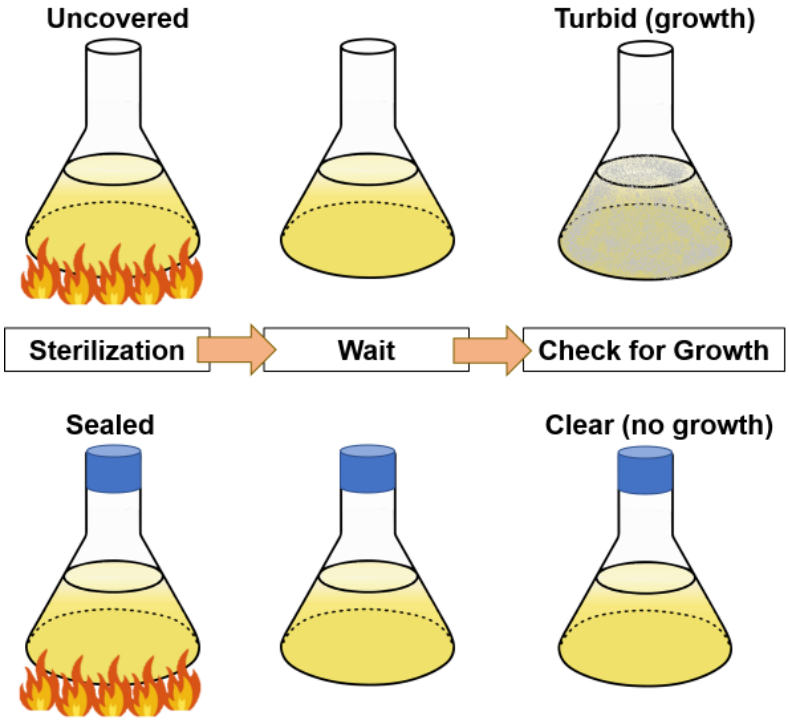


Figure 3: Diagram showing Spallanzani's experimental setup and results. (Top) An uncovered flask containing clear broth is sterilized by boiling. After a period of time (hours/days) the flask is re-examined and found to be turbid. This turbidity is microbial growth in the uncovered flask. Microbes from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

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

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

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

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

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

Tyndall's Discovery and Endospores

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

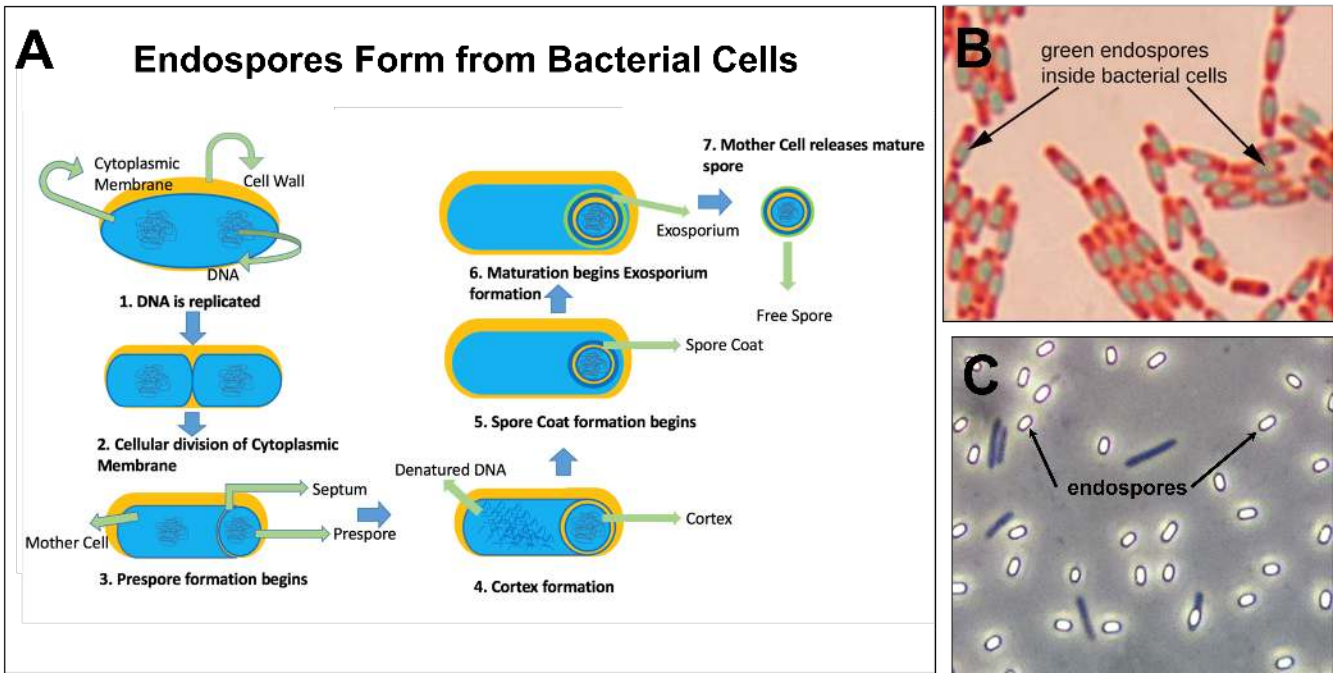


Figure 5: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore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

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



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

More recently, it has been found endospores can also be killed when medium is heated under pressure. The typical method of sterilization used in laboratories today uses an **autoclave**, a device that places materials to be sterilized under pressure while

heating. What used to take a few days to sterilize medium with Tyndall's process now can be sterilized in about an hour.

Re-creation of Lazzaro Spallanzani's Spontaneous Generation Experiment

In this laboratory activity you will re-create Spallanzani's experiment. debunk the myth of spontaneous generation... again!

Laboratory Instructions

1. Write your group name, experiment name, and the date on two pieces of tape and place on the outside of two test tubes (put tape about half way down on the test tube so you can add a cap onto one of the test tubes).
2. Use a graduated cylinder to measure TSB.
3. Put 8 mL of TSB into each of the two test tubes.
4. Put a cap onto one of the test tubes and leave the other test tube uncapped.
5. Put test tubes in a central location indicated by your instructor. Your test tubes will be autoclaved by your instructor to sterilize them (kill all microbes) and then placed onto a bench.
6. Create a hypothesis with your group:
    1. **Do you expect that the covered, sterilized test tube will show microbial growth? Why or why not?**
    2. **Do you expect that the uncovered, sterilized test tube will show microbial growth? Why or why not?**
    3. **If you said that microbial growth will occur in one of the test tubes, where did the microbes come from?**
7. You will examine your results next class. If the solution is **turbid** (foggy), that indicates microbial growth. If the solution is **clear**, that indicates no microbial growth.
8. **Results:**

1. 1. Covered, sterilized tube:
    2. Uncovered, sterilized tube:

9. Conclusions. What do the results you listed above mean about spontaneous generation? Consider your hypotheses above to help you answer.

Re-creation of Louis Pasteur's Spontaneous Generation Experiment

In this laboratory activity you will re-create Pasteur's experiment. Debunk the myth of spontaneous generation... again!

Laboratory Instructions

1. Write your group name, experiment name, and the date on two pieces of tape and place on the outside of two 125 mL flasks.
2. Use a graduated cylinder to measure TSB.
3. Add 50 mL of TSB to each of the 125 mL flasks.
4. You may or may not be asked to bend glass tubing into a swan-neck shape using a Bunsen burner.
5. Put one swan-neck glass tube into one stopper and a straight glass tube into another stopper.
6. Place the two stoppers securely into the 125 mL flasks.
7. Put the flasks in a central location indicated by your instructor. Your flasks will be autoclaved by your instructor to sterilize them (kill all microbes) and then placed onto a bench.
8. Create a hypothesis with your group:
    1. **Do you expect that the sterilized flask with the swan-neck glass tube will show microbial growth? Why or why not?**
    2. **Do you expect that the sterilized flask with the straight glass tube will show microbial growth? Why or why not?**
    3. **If you said that microbial growth will occur in one of the test tubes, where did the microbes come from?**
    4. **Why were scientists more convinced that spontaneous generation was incorrect by Pasteur's experiment and less convinced by Spallanzani's experiment?**
9. You will examine your results next class. If the solution is **turbid** (foggy), that indicates microbial growth. If the solution is **clear**, that indicates no microbial growth.
10. **Results:**

1. 1. Sterilized flask with swan-neck tube:
   2. Sterilized flask with straight tube:

11. Conclusions. What do the results you listed above mean about spontaneous generation? Consider your hypotheses above to help you answer.

Attributions

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1.4: 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 µm) in diameter; the head of a pin of 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 µm ("twenty micrometers"). To convert 20 µm to meters, multiply 20 µm by 0.000001:

$$20\ \mu m \times 0.000001 = 0.00002\ m$$

or

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

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:

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

or

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

? 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:

$$0.0003\ m \div 0.000001 = 300\ \mu m$$

or

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

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:

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

or

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

? Exercise 4.4

Convert 0.004 m to µm.

Answer

? Exercise 4.5

Convert 0.3 m to mm.

Answer

? Exercise 4.6

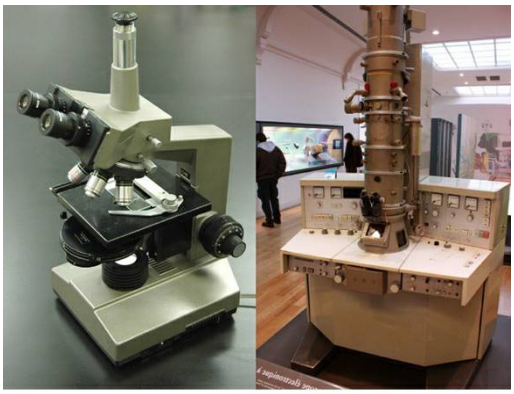
Convert 0.00000085 m to nm.

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.



(a) (b)

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 a 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.

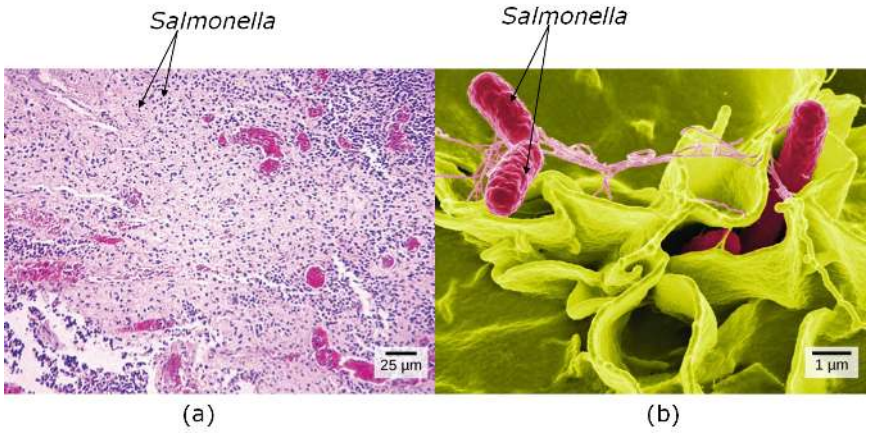


Figure 2: (a) These *Salmonella* bacteria appear as tiny purple dots when viewed with a light microscope (*Salmonella* are stained to appear purple). (b) This scanning electron microscope micrograph shows *Salmonella* bacteria (in red) invading human cells (yellow). Comparing *Salmonella* bacteria when 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 ~0.2 µm, 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 ≥ 0.2 µm, any objects closer than 0.2um 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.

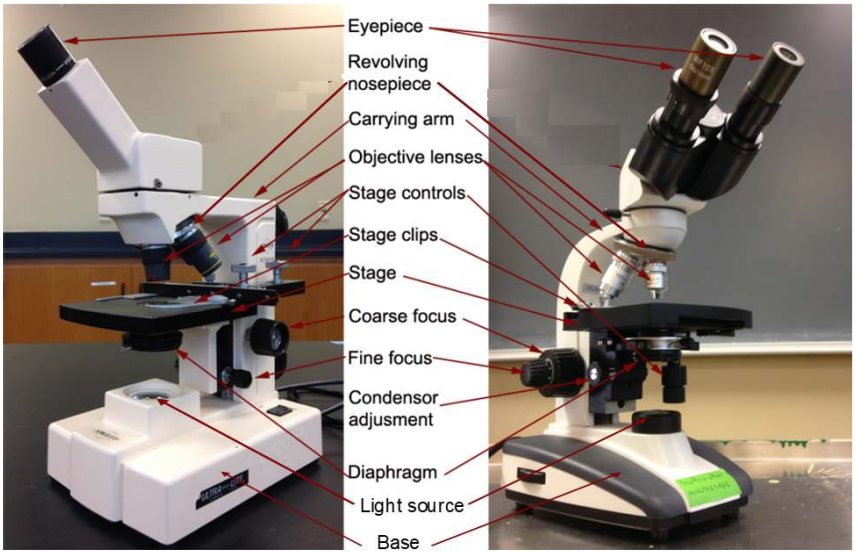


Figure 3: Parts of compound light microscopes. Some models are slightly different. Here, two different models are shown and labeled to indicate the parts of each.

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 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.

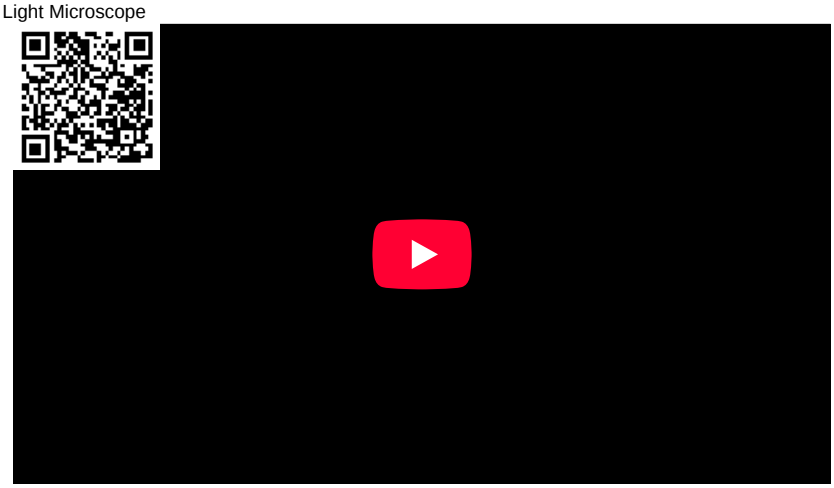
Focusing a Compound Light Microscope

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 course focus knob to bring the stage as close to the objective lens as possible.
7. Look through the microscope and use the course 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:

microscope resolution limit = (wavelength)/(numerical aperture x 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:

total magnification = (objective lens magnification) x (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).

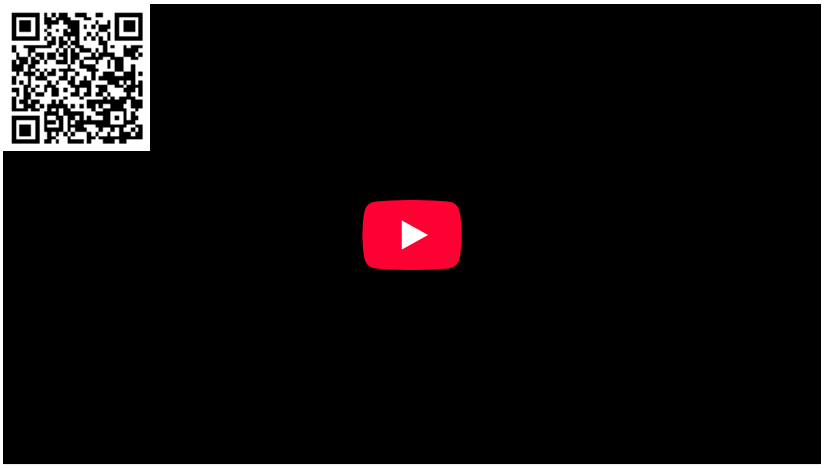
The use of objective and ocular lenses with different magnifications allows greater flexibility when using the compound microscope. Due to the size of most bacteria (ranges widely from ~1 µm to over 100 µm), generally we require the use of the 100X *oil immersion lens* with a 10X ocular lense to view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

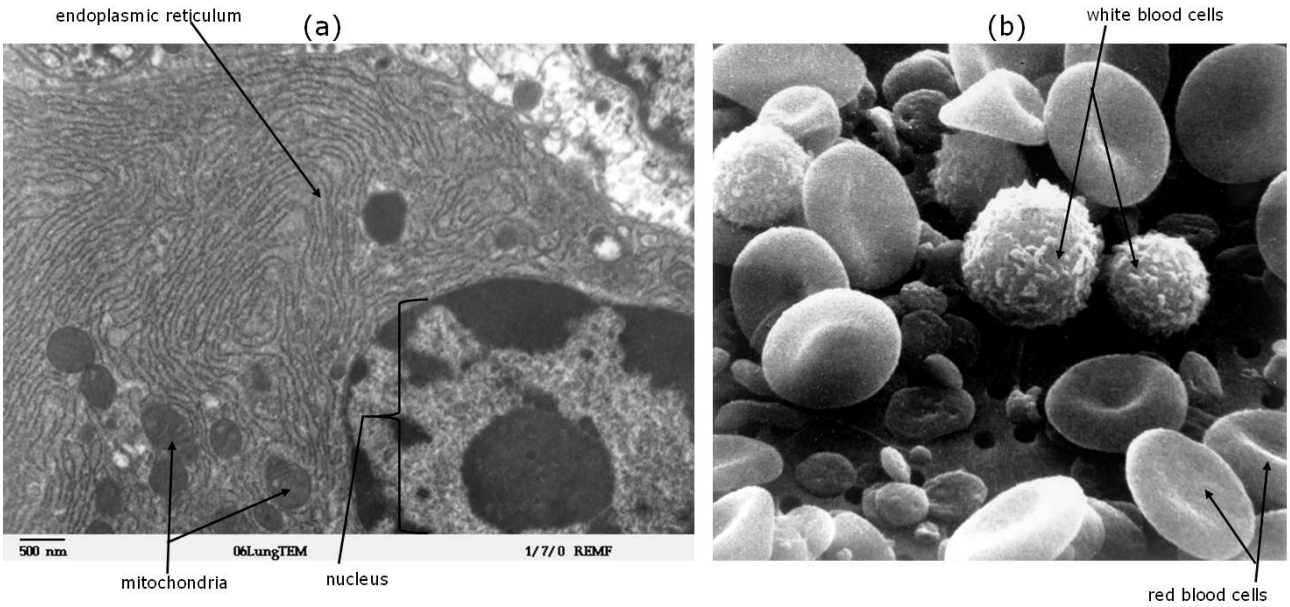


Figure 4: (a) A lung cell that has been thin-sliced and **imaged with TEM**. Thin-slicing a cell with TEM exposes the internal structures such as the nucleus and mitochondria. (b) Exterior of blood cells **imaged with SEM**. SEM images only allow surface visualizations of cells and tissues.

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1.5: Get to Know the Microscope and Microbes

Learning Objectives

- 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.
- Successfully examine different classifications of microbes using a light microscope.
- Illustrate different classifications of microbes.
- Compare the appearances of different classifications of microbes that were examined and illustrated using a light microscope.

Introduction

Learning to use a microscope is critical to your success in microbiology. This tool will provide the magnification and clarity necessary for you to observe and record quality images of microbes that cannot ordinarily be viewed with the naked eye.

Total Magnification

To better understand the compound light microscope (brightfield), it is important to have an understanding of magnification. Commonly, compound microscopes have 4 different **objective lenses**:

- 4X
- 10X
- 40X
- 100X

The object lens magnification is increased by the **ocular lenses** (located in the eyepieces), which has a 10X magnification. To calculate the **total magnification** at each objective, multiple the ocular lens magnification with the objective lens magnification.

Exercise 5.1

When the 4X objective lens is used, what is the **total magnification** of the light microscope?

Answer

Exercise 5.2

When the 10X objective lens is used, what is the **total magnification** of the light microscope?

Answer

Exercise 5.3

When the 40X objective lens is used, what is the **total magnification** of the light microscope?

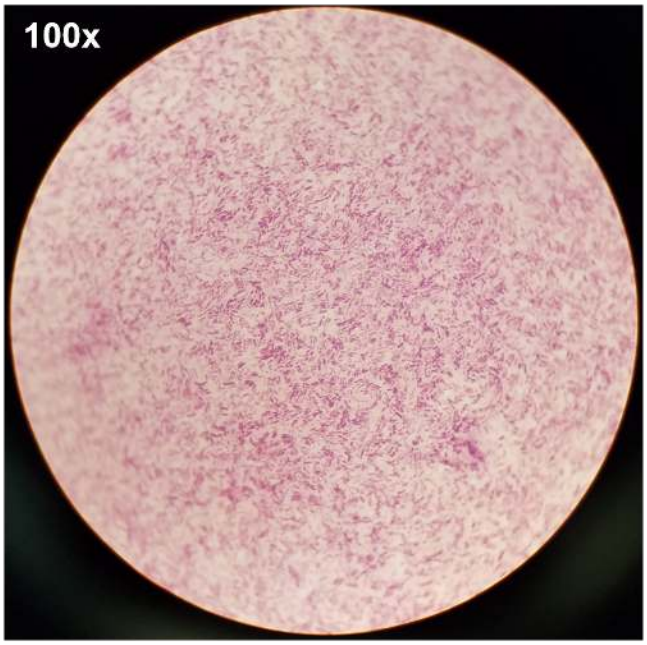
Answer

? Exercise 5.4

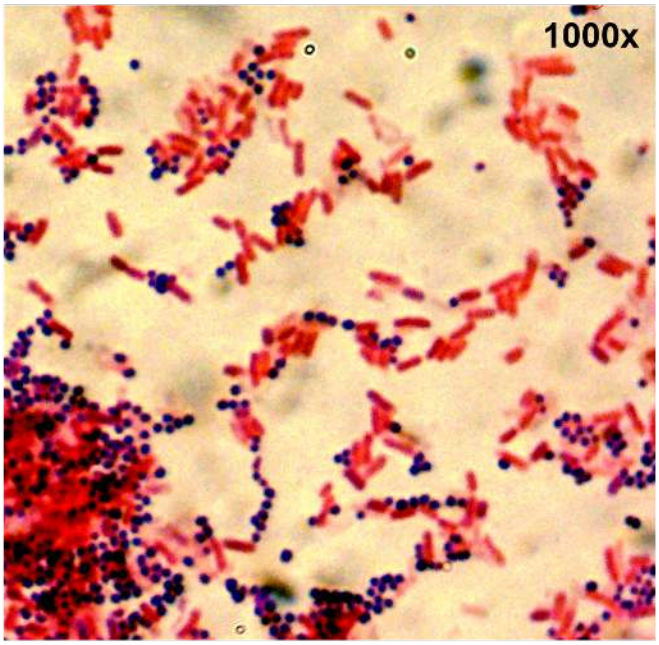
When the 100X objective lens is used, what is the **total magnification** of the light microscope?

Answer

Whenever you make an illustration or take a photo of a magnified specimen, the total magnification used should be given with the image. You can expect when you examine microscopic images that the magnification of the image is given as the total magnification (not the objective lens magnification).



E. coli magnified by 100x using a compound brightfield microscope



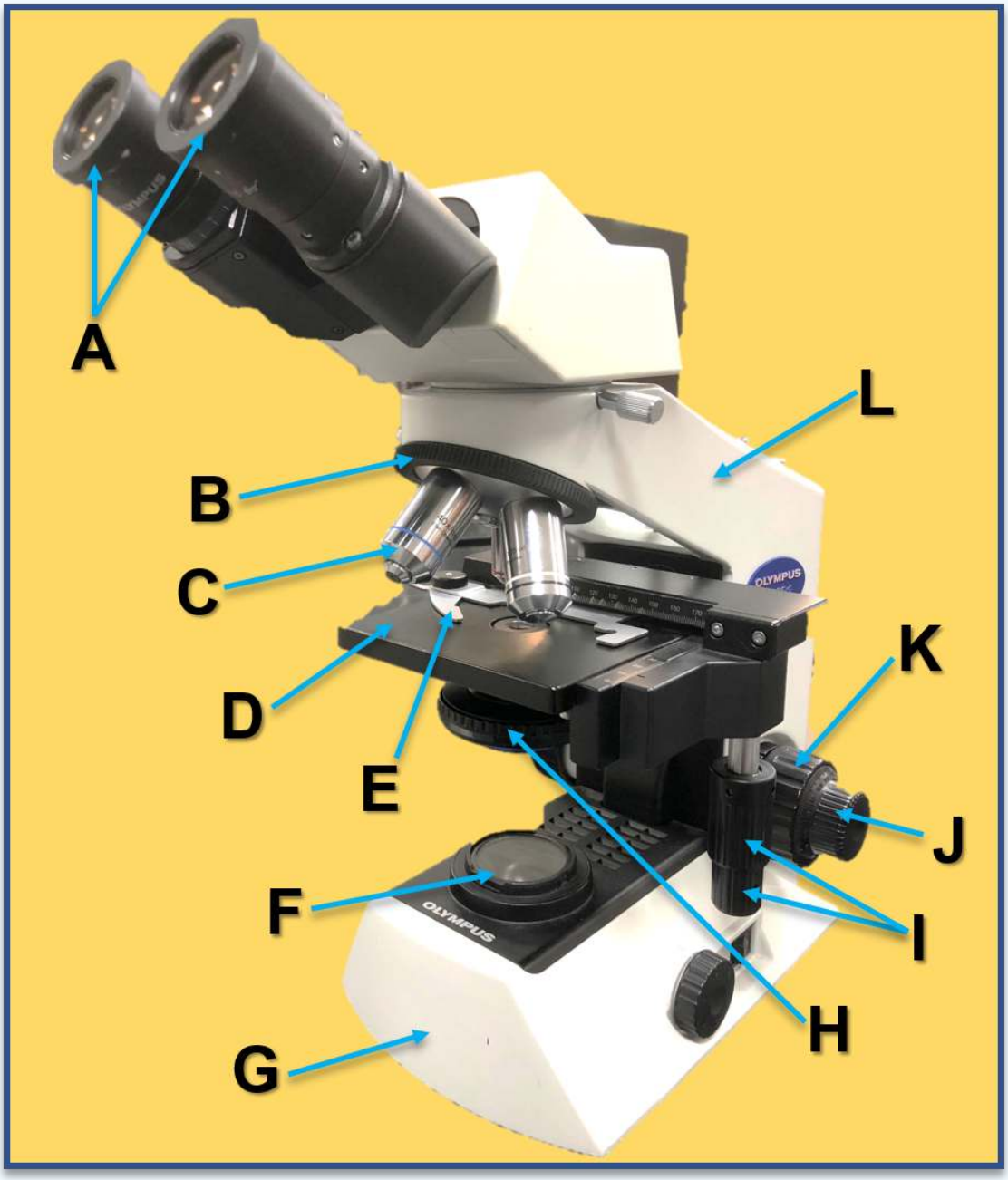
E. coli and *S. aureus* magnified by 1000x using a compound brightfield microscope

Figure 1: *Escherichia coli* is a bacterial species magnified by 100X using a compound microscope appears as tiny specks (left). The magnification reported is 100X, meaning that the 10X objective was used and in combination with the ocular lens (10X), the total magnification was 100X. (Right) *E. coli* (a bacterial species; appears pink and rod-shaped) and *Staphylococcus aureus* (a different bacterial species; appears purple and round) can be seen close enough to distinguish cell shapes at 1000X magnification. This magnification uses the 100X objective lens in combination with the ocular lens (10X) to produce a total magnification of 1000X.

Identify Parts of the Light Microscope

? Exercise 5.5

Name the components of a compound microscope labeled A-L.



Answers:

A
B
C
D
E
F
G
H
I
J
K
L

Match the Functions of the Parts of the Light Microscope

Exercise 5.6

Match the parts of the light microscope with their functions.

ocular lens	A. Adjusts the position of a slide on the stage.
revolving nosepiece	B. Adjusts the focus of the microscope by moving the stage up and down in large increments.
arm	C. A surface where a slide is positioned under the objective lens and over the light source.
stage control	D. A structure capable of rotating to change the objective lens being used to magnify the sample.
base	E. A magnifying lens located inside the microscope part where a person looks into the microscope.
coarse focus	F. Provides light that shines on the sample and carries the image of the specimen through the magnifying lenses.
fine focus	G. A structure capable of changing the amount of light passing through the specimen.
light source / illuminator	H. A structural component that serves to support the weight of the microscope from underneath.
objective lens	I. Secures a slide in place on the microscope.
stage	J. Where the microscope user looks into the microscope to view a magnified image of the specimen.
stage clip	K. Adjusts the focus of the microscope by moving the stage up and down in smaller increments.
diaphragm	L. A structural component that serves to support the eyepiece, revolving nosepiece, and stage.
eyepiece	M. A magnifying lens which increases magnification of the specimen that can easily be changed by rotating between lenses of different magnifications.

Answers:
ocular lens
revolving nosepiece
arm
stage control
base
coarse focus
fine focus
light source / illuminator
objective lens
stage
stage clip
diaphragm

Examine Specimen with Low Magnification

Place The letter ‘e’ slide on the microscope stage and focus the slide using the 4x objective.

1. Draw the ‘e’ as you see it on the slide with the naked eye (not looking through the microscope).
2. Look through the microscope and now draw the ‘e’ as you see.
3. In addition to being able to see the 'e' larger and with more detail, what else about the image of the 'e' has changed when comparing your illustration from 1. and from 2.?

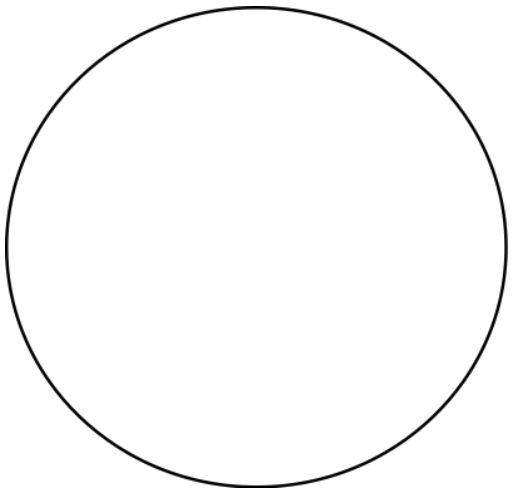
Examine Microbes at Higher Magnifications

"Microbes" include viruses, bacteria, archaea, fungi, protozoa, and helminths. Viruses are too small to see with the light microscope and must be imaged with an electron microscope (not used in class). Bacteria and archaea appear as tiny specks at the highest magnifications with a light microscope, protozoa and fungi and their details can be clearly imaged with a light microscope, and helminths (worms) can, depending on the species, be too long to see the entire worm at once with the light microscope.

Today, you will examine an example of a helminth, a fungus, protozoa, and bacteria. Choose the magnification the enables you to view the microbe well and clearly. Remember the relative sizes of these microbes. Smaller microbes will require higher magnifications.

Helminth

Examine an example of a species of helminth. Illustrate the magnified sample in detail, write down the name of the specimen, and indicate the total magnification you made the illustration at. The goal is to distinguish the different categories of microbes based on your illustrations.

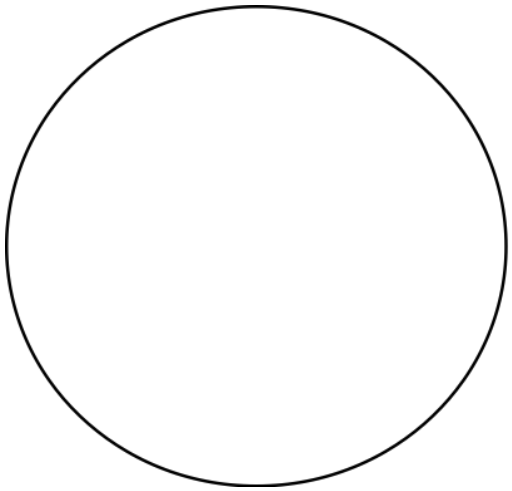


specimen: _____

total magnification: _____

Fungus

Examine an example of a species of fungus. Illustrate the magnified sample in detail, write down the name of the specimen, and indicate the total magnification you made the illustration at. The goal is to distinguish the different categories of microbes based on your illustrations.

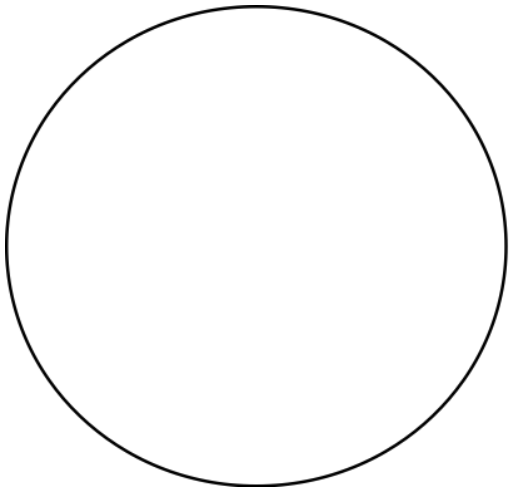


specimen: _____

total magnification: _____

Protozoa

Examine an example of a species of protozoa. Illustrate the magnified sample in detail, write down the name of the specimen, and indicate the total magnification you made the illustration at. The goal is to distinguish the different categories of microbes based on your illustrations.

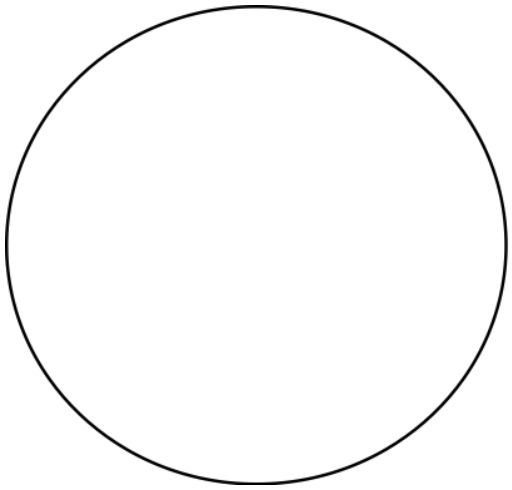


specimen: _____

total magnification: _____

Bacteria

Examine an example of a species of bacteria. Illustrate the magnified sample in detail, write down the name of the specimen, and indicate the total magnification you made the illustration at. The goal is to distinguish the different categories of microbes based on your illustrations.



specimen: _____

total magnification: _____

Compare and Contrast Different Types of Microbes

1. What were some notable similarities among the different microbe classifications examined with the light microscope?
2. What were some notable differences among the different microbe classifications examined with the light microscope?
3. Do all microbes have the same structure? Explain your answer.

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1.6: Molecules of Life

Learning Objectives

- Identify that all matter is composed of atoms and that atoms build molecules.
- Define organic molecules and inorganic molecules and be able to identify whether a molecule is organic or inorganic based on its chemical structure.
- List the four types of biological molecules, their monomers, and their functions.
- Define dehydration synthesis and hydrolysis and describe the importance of these reactions for biological molecules.
- Describe a burn test and interpret results of a burn test.
- Successfully build molecular molecules biological molecule monomers and conduct dehydration synthesis and hydrolysis reactions with these models.
- Test food items for a significant presence of proteins, carbohydrates, and lipids.

Atoms & Molecules

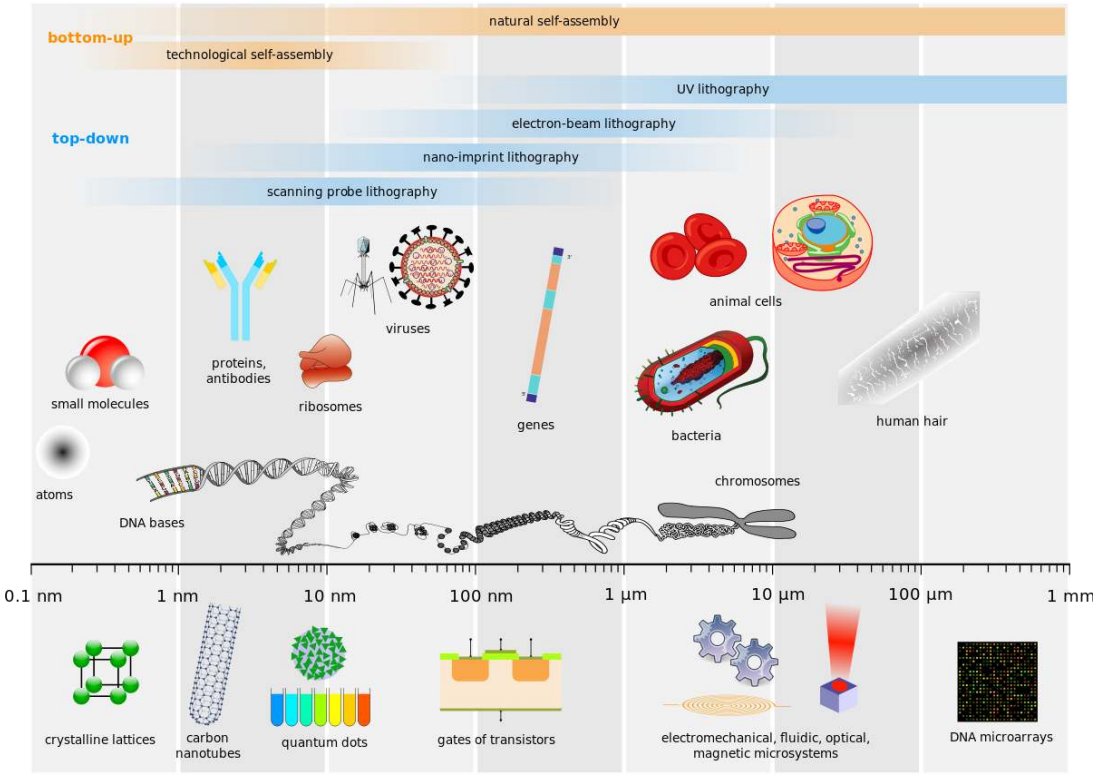


Figure 1: Anything in the universe that has physical substance is composed of atoms. Molecules are built from atoms. The molecules of life (biological molecules) are usually larger molecules (e.g. DNA, proteins). Biological molecules are the fundamental structures that make up living things (e.g. bacteria, animal cells) and non-living biological particles (e.g. viruses).

All **matter** (anything with physical substance) is made **atoms**; this includes all living things from microorganisms to humans to blue whales. Atoms, composed of protons, neutrons, and electrons, are the smallest structures that makes up **elements** of the periodic table. Therefore, the periodic table lists different types of atoms (also known as elements). Each type of element is different because the number of protons it has (the number of protons is the same as the **atomic number**).

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

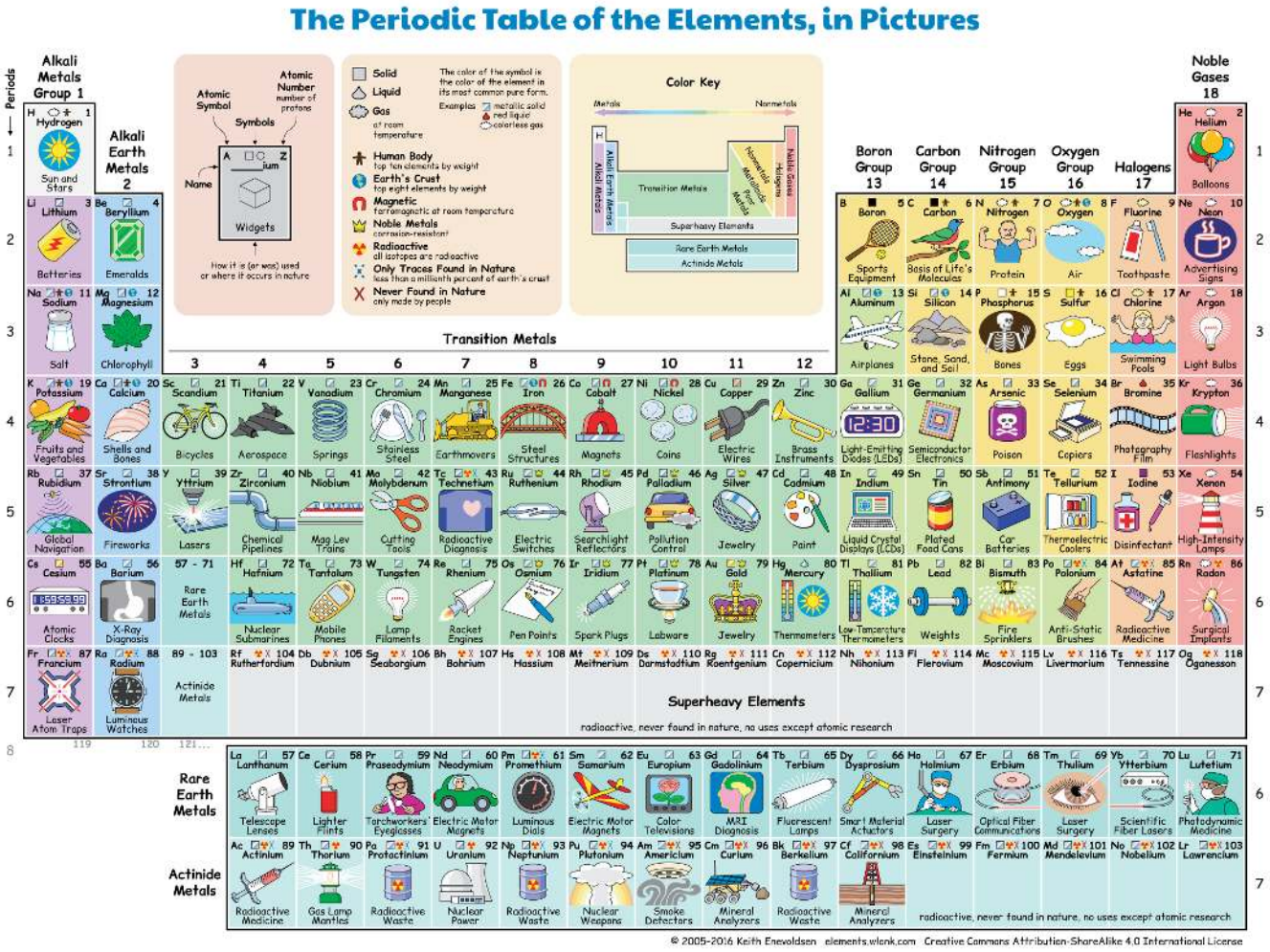


Figure 2: The periodic table of elements. Each element is a different type of atom that is defined based on the number of protons within that atom (the atomic number). For example, the atomic number of carbon (C) is 6. Carbon has six protons. Carbon will always have six protons. If you have an element that does not have six protons, it is not carbon.

Organic vs. Inorganic Molecules

All molecules can be classified as either organic or inorganic. **Organic molecules** are types of molecules typically found in living things and molecules that came from living things. In contrast, **inorganic molecules** are considered to be molecules not produced by living things. While organic molecules are considered the molecules of life/biological molecules, inorganic molecules can still be necessary for life. For example, water (H_2O) is a molecule that is required by all living things, yet it is considered inorganic. A straight-forward way to tell determine if a molecule is organic or inorganic is based on the types of elements it has:

- organic molecules always contain carbon atoms (C) ***and*** hydrogen atoms (H).
- inorganic molecules *can* contain carbon atoms (C) and *can* contain hydrogen atoms (H), ***but inorganic molecules will not contain both*** carbon atoms (C) and hydrogen atoms (H).

Note

Inorganic molecules usually do not contain carbon atoms (C). Also, organic molecules are formed only by covalent bonds, whereas inorganic molecules can be formed by covalent bonds or ionic bonds.

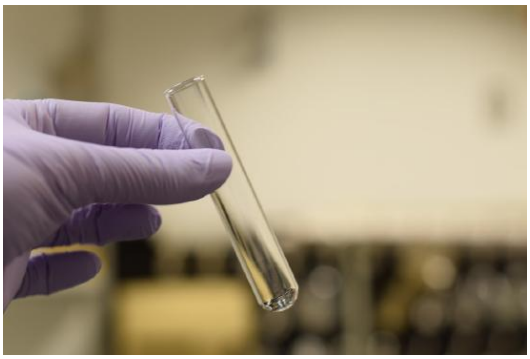
Exercise 6.1

Below are empirical formulas for a variety of different molecules. Based on the elements found in each molecule, identify whether each is an organic molecule or an inorganic molecule:

1. CH_4
2. $C_6H_{12}O_6$
3. $NaCl$
4. $CaCl_2$
5. H_2SO_4
6. $C_{60}H_{92}O_6$
7. $C_2H_5NO_2$
8. CO_2

Click each above for the answer.

What if we have a sample of a substance (and perhaps do not know the elements it contains) and would like to determine if the substance is organic or inorganic?



A simple laboratory test that can determine if a sample is organic or inorganic is called the **burn test**. This test is very simple: place the substance over heat - if it burns/turns black, it is likely organic. If it does not burn/does not turn black, it is likely inorganic.

Molecules that Make up Living Things

Biological molecules are classified into four types:

- **proteins**
- **carbohydrates**
- **lipids**
- **nucleic acids**

Each category of biological molecule has distinct features, including their building blocks and the types of functions they can serve. Biological molecules tend to be very large and are built of repeating units of smaller molecules. These small subunits that make up larger biological molecules are called **monomers**. The monomers of proteins are called **amino acids**, the monomers of carbohydrates are called **monosaccharides**, the monomers of lipids are called **fatty acids**, and the monomers of nucleic acids are called **nucleotides**.

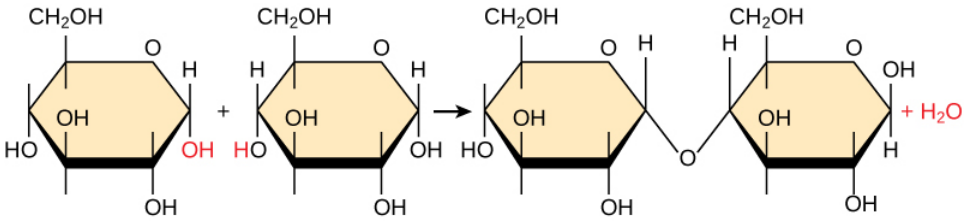


Figure 3: In the dehydration synthesis reaction depicted above, two molecules of glucose are linked together to form the disaccharide maltose. In the process, a water molecule is formed. The arrow represents the chemical reaction. At the butt end of the arrow are the reactants. Notice that there are two separate molecules that are not touching each other on the reactant side. These are two small monomers. On the pointy end of the arrow are the products. Notice that there is one larger molecule on the product side. The reaction changed two monomers into a dimer (two monomers bonded together).

Monomers are joined together by covalent bonds through **dehydration synthesis reactions** to form larger biological molecules made of multiple monomers. The number of monomers bonded together is apparent in the generalized naming scheme for biological molecules:

number of monomers bonded together	commonly used prefix	protein name	carbohydrate name	lipid name	nucleic acid name
1 (no bonds between monomers)	mono-	amino acid	monosaccharide	fatty acid	nucleotide
2	di-	dipeptide	disaccharide	diglyceride	dinucleotide
3	tri-	tripeptide	trisaccharide	triglyceride	trinucleotide
"a few" (2, 3, or 4)	oligo-	oligopeptide	oligosaccharide	n/a	oligonucleotide
many (could be hundreds or more)	poly-	polypeptide	polysaccharide	n/a	polynucleotide

Note

Lipids are limited to triglycerides and cannot get bigger. This is due to the glycerol structure fatty acids bond with that can only attach to three fatty acids. There are other types of lipids that do not fit this model, such as steroid molecules like cholesterol.

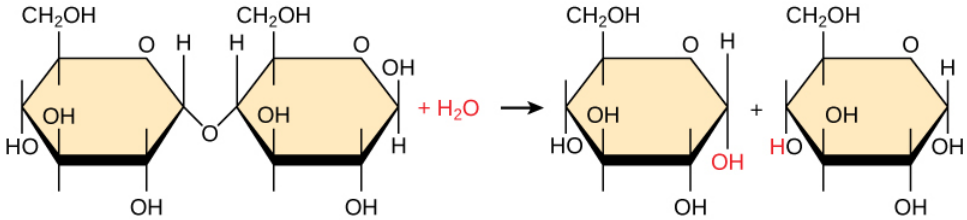


Figure 4: In the hydrolysis reaction shown here, the disaccharide maltose is broken down to form two glucose monomers with the addition of a water molecule. Note that this reaction is the reverse of the synthesis reaction shown in Figure 3.

Polymers and other monomers bonded together can also be broken down into smaller pieces by breaking covalent bonds through **hydrolysis reactions**.



Proteins

Proteins are one of the most abundant organic molecules in living systems and have the most diverse range of functions of all macromolecules. Proteins may be structural, regulatory, contractile, or protective; they may serve in transport, storage, or membranes; or they may be toxins or enzymes. Each cell in a living system may contain thousands of proteins, each with a unique function. Their structures, like their functions, vary greatly. They are all, however, polymers of amino acids, arranged in a linear sequence.

Type	Examples	Functions
digestive enzymes	amylase, lipase, pepsin, trypsin	Help in digestion of food by catabolizing nutrients into monomeric units
transport	hemoglobin, albumin	Carry substances in the blood or lymph throughout the body
structural	actin, tubulin, keratin	Construct different structures, like the cytoskeleton
hormones	insulin, thyroxine	Coordinate the activity of different body systems
defense	immunoglobulins	Protect the body from foreign pathogens
contractile	actin, myosin	Effect muscle contraction
storage	legume storage proteins, egg white (albumin)	Provide nourishment in early development of the embryo and the seedling

Proteins have different shapes and molecular weights; some proteins are globular in shape whereas others are fibrous in nature. For example, hemoglobin is a globular protein, but collagen, found in our skin, is a fibrous protein. Protein shape is critical to its function, and this shape is maintained by many different types of chemical bonds. Changes in temperature, pH, and exposure to chemicals may lead to permanent changes in the shape of the protein, leading to loss of function, known as denaturation. All proteins are made up of different arrangements of the same 20 types of amino acids.

Amino acids are the monomers that make up proteins. Each amino acid has the same fundamental structure, which consists of a central carbon atom, also known as the alpha (α) carbon, bonded to an amino group (NH_2), a carboxyl group (COOH), and to a

hydrogen atom. Every amino acid also has another atom or group of atoms bonded to the central atom known as the R group.

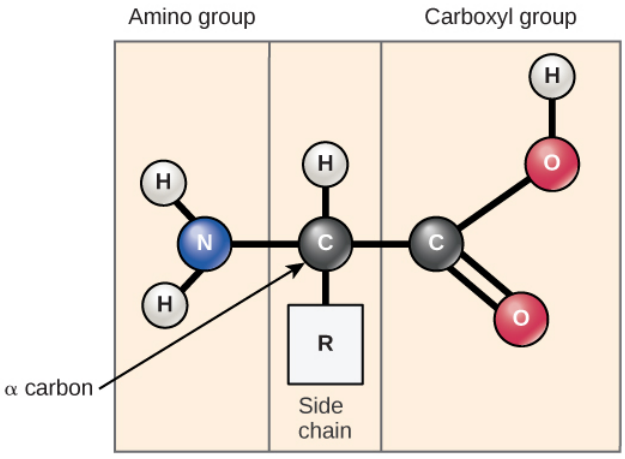


Figure 5: Amino acids have a central asymmetric carbon to which an amino group, a carboxyl group, a hydrogen atom, and a side chain (R group) are attached.

The name "amino acid" is derived from the fact that they contain both amino group (NH_2) and carboxylic acid group (COOH) in their basic structure. As mentioned, there are 20 amino acids present in proteins. Ten of these are considered essential amino acids in humans because the human body cannot produce them and they are obtained from the diet. For each amino acid, the R group (or side chain) is different.

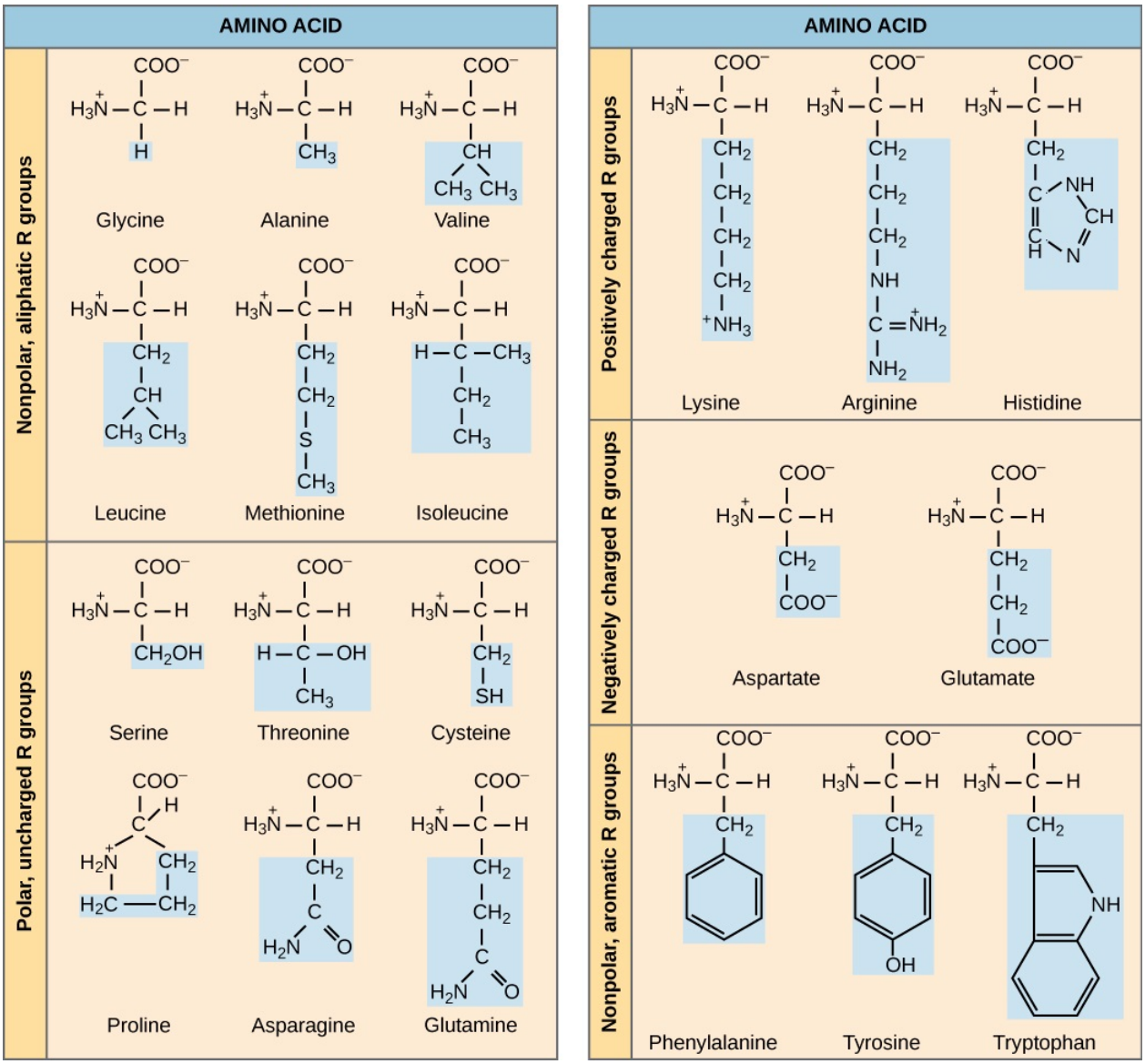


Figure 6: There are 20 common amino acids commonly found in proteins, each with a different R group (variant group) that determines its chemical nature.

The chemical nature of the side chain determines the nature of the amino acid (that is, whether it is acidic, basic, polar, or nonpolar). For example, the amino acid glycine has a hydrogen atom as the R group. Amino acids such as valine, methionine, and alanine are nonpolar or hydrophobic in nature, while amino acids such as serine, threonine, and cysteine are polar and have hydrophilic side chains. The side chains of lysine and arginine are positively charged, and therefore these amino acids are also known as basic amino acids. Proline has an R group that is linked to the amino group, forming a ring-like structure. Proline is an exception to the standard structure of an animo acid since its amino group is not separate from the side chain.

The sequence and the number of amino acids ultimately determine the protein's shape, size, and function. Each amino acid is attached to another amino acid by a covalent bond, known as a peptide bond, which is formed by a dehydration reaction. The carboxyl group of one amino acid and the amino group of the incoming amino acid combine, releasing a molecule of water. The resulting bond is the **peptide bond**.

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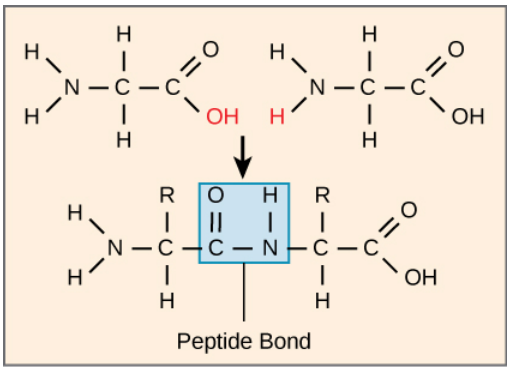


Figure 7: Peptide bond formation is a dehydration synthesis reaction. The carboxyl group of one amino acid is linked to the amino group of the incoming amino acid. In the process, a molecule of water is released.

The products formed by such linkages are called peptides. As more amino acids join to this growing chain, the resulting chain is known as a polypeptide. Each polypeptide has a free amino group at one end. This end is called the N terminal, or the amino terminal, and the other end has a free carboxyl group, also known as the C or carboxyl terminal. While the terms polypeptide and protein are sometimes used interchangeably, a polypeptide is technically a polymer of amino acids, whereas the term protein is used for a polypeptide or polypeptides that have combined together, often have bound non-peptide prosthetic groups, have a distinct shape, and have a unique function. After protein synthesis (translation), most proteins are modified. These are known as post-translational modifications. They may undergo cleavage, phosphorylation, or may require the addition of other chemical groups. Only after these modifications is the protein completely functional.

Carbohydrates

Carbohydrates are a group of macromolecules that are a vital energy source for the cell and provide structural support

Most people are familiar with carbohydrates, one type of macromolecule, especially when it comes to what we eat. To lose weight, some individuals adhere to “low-carb” diets. Athletes, in contrast, often “carb-load” before important competitions to ensure that they have enough energy to compete at a high level. Carbohydrates are, in fact, an essential part of our diet; grains, fruits, and vegetables are all natural sources of carbohydrates. Carbohydrates provide energy to the body, particularly through glucose, a simple sugar that is a component of starch and an ingredient in many staple foods. Carbohydrates also have other important functions in humans, animals, and plants.

Carbohydrates can be represented by the stoichiometric formula $(CH_2O)_n$, where n is the number of carbons in the molecule. In other words, the ratio of carbon to hydrogen to oxygen is 1:2:1 in carbohydrate molecules. This formula also explains the origin of the term “carbohydrate”: the components are carbon (“carbo”) and the components of water (hence, “hydrate”). Carbohydrates are classified into three subtypes: monosaccharides, disaccharides, and polysaccharides.

Monosaccharides (mono- = “one”; sacchar- = “sweet”) are simple sugars, the most common of which is glucose. In monosaccharides, the number of carbons usually ranges from three to seven. Most monosaccharide names end with the suffix -ose. If the sugar has an aldehyde group (the functional group with the structure R-CHO), it is known as an aldose, and if it has a ketone group (the functional group with the structure RC(=O)R'), it is known as a ketose. Depending on the number of carbons in the sugar, they also may be known as trioses (three carbons), pentoses (five carbons), and or hexoses (six carbons).

Disaccharides (di- = “two”) form when two monosaccharides undergo a dehydration reaction (also known as a condensation reaction or dehydration synthesis). During this process, the hydroxyl group of one monosaccharide combines with the hydrogen of another monosaccharide, releasing a molecule of water and forming a covalent bond. A covalent bond formed between a carbohydrate molecule and another molecule (in this case, between two monosaccharides) is known as a glycosidic bond. Glycosidic bonds (also called glycosidic linkages) can be of the alpha or the beta type.

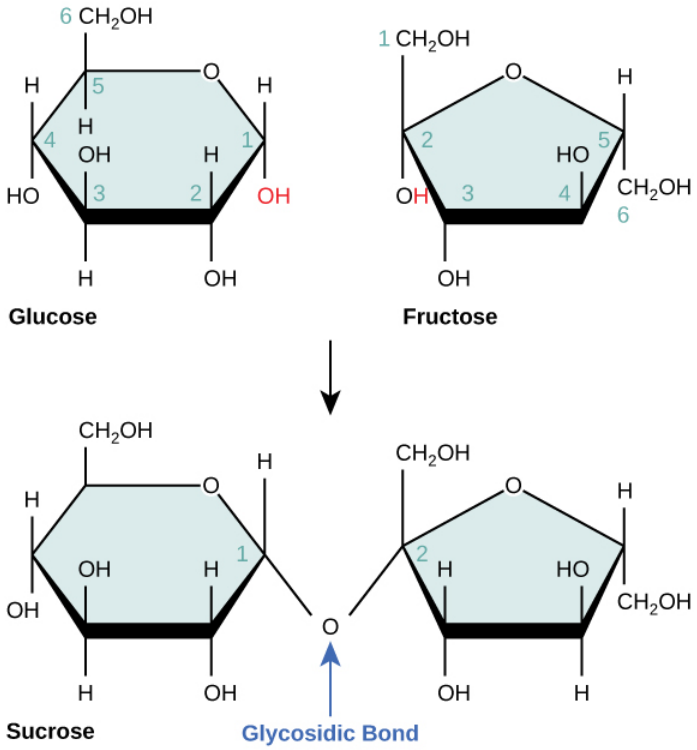


Figure 8: Sucrose is formed when a monomer of glucose and a monomer of fructose are joined in a dehydration reaction to form a glycosidic bond. In the process, a water molecule is lost. By convention, the carbon atoms in a monosaccharide are numbered from the terminal carbon closest to the carbonyl group. In sucrose, a glycosidic linkage is formed between carbon 1 in glucose and carbon 2 in fructose.

Common disaccharides include lactose, maltose, and sucrose. Lactose is a disaccharide consisting of the monomers glucose and galactose. It is found naturally in milk. Maltose, or malt sugar, is a disaccharide formed by a dehydration reaction between two glucose molecules. The most common disaccharide is sucrose, or table sugar, which is composed of the monomers glucose and fructose.

A long chain of monosaccharides linked by glycosidic bonds is known as a polysaccharide (poly- = “many”). The chain may be branched or unbranched, and it may contain different types of monosaccharides. The molecular weight may be 100,000 daltons or more depending on the number of monomers joined. Starch, glycogen, cellulose, and chitin are primary examples of polysaccharides.

Lipids

Lipids include a diverse group of compounds that are largely nonpolar in nature. This is because they are hydrocarbons that include mostly nonpolar carbon–carbon or carbon–hydrogen bonds. Non-polar molecules are hydrophobic (“water fearing”), or insoluble in water. Lipids perform many different functions in a cell. Cells store energy for long-term use in the form of fats. Lipids are also the building blocks of many hormones and are an important constituent of all cellular membranes. Lipids include fats, oils, waxes, phospholipids, and steroids.

A fat molecule consists of two main components—glycerol and fatty acids. Glycerol is an organic compound (alcohol) with three carbons, five hydrogens, and three hydroxyl (OH) groups. Fatty acids have a long chain of hydrocarbons to which a carboxyl group is attached, hence the name “fatty acid.” The number of carbons in the fatty acid may range from 4 to 36; most common are those containing 12–18 carbons. In a fat molecule, the fatty acids are attached to each of the three carbons of the glycerol molecule with an ester bond through an oxygen atom.

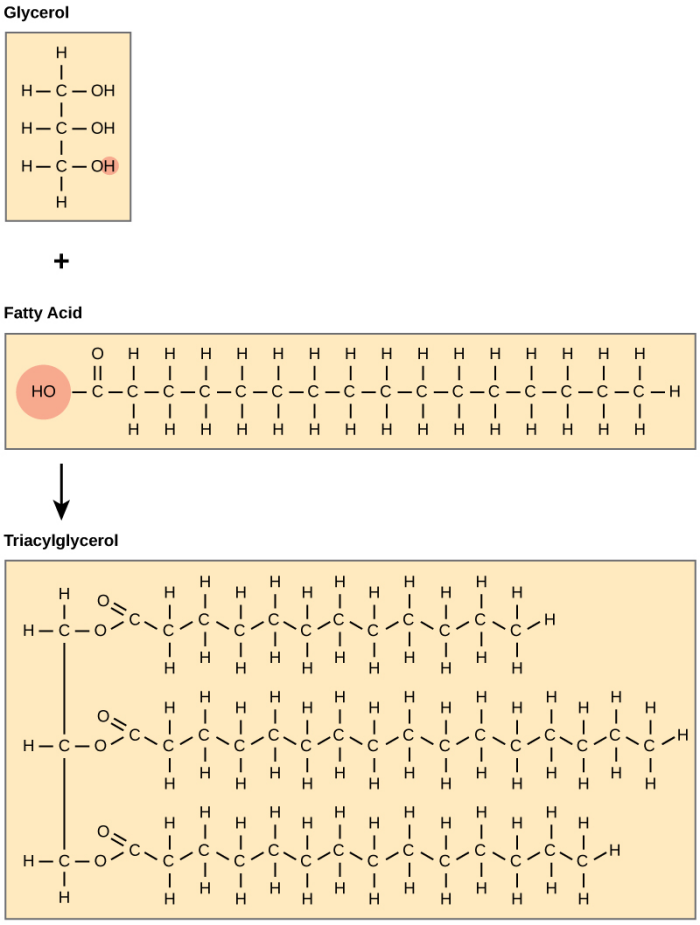


Figure 9: Triacylglycerol (also known as a triglyceride) is formed by the joining of three fatty acids to a glycerol backbone in a dehydration reaction. Three molecules of water are released in the process.

Phospholipids are major constituents of the plasma membrane, the outermost layer of animal cells. Like fats, they are composed of fatty acid chains attached to a glycerol or sphingosine backbone. Instead of three fatty acids attached as in triglycerides, however, there are two fatty acids forming diacylglycerol, and the third carbon of the glycerol backbone is occupied by a modified phosphate group. A phosphate group alone attached to a diaglycerol does not qualify as a phospholipid; it is phosphatidate (diacylglycerol 3-phosphate), the precursor of phospholipids. The phosphate group is modified by an alcohol. Phosphatidylcholine and phosphatidylserine are two important phospholipids that are found in plasma membranes.

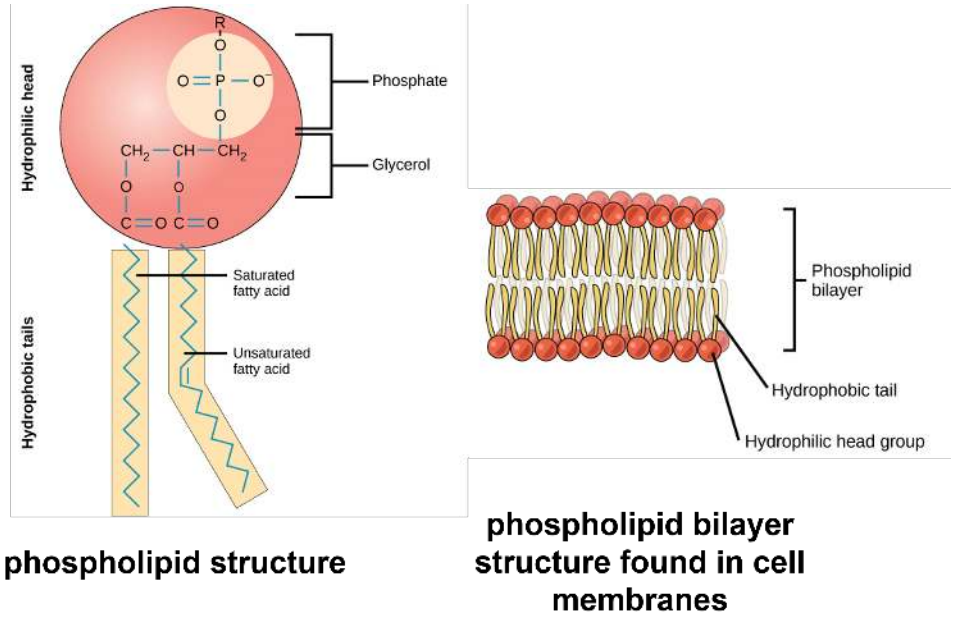


Figure 10: (Left) A phospholipid is a molecule with two fatty acids and a modified phosphate group attached to a glycerol backbone. The phosphate may be modified by the addition of charged or polar chemical groups. Two chemical groups that may modify the phosphate, choline and serine, are shown here. Both choline and serine attach to the phosphate group at the position labeled R via the hydroxyl group indicated in green. (Right) The phospholipid bilayer is the major component of all cellular membranes. The hydrophilic head groups of the phospholipids face the aqueous solution. The hydrophobic tails are sequestered in the middle of the bilayer.

Unlike the phospholipids and fats discussed earlier, steroids have a fused ring structure. Although they do not resemble the other lipids, they are grouped with them because they are also hydrophobic and insoluble in water. All steroids have four linked carbon rings and several of them, like cholesterol, have a short tail.

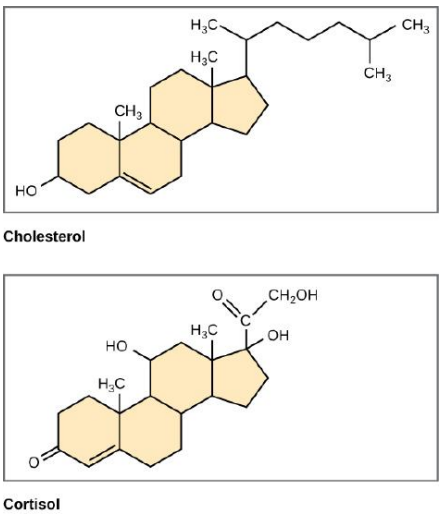


Figure 11: Steroids such as cholesterol and cortisol are composed of four fused hydrocarbon rings.

Nucleic Acids

Nucleic acids are the most important macromolecules for the continuity of life. They carry the genetic blueprint of a cell and carry instructions for the functioning of the cell.

The two main types of nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is the genetic material found in all living organisms, ranging from single-celled bacteria to multicellular mammals. It is found in the nucleus of eukaryotes and in the organelles, chloroplasts, and mitochondria. In prokaryotes, the DNA is not enclosed in a membranous envelope.

DNA and RNA are made up of monomers known as **nucleotides**. The nucleotides combine with each other to form a polynucleotide, DNA or RNA. Each nucleotide is made up of three components: a nitrogenous base, a pentose (five-carbon) sugar, and a phosphate group. Each nitrogenous base in a nucleotide is attached to a sugar molecule, which is attached to one or more phosphate groups.

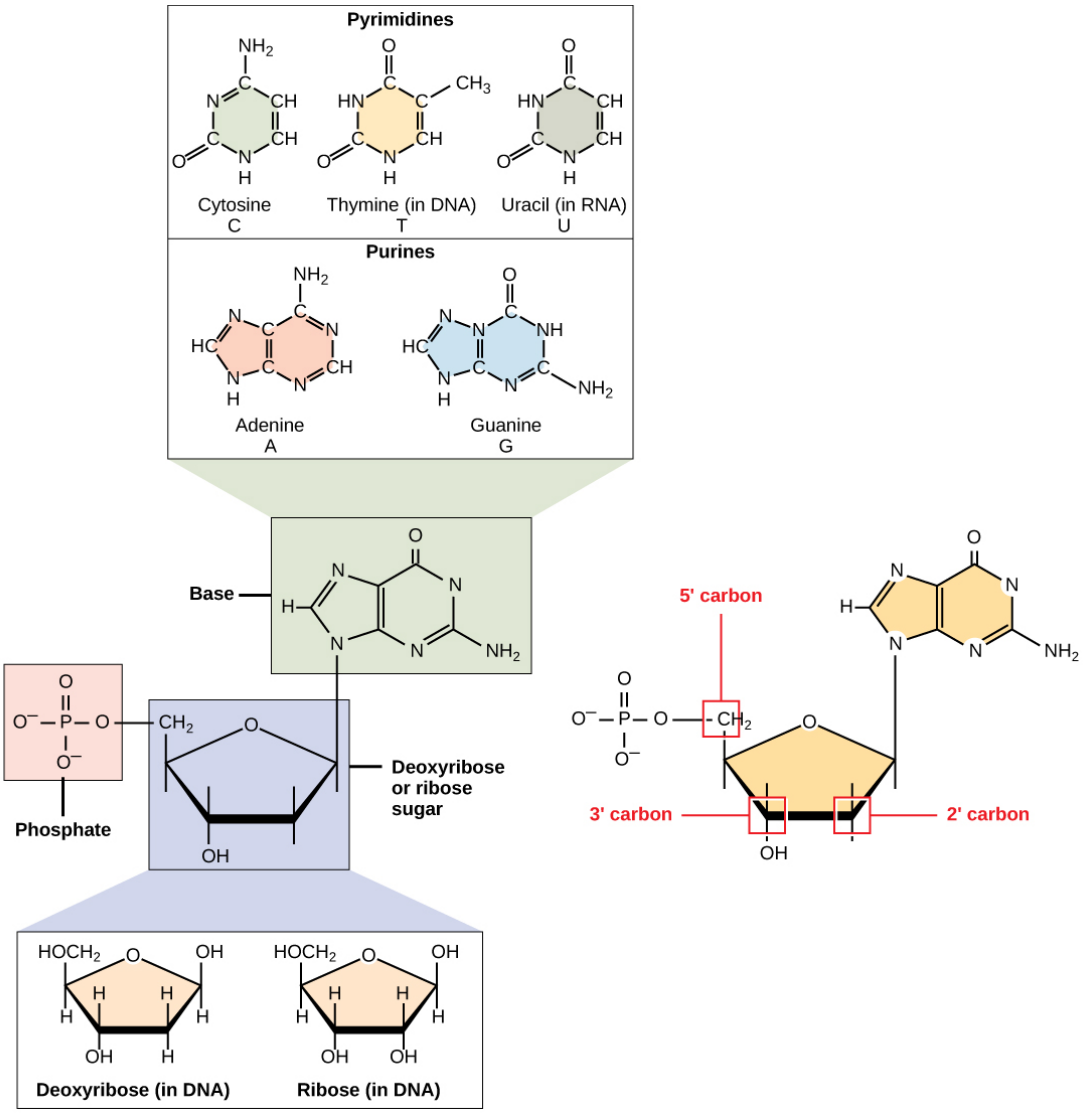


Figure 12: A nucleotide is made up of three components: a nitrogenous base, a pentose sugar, and one or more phosphate groups. Carbon residues in the pentose are numbered 1′ through 5′ (the prime distinguishes these residues from those in the base, which are numbered without using a prime notation). The base is attached to the 1′ position of the ribose, and the phosphate is attached to the 5′ position. When a polynucleotide is formed, the 5′ phosphate of the incoming nucleotide attaches to the 3′ hydroxyl group at the end of the growing chain. Two types of pentose are found in nucleotides, deoxyribose (found in DNA) and ribose (found in RNA). Deoxyribose is similar in structure to ribose, but it has an H instead of an OH at the 2′ position. Bases can be divided into two categories: purines and pyrimidines. Purines have a double ring structure, and pyrimidines have a single ring.

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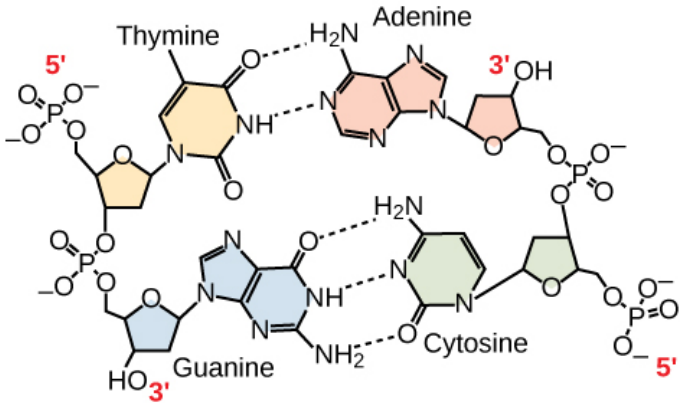


Figure 13: In a double stranded DNA molecule, the two strands run antiparallel to one another so that one strand runs 5′ to 3′ and the other 3′ to 5′. The phosphate backbone is located on the outside, and the bases are in the middle. Adenine forms hydrogen bonds (or base pairs) with thymine, and guanine base pairs with cytosine.

Overview Video of Biological Molecules



Laboratory Instructions

Burn Test

Conduct the Burn Test

1. Put safety goggles on.
2. Put a small amount of baking soda into a test tube.
3. Hold the test tube with a test tube holder.
4. Ignite a Bunsen burner.
5. Angle the test tube so the open end is not facing yourself or any other person. Place the bottom of the test tube into the flame to heat the baking soda.
6. Continue burning until the baking soda is clearly very hot and smoking.
7. Observe the baking soda color.
8. Write results in the table below.
9. Repeat steps 2-8 for each of the following substances (with new test tubes each time): hair, salt, starch, sugar, vegetable oil, water.

Alternatively, your instructor may have completed these burn tests for you. Observe the results and record the results in the table below.

Burn Test Results & Questions

Substance Tested in the Burn Test	Appearance of Substance after Burn Test	Is the Substance Organic or Inorganic?	If Substance is Organic, Give the Type of Biological Molecule (protein, carbohydrate, lipid, or nucleic acid)
baking soda			
hair			
salt			
starch			
sugar			
vegetable oil			
water			

1. Complete the table above based on results of the burn test.
2. What type(s) of biological molecules were not tested using the burn test? Give examples of these types of biological molecules.
3. True or False: Every type of molecule found within a living thing is organic. Explain your answer.
4. True or False: It is possible for inorganic molecules to be essential for life.
5. What is the burn test and what can it show us?

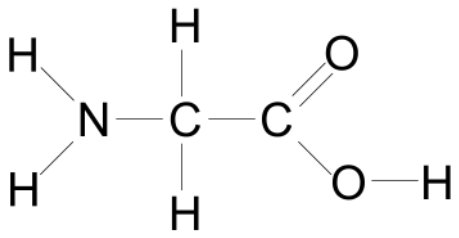
Building Monomers of Biological Molecules

To become familiar with the molecular modeling kits, examine the colored balls that represent different elements common in biological molecules. Each colored ball has one or more holes. These holes represent the ability of these elements to form bonds:

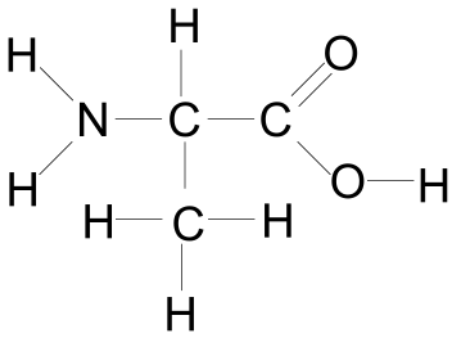
element	element abbreviation	molecular model ball color	bonding capacity
carbon	C	black	
oxygen	O	red	
hydrogen	H	white	
nitrogen	N	blue	
phosphorus	P	orange	

Based on the holes in each of the model element balls, fill in the column that indicates the bonding capacity of the element. Bonding capacity is based on the number of protons the element has and the number of valence electrons the element has.

Protein Monomers: Amino Acids



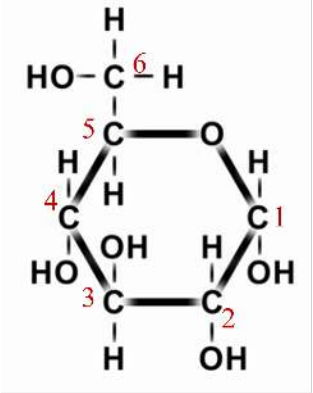
glycine



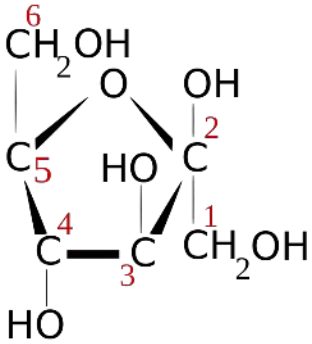
alanine

1. Your instructor will assign you to build one of the two amino acids shown above.
2. Use the diagram above and the table indicating the molecular model element ball colors to build the amino acid you are assigned.
3. With your partner, conduct a dehydration synthesis reaction as directed by your instructor to form a dipeptide from your amino acids. This will form a molecule of water.
4. Conduct a hydrolysis reaction by breaking the water molecule to break down the dipeptide into the two amino acids.

Carbohydrate Monomers: Monosaccharides



glucose



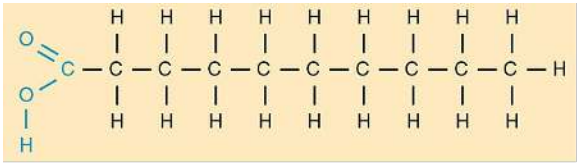
fructose

1. Your instructor will assign you to build one of the two monosaccharides shown above.
2. Use the diagram above and the table indicating the molecular model element ball colors to build the monosaccharide you are assigned.
3. With your partner, conduct a dehydration synthesis reaction as directed by your instructor to form a disaccharide from your monosaccharides. This will form a molecule of water.
4. Conduct a hydrolysis reaction by breaking the water molecule to break down the disaccharide into the two monosaccharides.

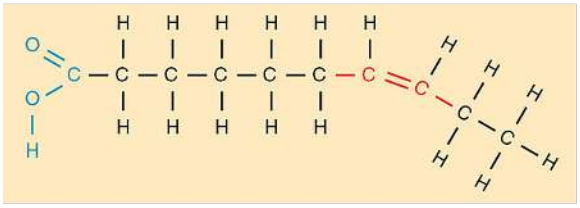
Hint

Start this structure by building the circle of carbons (they have red numbers 1-5 in glucose and 2-5 in fructose) with the one oxygen that helps make up the ring. Once you have the circle, then build off of that circle.

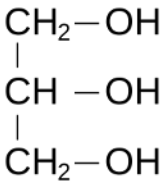
Lipids: Fatty Acids



saturated fatty acid



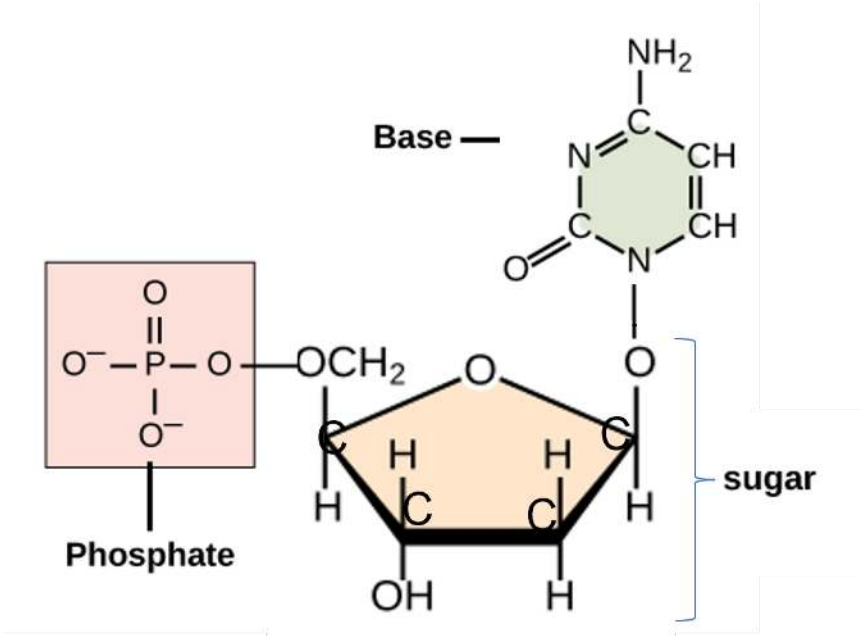
unsaturated fatty acid



glycerol

1. Build the fatty acid shown above.
2. Work with your partner to build one molecule of glycerol.
3. With your partner, conduct two dehydration synthesis reactions as directed by your instructor to form a diglyceride from your two fatty acids and the glycerol. This will form two molecules of water.
4. Conduct a hydrolysis reaction by breaking the water molecules to break down the diglycerides into the two fatty acids and one glycerol.

Nucleic Acids: Nucleotides



1. Work together with your partner to build the nucleotide above.
    - You can each build one of the pieces of the nucleotide: phosphate group, sugar, or base.
    - When the three parts are completed, form bonds between the parts as shown in the diagram above.

Building Monomers of Biological Molecules Questions

classification of biological molecule	monomer of this classification of biological molecule	elements found in this type of biological molecule	function(s) of this type of biological molecule	name a specific example of a molecule in this category of biological molecules

1. Fill out the table above to summarize information about the four categories of biological molecules.
2. Define the following:
    - atom:
    - element:
    - organic molecule:
    - inorganic molecule:
    - monomer:
    - polymer:
    - dehydration synthesis:
    - hydrolysis:
3. **Catabolic reactions** break down larger biological molecules into smaller biological molecules. **Anabolic reactions** form bonds to join together smaller biological molecules into larger biological molecules.
    - Fill in the blanks with the bold words above: Dehydration synthesis is a type of reaction that must be a(n) _____ and hydrolysis is a type of reaction that must be a(n) _____.

Foods and their Biological Molecules

Foods are Made of Biological Molecules

1. Discuss with your group the types of foods you are familiar with that fall into the categories of biological molecules listed above. Fill in the table.

proteins (list protein-rich food below)	**carbohydrates** (list carbohydrate-rich foods below)	**lipids (fats)** (list lipid-rich foods/cooking ingredients below)

Note

Just like humans need food, so do microorganisms. Microorganisms also "eat." They, like us humans, require nutrients to survive.

Note

Pathogenic (disease-causing) microorganisms use their host (e.g. humans) as a source of nutrients.

Test Food Samples for Proteins

1. Put on laboratory safety goggles/glasses.
2. Label five test tubes with the samples to be tested: green banana, black banana, muscle, egg, DI or distilled water.
3. To each test tube, add 5 drops of the samples to be tested as labeled on the test tubes.
4. Add 3 drops of biuret reagent and mix well by swirling.
5. Wait 2 minutes.
6. Blue color is negative for significant protein content; purple/violet is positive for significant protein content
7. Record results in the table below.

Test Food Samples for Sugars (Small Carbohydrates)

1. Empty and thoroughly rinse the test tubes after the protein test.
2. To each test tube, add 5 drops of the samples to be tested as labeled on the test tubes.
3. Add 3 drops of Benedict's solution and mix well by swirling.
4. Gently place test tubes in a beaker of boiling or nearly boiling water.
5. Wait 2 minutes.
6. Use a test tube holder to remove the test tubes from the hot water and allow to cool.
7. Examine the liquid in the test tubes and interpret the results:
    - **green to yellow** - very low to low levels (positive) of monosaccharides and disaccharides
    - **yellow-orange to orange** - moderate to high levels (positive) of monosaccharides and disaccharides
    - **orange-red** - very high levels (positive) of monosaccharides and disaccharides
    - **blue, grey, or any other color** - negative for detectable monosaccharides and disaccharides
8. Record results in the table below.

Test Food Samples for Starch (A Large Carbohydrate)

1. Empty and thoroughly rinse the test tubes after the sugars test.
2. To each test tube, add 5 drops of the samples to be tested as labeled on the test tubes.
3. Add 3 drops of iodine and mix well by swirling.
4. Examine the liquid in the test tubes and interpret the results:
    - **black liquid or amber liquid with black chunks** - positive for starch
    - **amber liquid (no black color)** - negative for starch
5. Record the color of the test result in the table below.

Test Food Samples for Lipids (Fats)

1. On a piece of brown paper, draw five circles and label with with the samples to be tested: green banana, black banana, muscle, egg, DI or distilled water.
2. Drop ***one drop*** of each substance to be tested into the appropriate circle and allow to dry for at least 15 minutes.
3. Examine the dried spots. An oily spot (similar to the oil you would see on a brown napkin after french fries or potato chips) on the paper indicates a positive result for lipids.
4. Record your results in the table below.

Type of Cells Blended with DI or distilled Water	protein (+/-)	sugar (small carbohydrates) (+/-)	starch (large carbohydrates) (+/-)	lipid (fats) (+/-)
green (under-ripe) banana cells				
black (over-ripe) banana cells				
muscle cells (chicken breast)				
egg (chicken egg)				
DI or distilled water (the control)				

1. Complete the table above to summarize results from testing food items for biological molecules.
2. Of the four categories of biological molecules, which ones did we test for in these experiments?
3. Which category of biological molecule was not tested in these experiments?
4. Nucleic acids include DNA molecules. All cells have DNA (with only very few exceptions). If we tested the following, should the be positive or negative for nucleic acids? Briefly explain your answer for each.
   - banana cells:
   - muscle cells:
   - egg cells:
   - DI water or distilled water:
5. Each of the samples tested were prepared by mixing the food item with DI water or distilled water and mixing it in a blender. Think about the integrity of the experiment. Why did we need to also separately test the DI water or distilled water?

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1.7: 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)

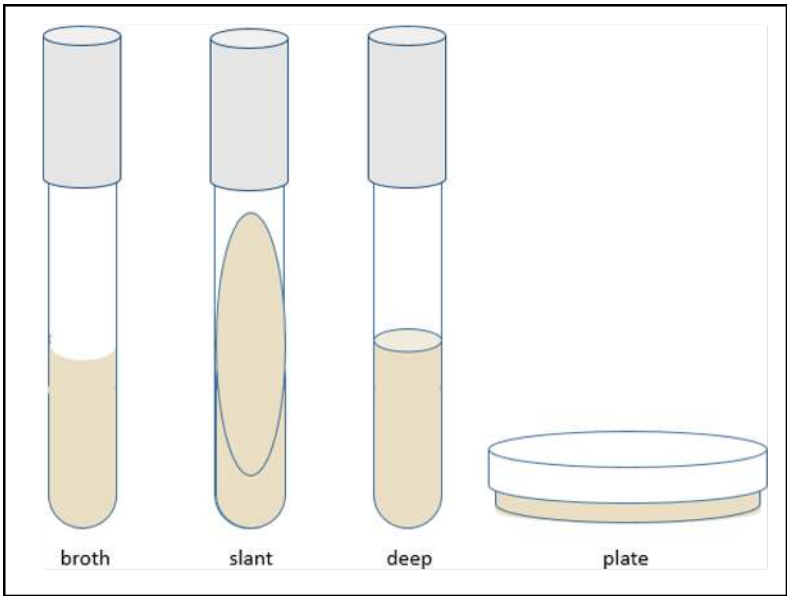


Figure 1: Illustration showing the different forms microbiological media can be prepared as.

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**. Deeps are often used to for certain differential metabolic tests.

In contrast to deeps 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./in^{2}, 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 chorine-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 show be blue and a moderate height (not to 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.

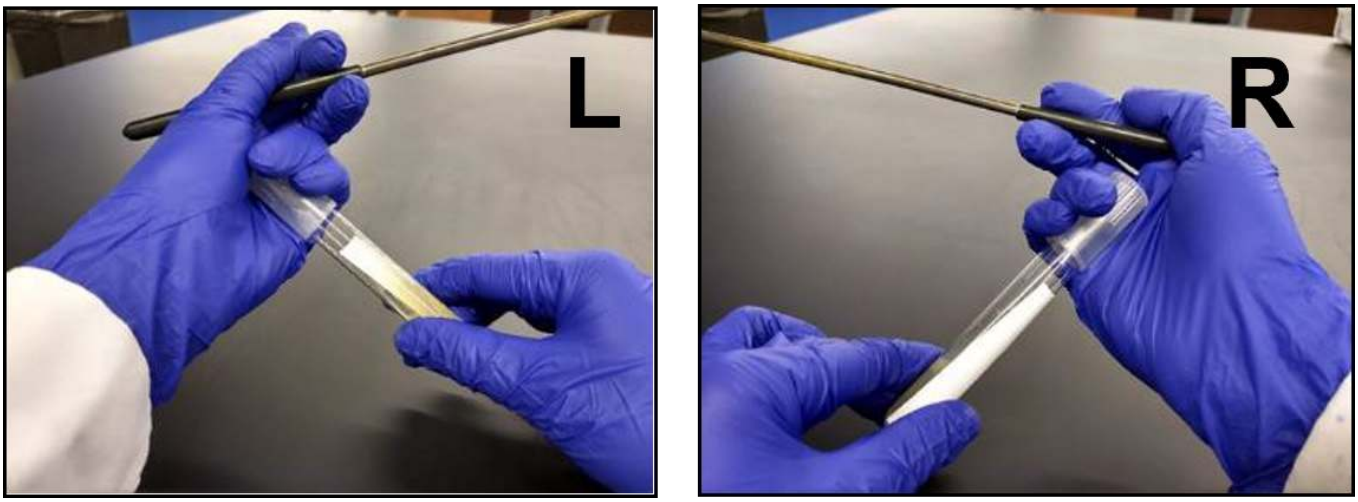


Figure 3: Grasping, removing, and hold a test tube cap while holding an inoculation loop or needle. The cap should never be placed on the bench top and the open end of the cap should not tough anything to avoid contamination.

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.

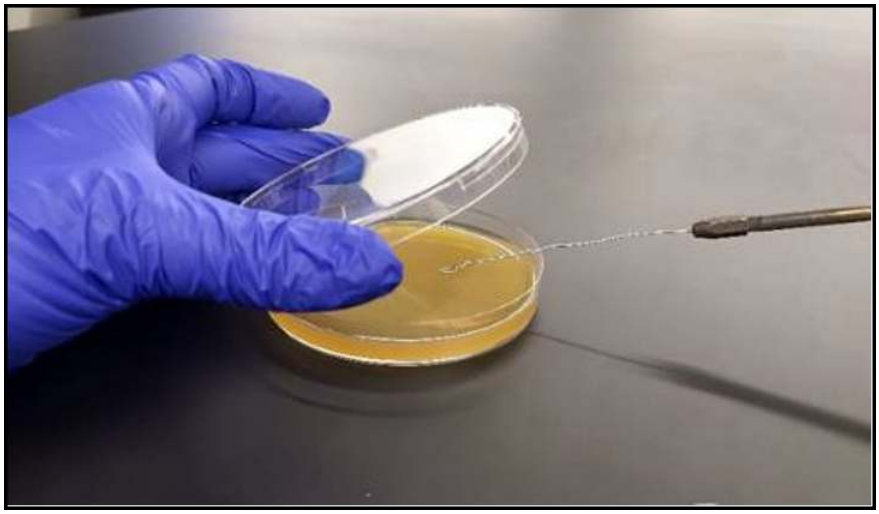


Figure 4: Whenever transferring bacteria to or from a petri plate, the lid should only be lifted just enough to allow the loop to reach the agar. The lid of the petri plate should never be set down on the bench and should never touch anything to avoid contamination.

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.

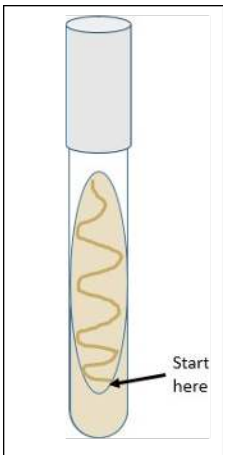


Figure 5: Inoculating a slant. Begin with the loop at the bottom of the slant you are transferring bacteria to and snake the loop up the surface of the slant.

Inoculating a broth

1. With your non-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.
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. Record your results in the table below.

	Growth (+) or No Growth (-)
TSB culture tube 1	
TSB culture tube 2	

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?
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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1.8: 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).

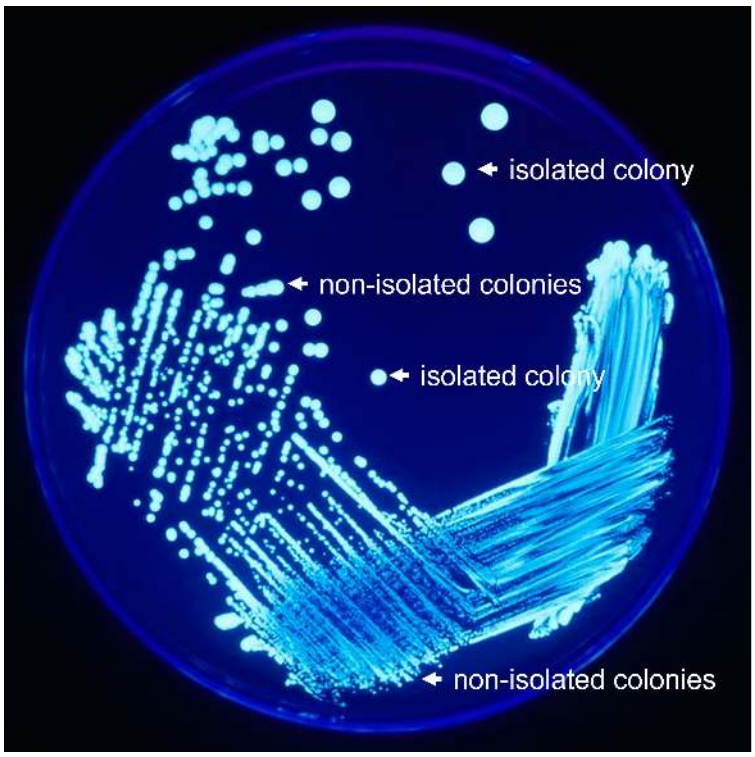


Figure 1: A petri plate with bacterial growth showing the distinction between isolated colonies and non-isolated colonies.

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.

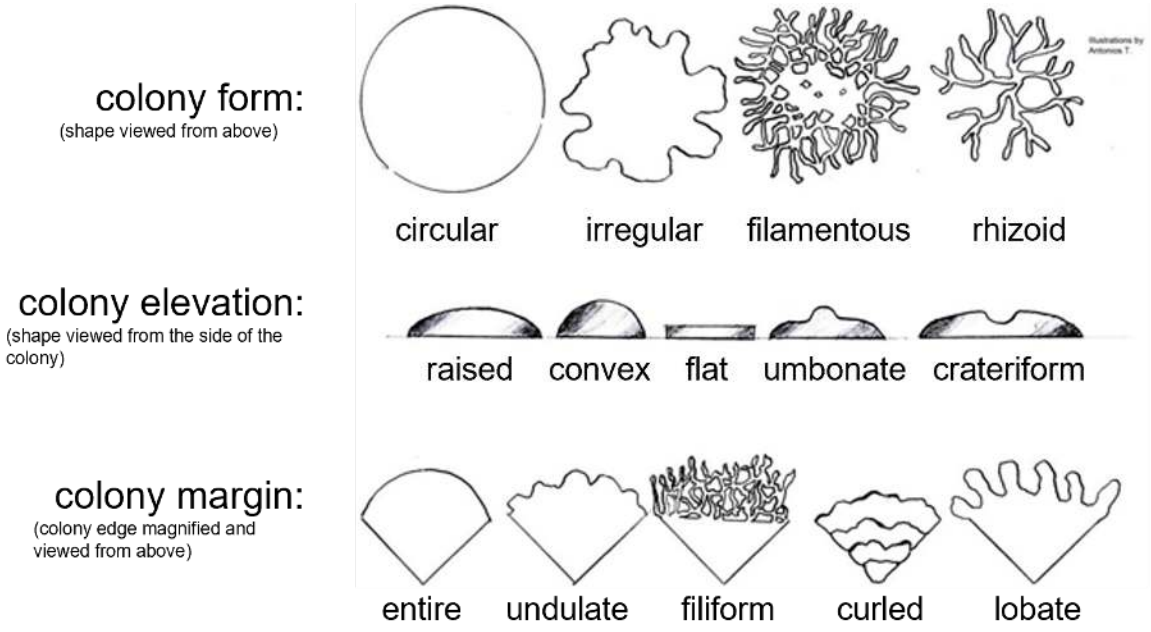


Figure 2: A guide for classifying colony characteristics. Colony form is the overall shape of the colony (circular, irregular, filamentous, or rhizoid) when viewed from above. Colony elevation examines the height and shape of the growth above the surface of the petri plate agar (raised, convex, flat, 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.

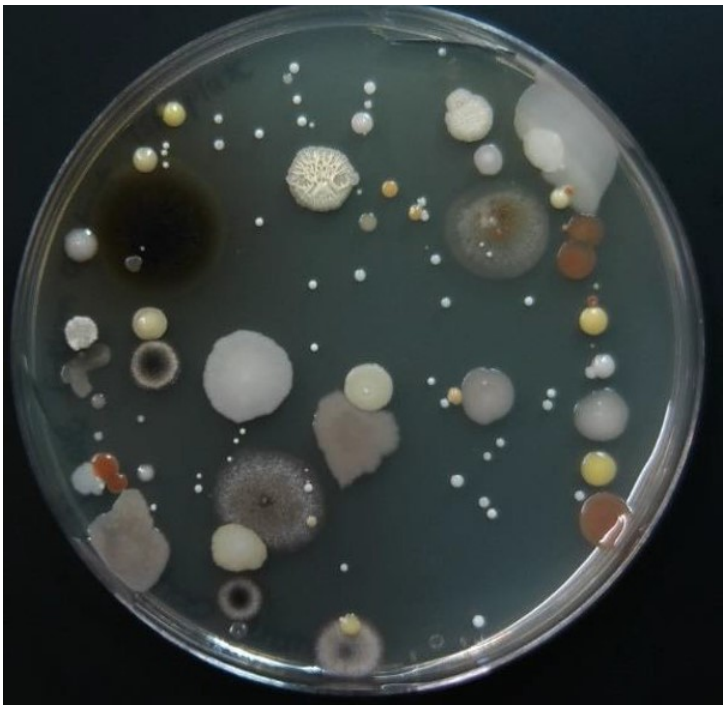


Figure 3: Zig zag streak plate of an environmental sample. The colonies have diverse colors, textures, and forms. This diversity is because different microbial species produce different colony characteristics. Environmental samples will contain mixtures of microbial species.

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.

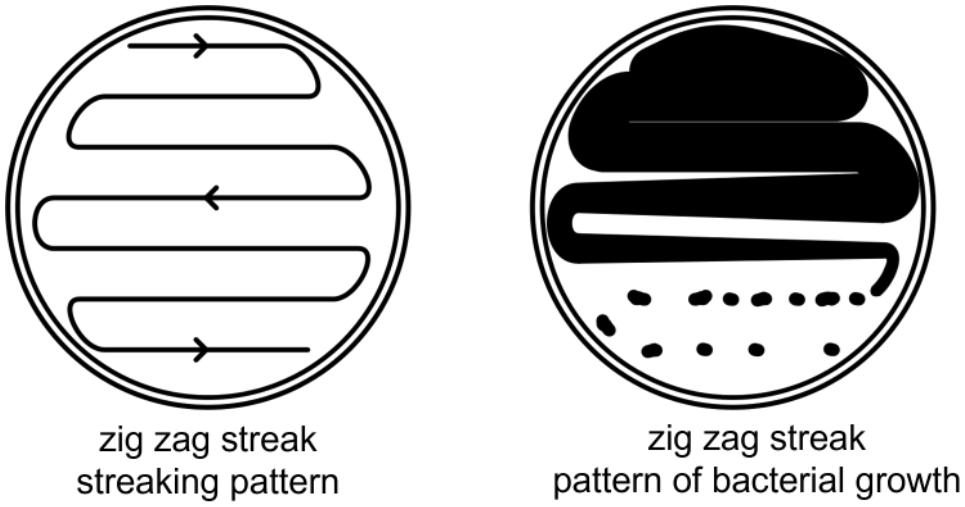


Figure 4: The zig zag streaking pattern uses streak directions moving left to right, then right to left by snaking across the petri plate. The entire petri plate surface is used. The result is bacterial growth that is dense (confluent) at the location of the first "zig" and becomes successively less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

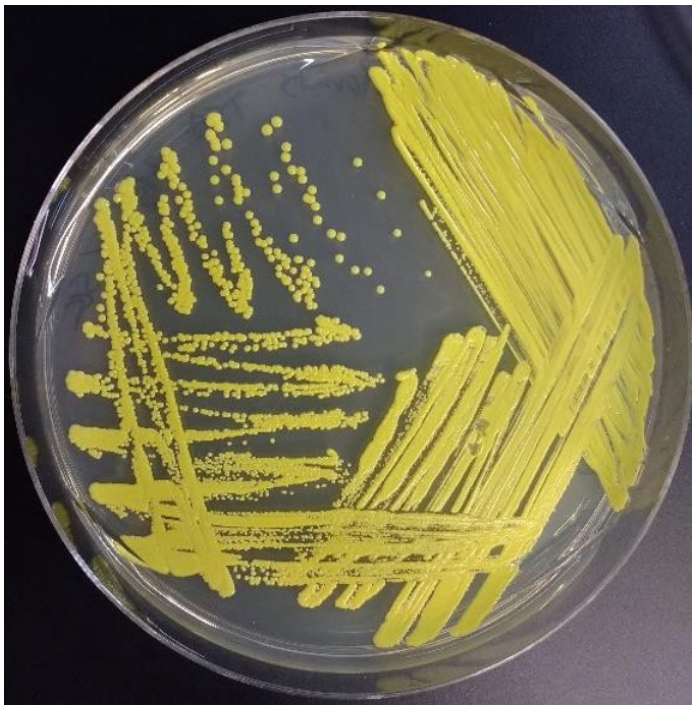


Figure 5: Bacteria growth on a petri plate streaked using the quadrant streak plate technique. Four zones of growth are apparent with successively less growth in each area/zone. Isolated colonies are present in the fourth area/zone streaked.

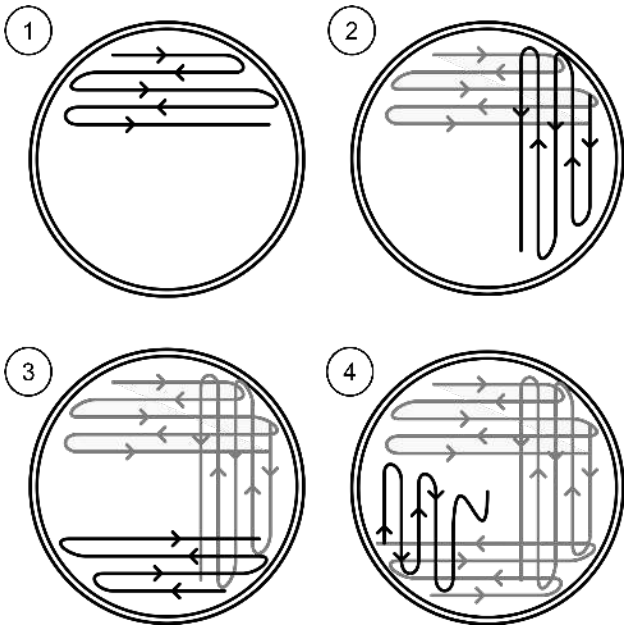


Figure 6: Quadrant streak plate technique to create four areas/zones with successively less growth. (1) Zone one occupies one fourth of the petri plate with the inoculating loop snaking back and forth in that quadrant. After zone one is completed, the inoculating loop is flamed to kill all microbes. (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.

Streak Plate Results

1. Fill out the table below to describe the types of colonies observed:

			Escherichia coli & Serratia marcescens	
	Escherichia coli	*Serratia marcescens*	Colony type 1	Colony type 2
Colony form				
Colony elevation				
Colony margin				
Colony color				
Colony diameter (mm)				

2. Make detailed drawings of your plates (using color). Alternatively, you can take images of the plates and attach them below.

3. Did you obtain isolated colonies on each of the plates?

    Escherichia coli:

Serratia marcescens:

Escherichia coli & Serratia marcescens (mixture):

4. Explain how the streak-plate technique dilutes and spreads out microorganisms to form individual colonies.
5. Considering how you streaked the plates, which area of the streak plate will always contain:

   the greatest amount of growth:

   the least amount of growth:

6. Does each individual colony represent the growth of one cell? Explain your answer.
7. 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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1.9: 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 distingushed using a simple stain.

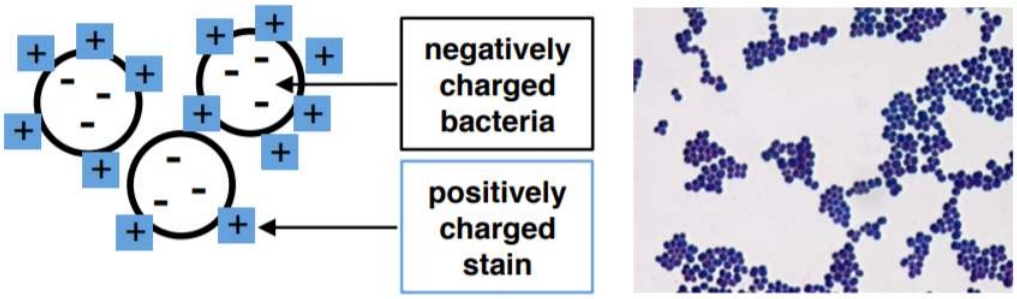
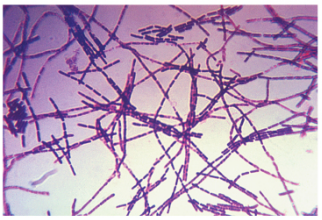


Figure 1: Methylene blue is a simple and direct stain used for determining bacterial morphology (shape and arrangement). It is a cationic dye (positive charge) which stains the cell a blue color. The presence of negatively charged molecules in the cell (like DNA & RNA) causes the cell to stain blue. 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.

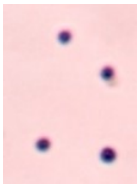
SIMPLE STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Basic stains	Methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	
Acidic stains	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	

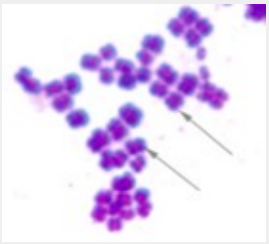
A good stained smear should be somewhat difficult to see with the naked eye on the surface of a microscope slide. If there is a dark spot of color visible, it means that the bacteria are stacked too densely for adequate evaluation. This is a common mistake made by students learning to make bacterial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

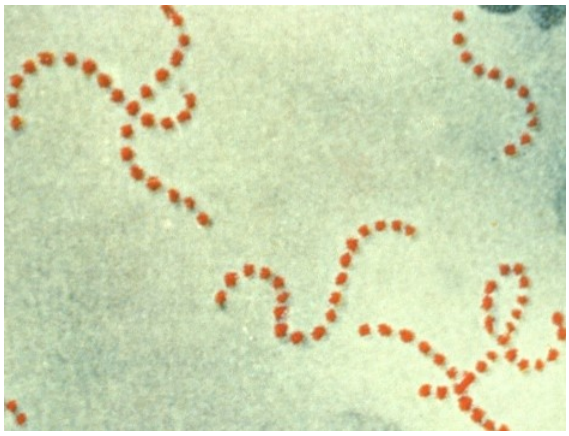
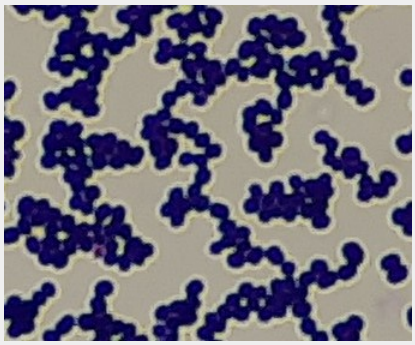
Bacterial Cell Morphology & Arrangement

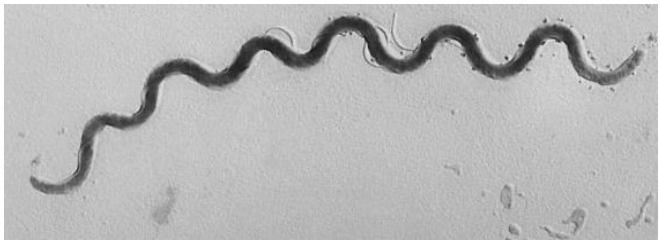
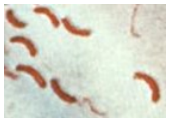
Two characteristics that can be used to distinguish different types of bacteria is their microscopic shapes and their microscopic arrangements. Identification of these features can lead to a diagnosis and aid in treatment of a patient. The cell shape describes the overall shape of a single bacterial 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.

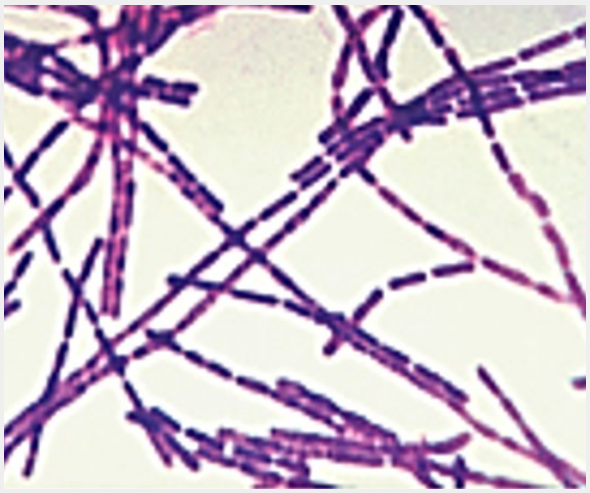
Cell Arrangement (unstained unless otherwise specified)	Description	Image (color of cells is based on the type of stain used)
single	Cells are alone and round or spherical.	

diplococcus/diplococci	Cells are in pairs and round or spherical. 
coccus tetrad/cocci	Cells are grouped in fours and are round or spherical. 
coccus sarcina/cocci	Cells are group in eights as two groups of four on top of each other. Cells are round. 

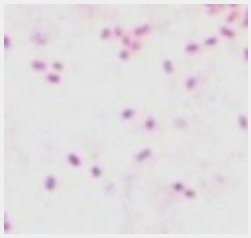
streptococcus / streptococci	Cells are in a chain and are round or spherical. 
staphylococcus / staphylococci	Cells are in bunches or clusters and are round or spherical. 

coccus	
spirillum	Cells are alone and long and thin with a three-dimensional corkscrew forming a helical shape. 
spirochete	Cells are alone and long and thin with a wavy appearance but are not helical in shape. 
vibrio	Cells are alone and have a rod shape with an arch or curve. 

streptobacillary	
bacillus	Cells are alone and rod shaped. 
diplobacillus / diplobacillary	Cells are in pairs and are rod shaped. 

streptobacillus / streptobacilli	Cells are in chains and are rod shaped. 
bacillus / bacilli	Cells are rod shaped and lined up with each other long-ways resembling the pickets in a picket fence. 

coccobacillus coccobacillus coccobacillus coccobacillus coccobacillus	Cells are alone and are short rods.

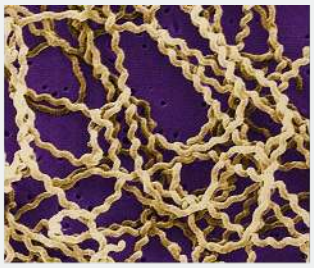


Note

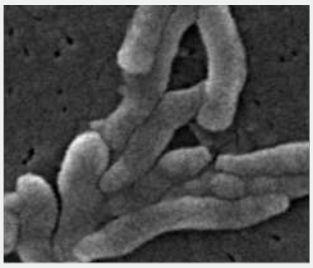
There are other less common bacterial morphologies (e.g., filamentous, squares, etc.) that are not shown here.

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.

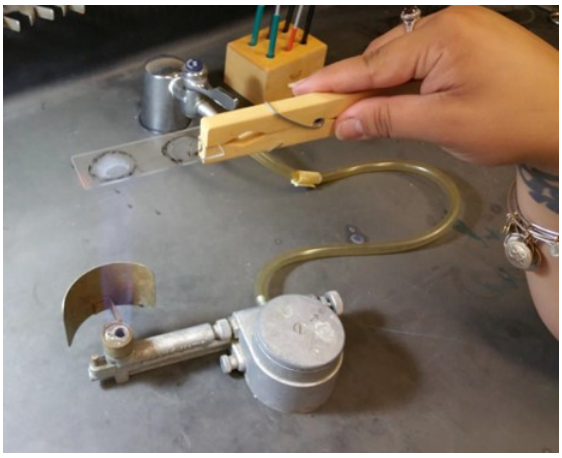


Figure 2: A microscope slide with a bacterial smear is being heat fixed. The underside of the microscope slide is passed briefly into the flame of a Bunsen burner to heat fix. For safety, the person who is heat fixing the smear is holding a wooden clip that grips the microscope slide. This way the person 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.

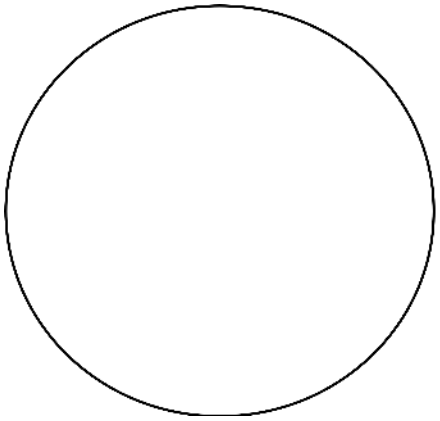
> **Note**
>
> If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them.

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.

Results & Questions



specimen: _____

total magnification: _____

1. Draw the cells you observe after the simple stain in the space above.
2. Critique your cell drawings:
   - Does the drawing accurately show how the cells appear when you look into the microscope (including cell shape and arrangement)?
   - Does the drawing accurately show the cell size you see in the microscope?
   - Does the drawing accurately show the color(s) you see in the microscope?
3. What microbial characteristics can one ascertain from a simple stain?
4. What were the shapes of the cells you observed? Use microbiological terminology.
5. What was the arrangement of the cells you observed? Use microbiological terminology.
6. A successful simple stain would enable you to observe individual cells (cells not on top of each other) and a sample where you can clearly see the cells, their undistorted shapes, and their undistorted arrangements. Reflect on your results. In what way(s) could your simple stain be improved?
7. What might you do to improve the results of the simple stain as mentioned in 6.
8. Fixing a bacterial smear is an essential step in preparing for staining. Why? What might happen if cells are not fixed to the microscope slide?
9. Fixing also has another effect on the cells other than your answer to 8. What is it?
10. Define simple stain.
11. How is the procedure different when taking cells from a solid medium compared to taking cells from a liquid medium? Why is it important that your smear be thin?

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1.10: Gram Stain

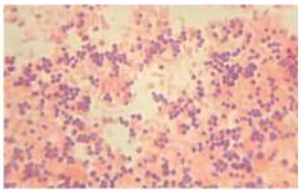
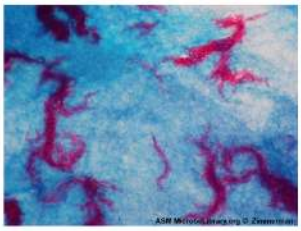
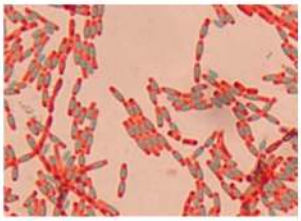
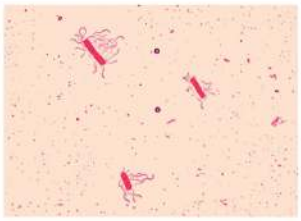
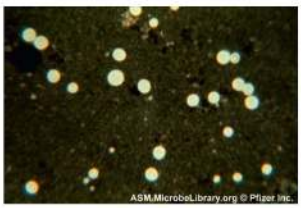
Learning Objectives

- 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 interacts 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 read 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, pararosaline 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 *M. tuberculosis*, 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 pararosaline 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.

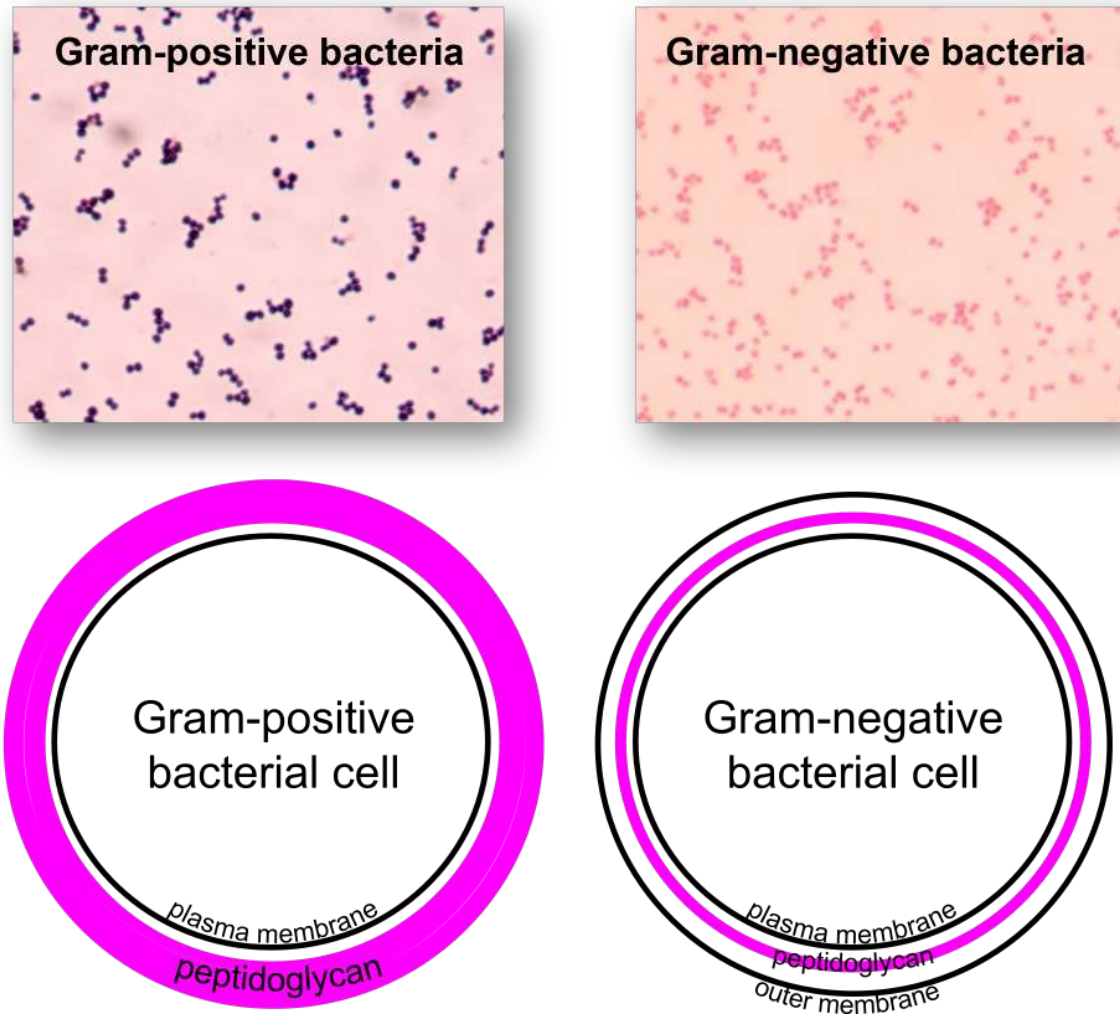


Figure 1: (Top) Microscopic images of bacteria stained with the Gram stain. (Top right) Gram-positive bacterial species stain purple with the Gram stain. (Top left) Gram-negative bacterial species stain pink with the Gram stain. (Bottom) Diagrams illustrating major structural components of Gram-positive 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.

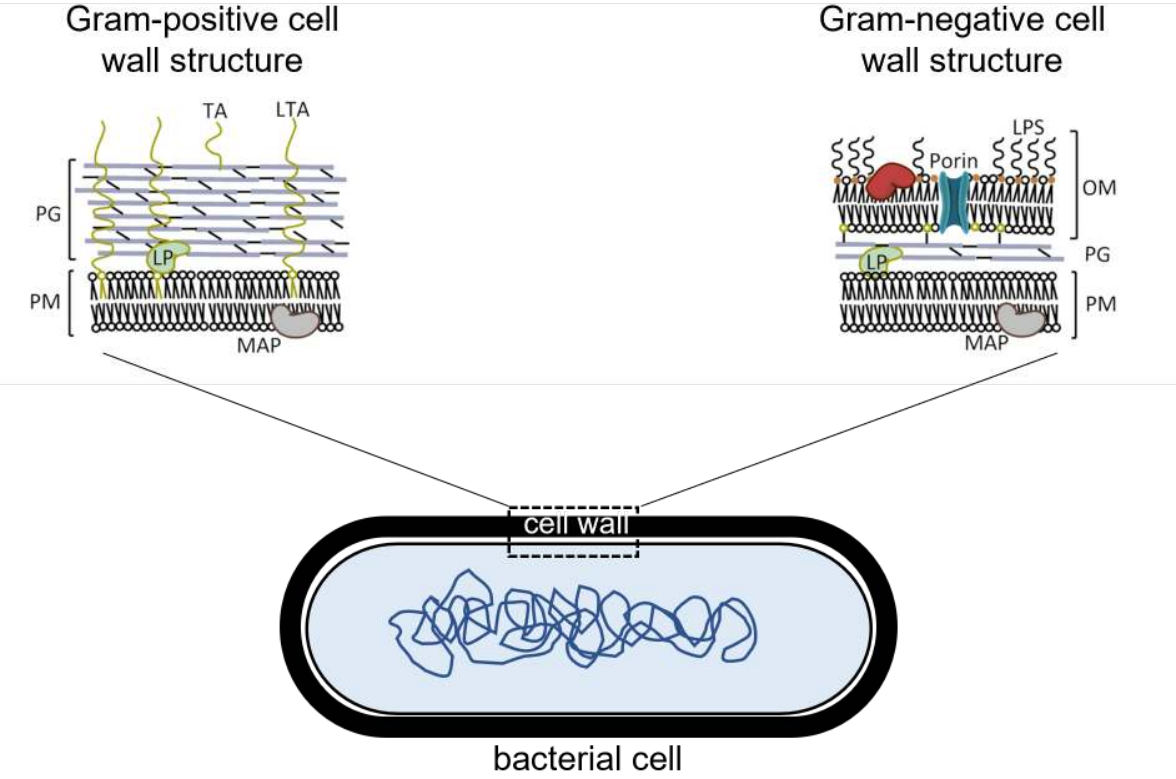


Figure 2: Detailed view of the structure of bacterial cell walls for Gram-positive species and for Gram-negative species. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across 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.

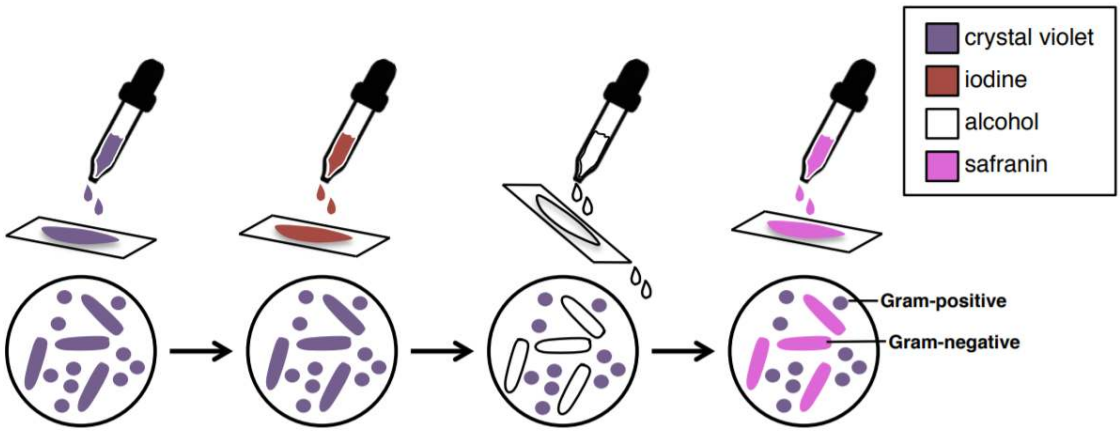


Figure 3: Overview of the Gram stain procedure and how each step changes cell color. (1) Applying crystal violet turns all cells purple. (2) Applying Gram's iodine does not change cell colors (all cells stay purple), but it will cause the crystal violet to firmly adhere to Gram-positive cell walls, but 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

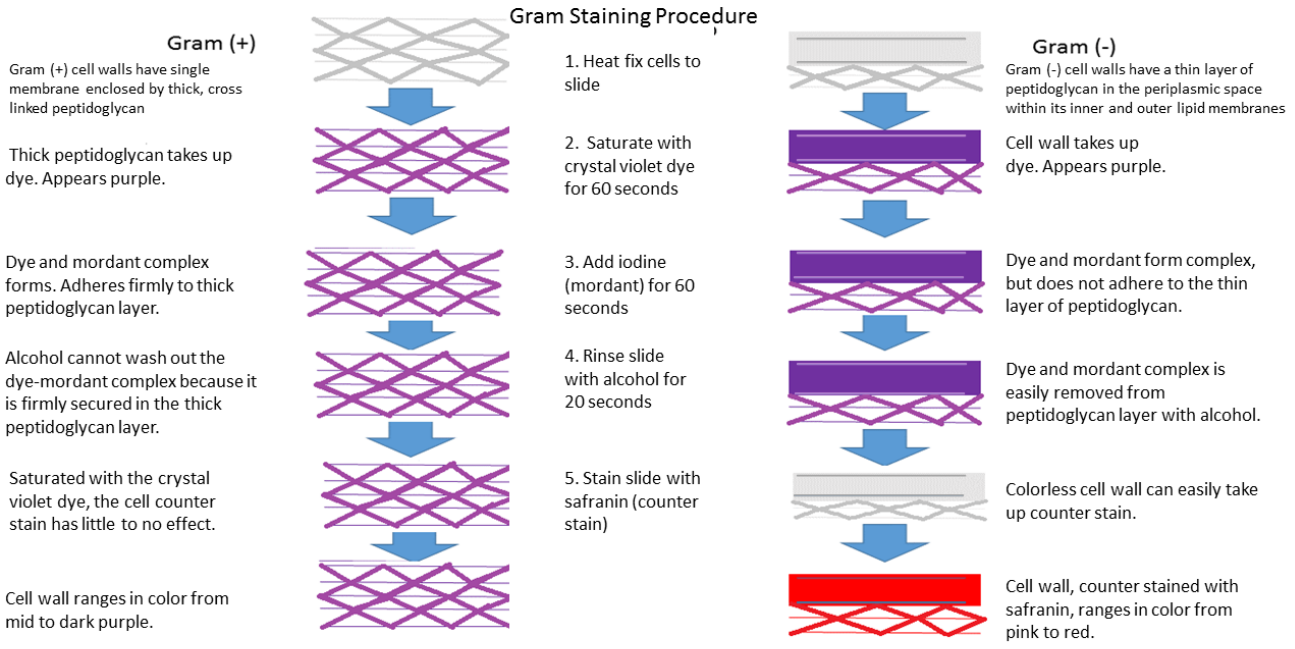
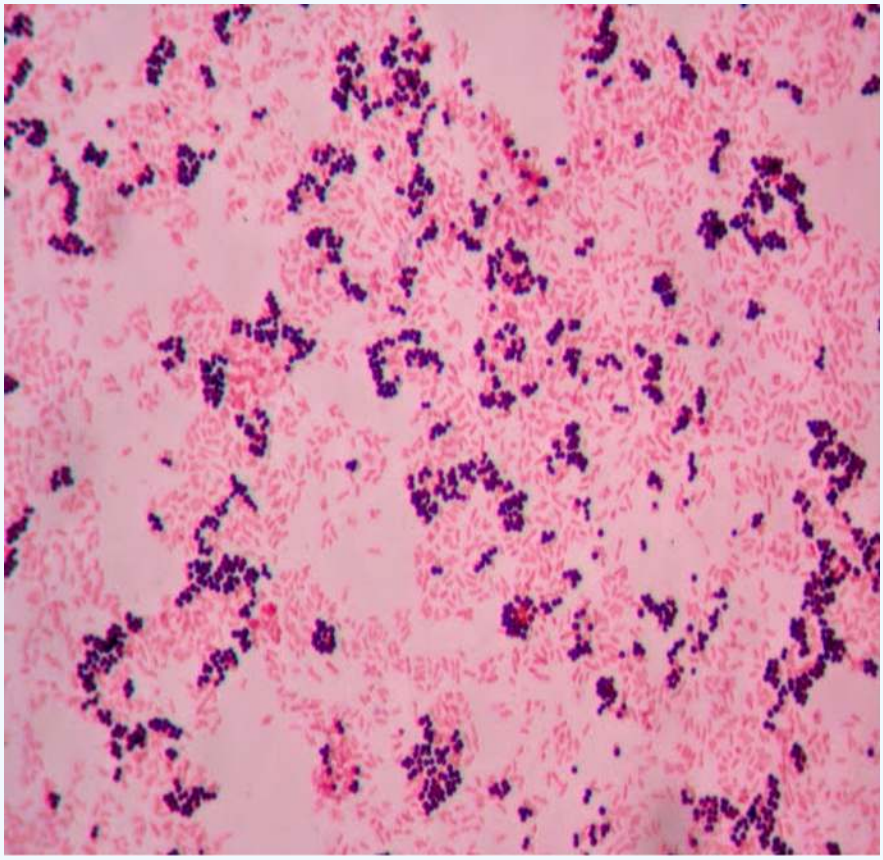


Figure 4: Gram-positive cell walls have a single membrane (the plasma membrane) enclosed by a thick, cross-linked peptidoglycan layer. Gram-negative cell walls have a thin layer of peptidoglycan between the plasma membrane and an outer membrane. When the Gram-positive cells and Gram-negative cells are 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.

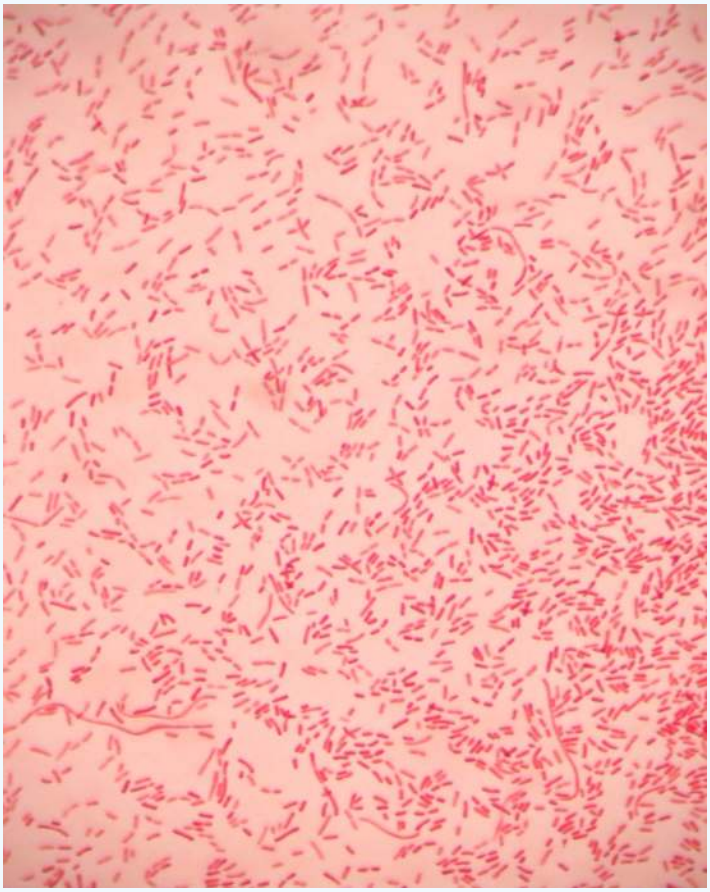
Exercise 10.4



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

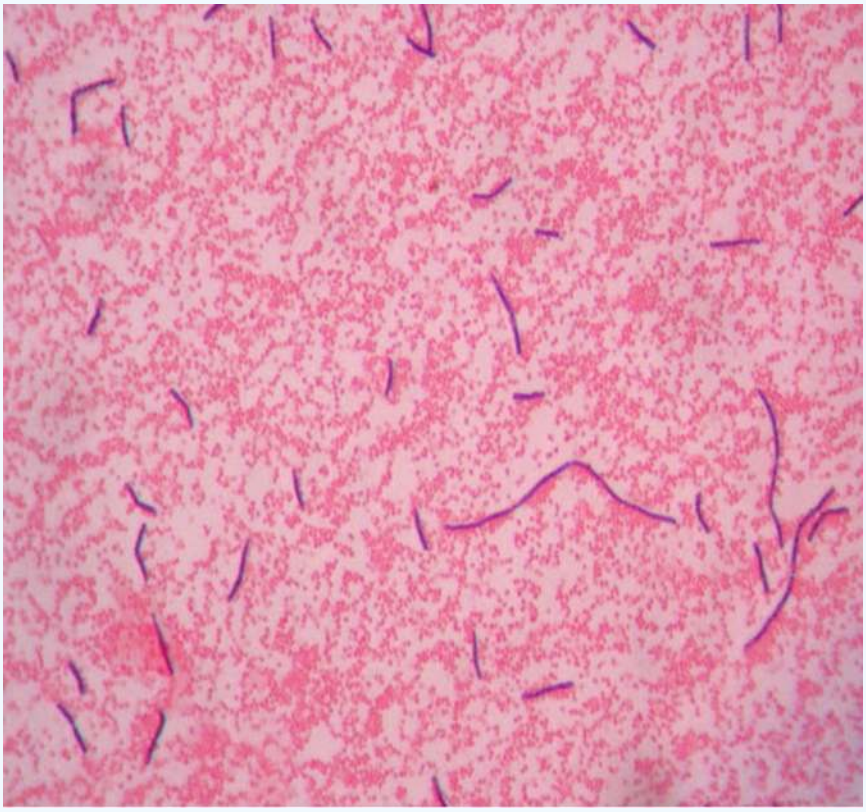


1. How many species can you see in this Gram stain?
2. What is the Gram (positive or negative) of the cells?
3. What is the cell shape of the cells (see Bacterial Cell Morphology & Arrangement for assistance)?

Answer

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? 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.

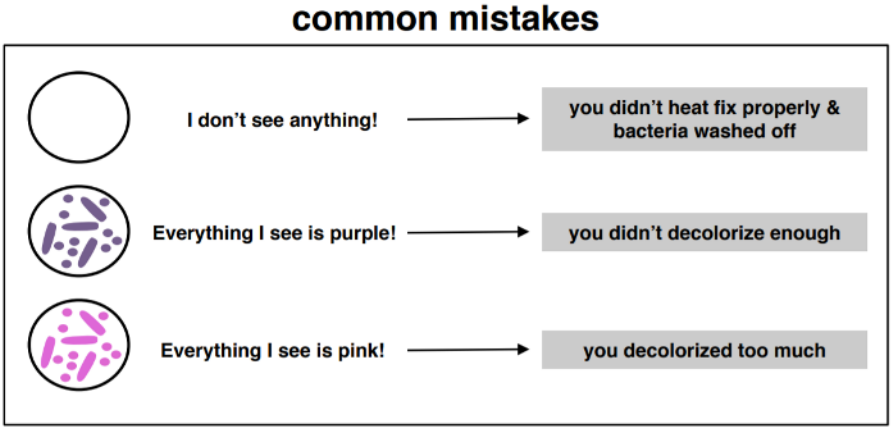


Figure 5: If you are staining a mixture of Gram-negative and Gram-positive bacteria and you do not see cells, see all cells purple, or see all cells pink, it is likely due to one of these common errors. (1) If you do not see anything (no cells are visible), it is likely because the smear was not properly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

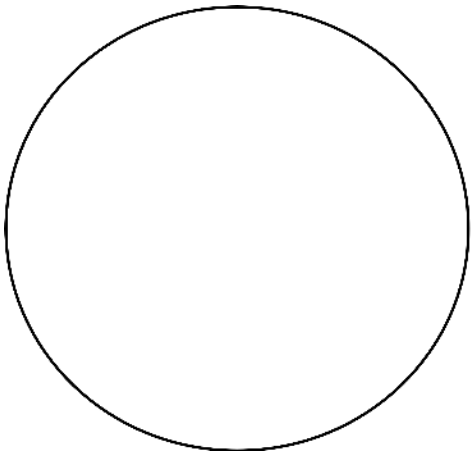
If you heat fix too little, the bacteria will wash off the slide. If you heat fix too much, you will cook the bacteria and denature them.

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.

Results & Questions



specimen: _____

total magnification: _____

1. Draw the cells you observe after the Gram stain in the space above.
2. Critique your cell drawings:
   - Does the drawing accurately show how the cells appear when you look into the microscope (including cell shape and arrangement)?
   - Does the drawing accurately show the cell size you see in the microscope?
   - Does the drawing accurately show the color(s) you see in the microscope?
3. After you examined your slide, did it show the Gram stain was successful? If you saw both Gram-positive and Gram-negative cells in the mixture of bacteria then your Gram stain was successful. If not, what did you see (or not see)?
4. If your answer to question 3. indicated that your Gram stain was not successful, what might have occurred? See the section Improving and Troubleshooting your Gram Stain Approach for assistance.
5. What was the cell shape of the Gram-positive cells you observed (see Bacterial Cell Morphology & Arrangement for assistance)?
6. What was the cell shape of the Gram-negative cells you observed (see Bacterial Cell Morphology & Arrangement for assistance)?
7. *Staphylococcus aureus* is the Gram-positive species you observed today. What does being "Gram-positive" mean about the cell wall structure of *S. aureus*?
8. *Escherichia coli* is the Gram-negative species you observed today. What does being "Gram-negative" mean about the cell wall structure of *E. coli*?
9. Some species of bacteria are Gram-variable. What does this mean?
10. What might occur if the heat fixation step is skipped?
11. What might occur if decolorization is too long?
12. What might occur if decolorization is too short?
13. What is the difference between a simple stain and a differential stain?
14. Is the Gram stain a simple stain or a differential stain?

Attributions

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1.11: 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.

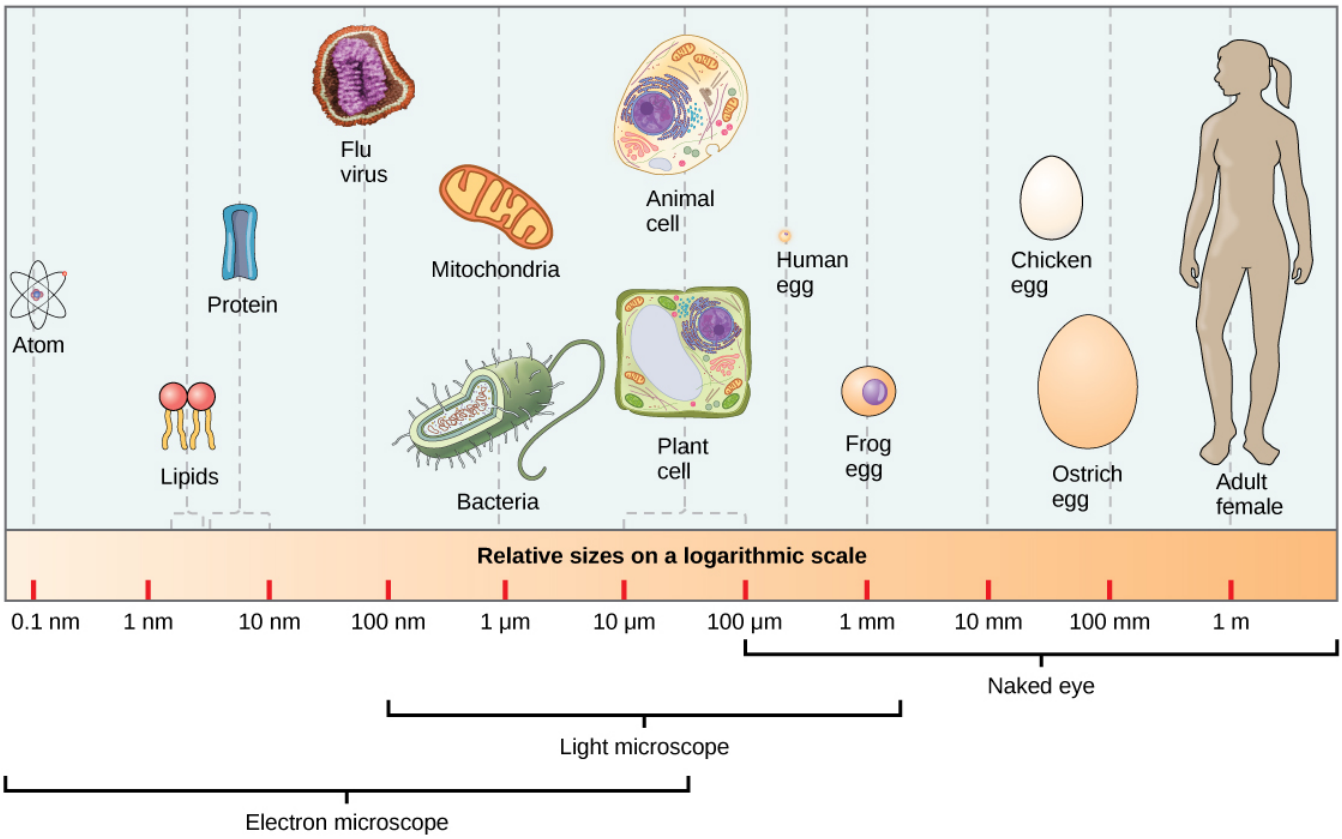


Figure 1: This figure shows relative sizes of microbes on a logarithmic scale (recall that each unit of increase in a logarithmic scale represents a 10-fold increase in the quantity being measured). The smallest cells in the world are prokaryotic cells (including bacteria) that tend to be between 1 µm 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**

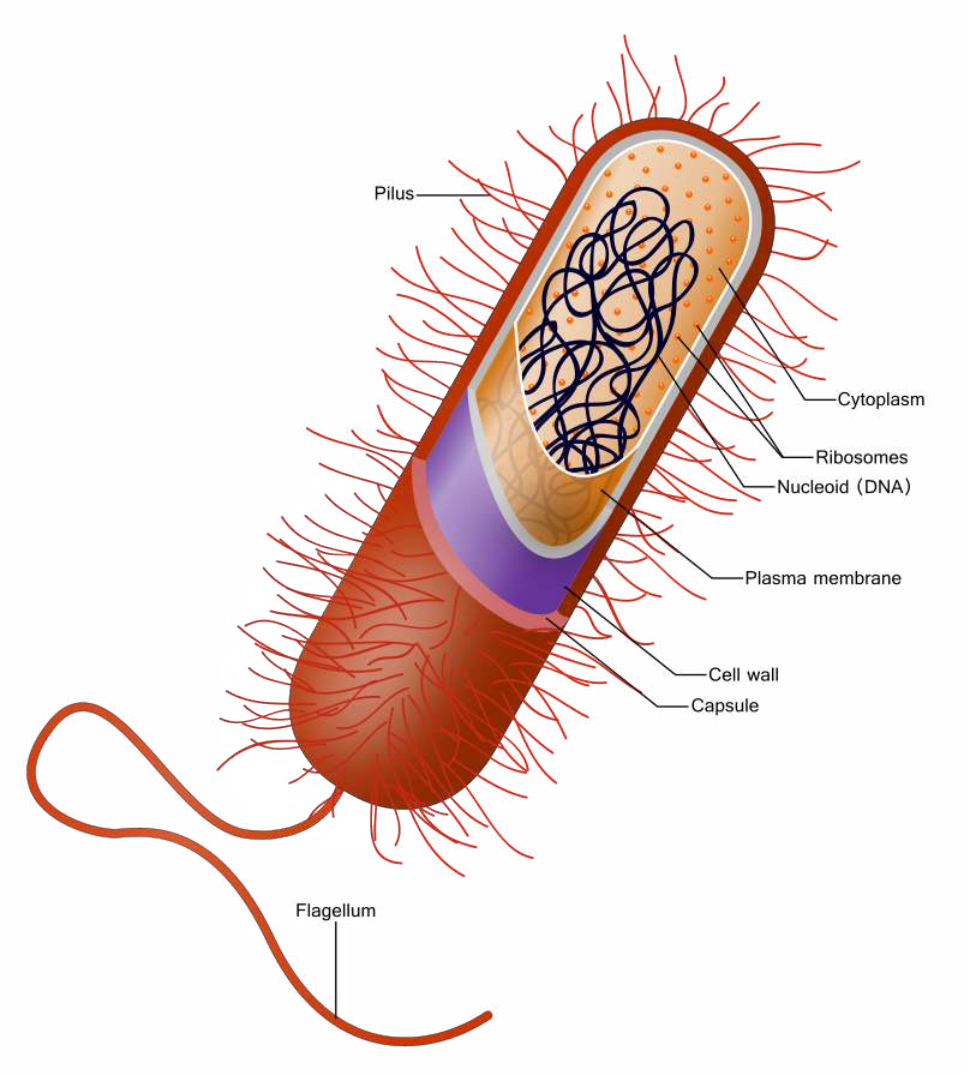


Figure 2: This figure shows the generalized structure of a prokaryotic cell. Prokaryotes have chromosomal DNA localized in a nucleoid, ribosomes, a cell membrane, and a cell wall. The other structures shown are present in some, but not all, bacteria (flagellum, pili, capsule).

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.

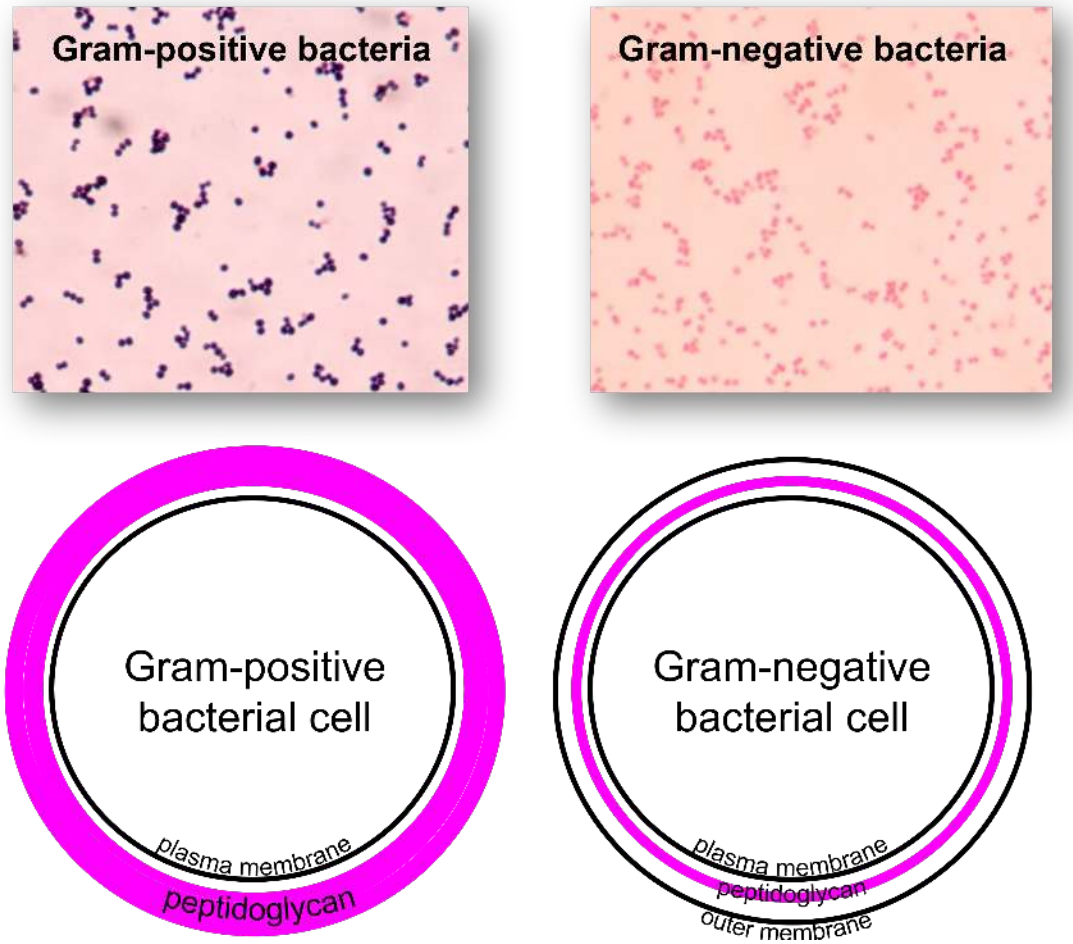


Figure 3: (Top) Microscopic images of bacteria stained with the Gram stain. (Top right) Gram-positive bacterial species stain purple with the Gram stain. (Top left) Gram-negative bacterial species stain pink with the Gram stain. (Bottom) Diagrams illustrating major structural components of Gram-positive 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.

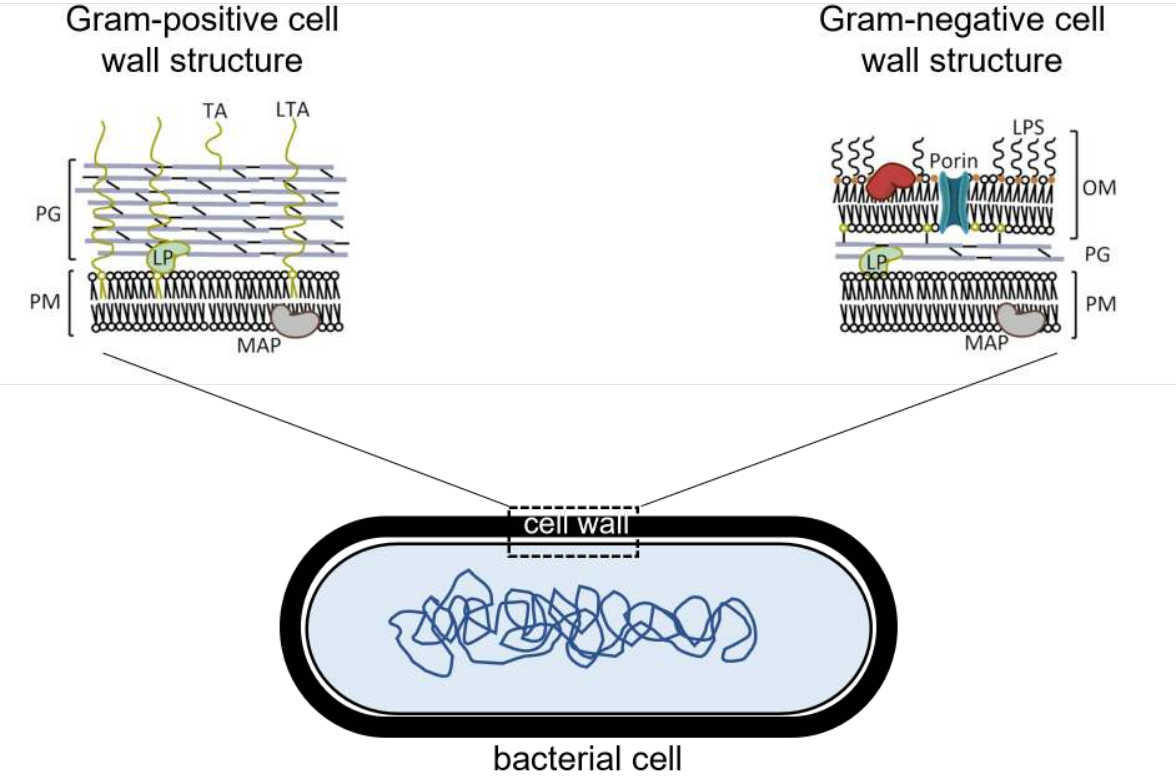


Figure 4: Detailed view of the structure of bacterial cell walls for Gram-positive species and for Gram-negative species. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across 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 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.

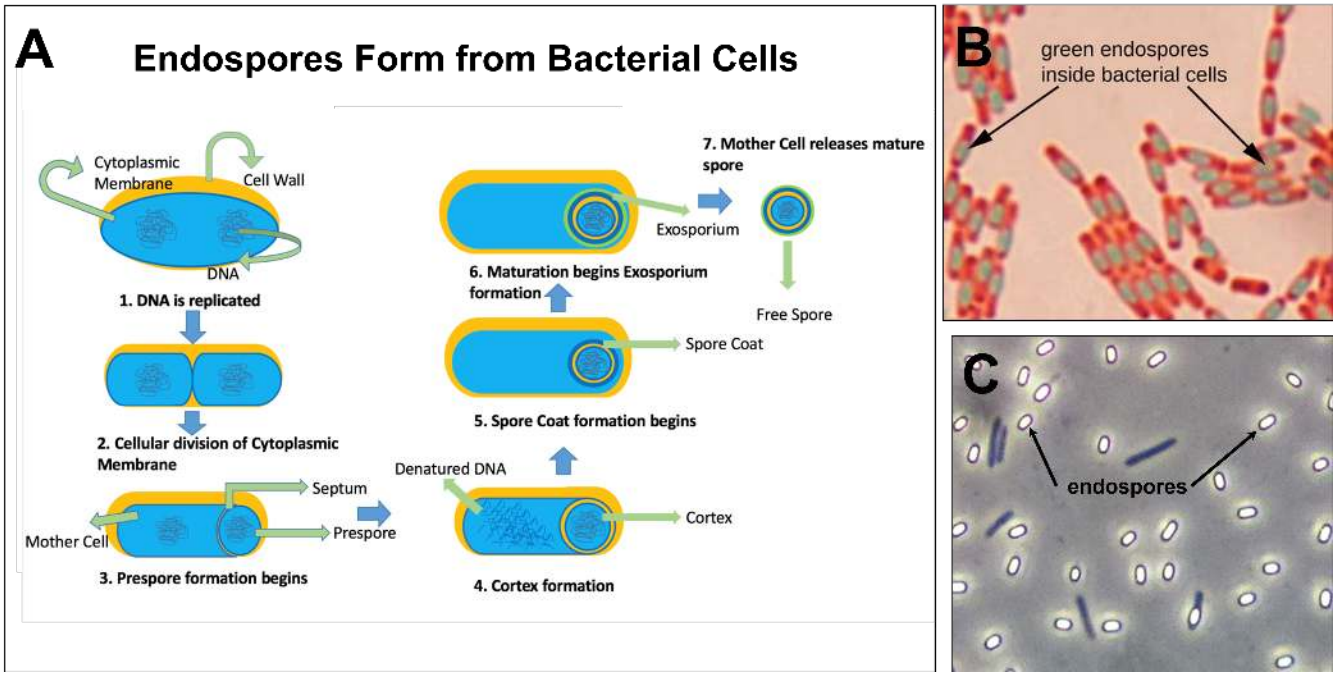


Figure 5: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore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

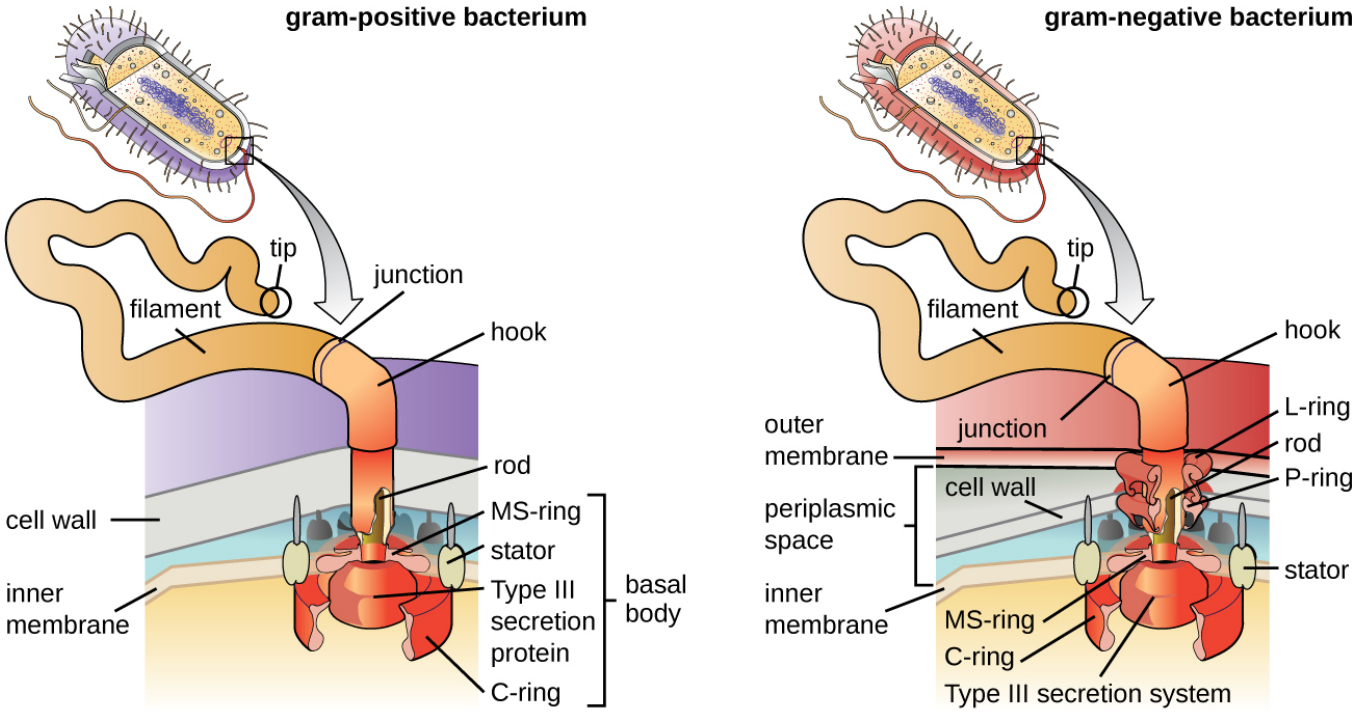


Figure 6: The basic structure of a bacterial flagellum consists of a basal body, hook, and filament. The basal body composition and arrangement differ between gram-positive and gram-negative bacteria. (credit: modification of work by "LadyofHats"/Mariana Ruiz Villareal)

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.

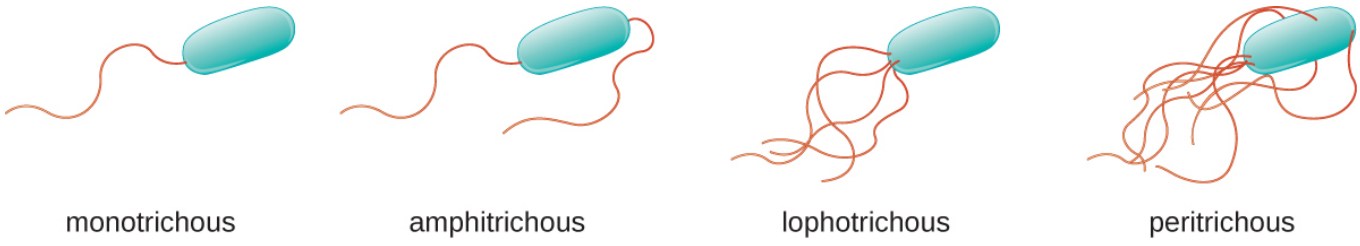
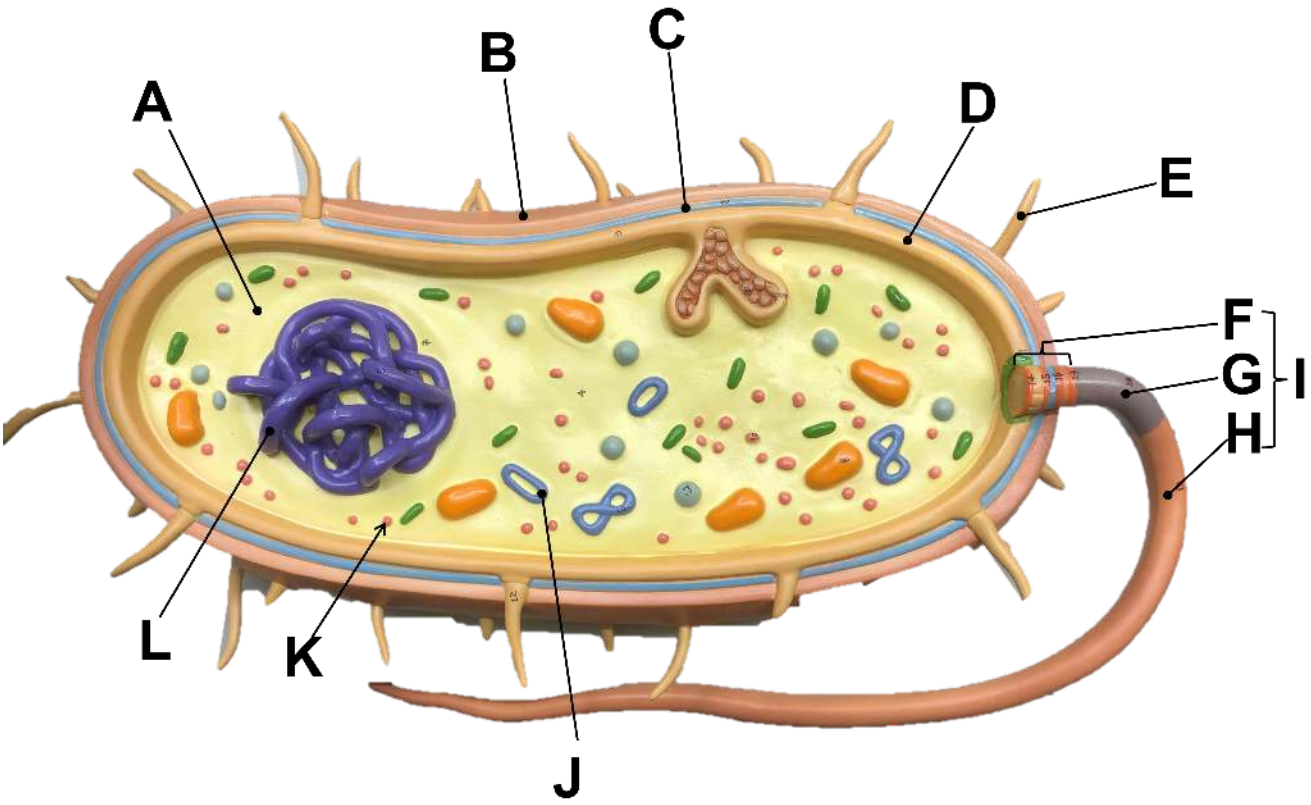
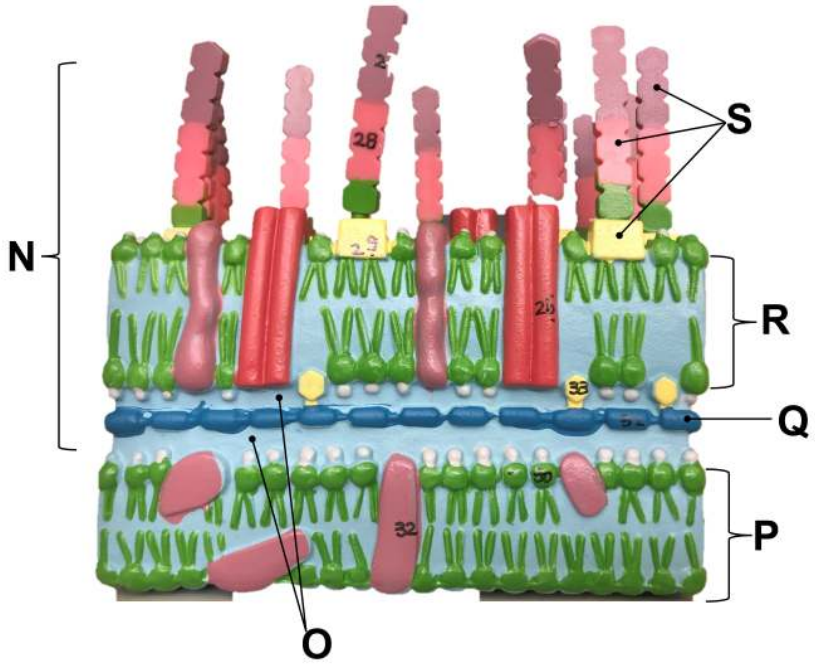


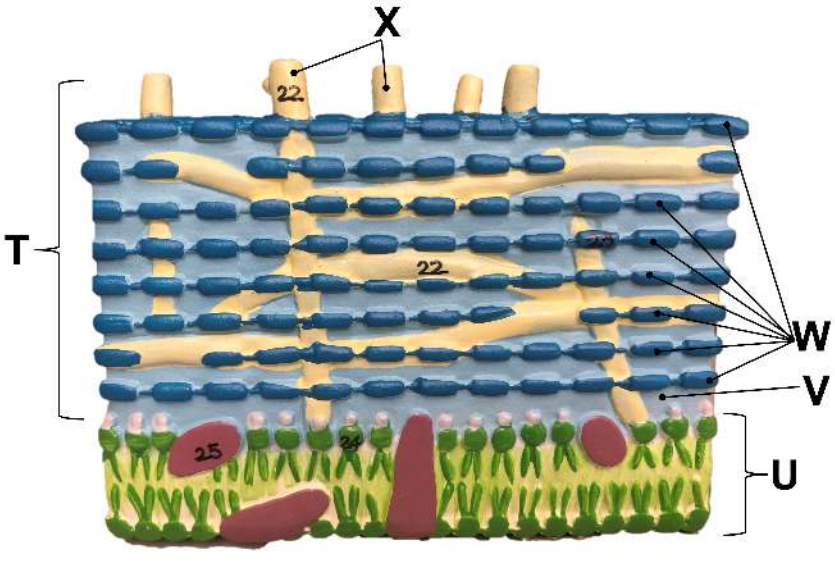
Figure 7: Flagellated bacteria may exhibit multiple arrangements of their flagella. Common arrangements include monotrichous, amphitrichous, lophotrichous, or peritrichous.

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	

Collaboratively Study Prokaryotic Cell Structures

1. Work with your group. You will point to each structure on the prokaryotic cell model to ask each member of your group its name. Wait for the person who you are asking to come up with the correct name of the structure on the model.
2. Your group members will conduct the same process in step 1 so you and the rest of the group get practice recalling the prokaryotic cell structures on the model.
3. Continue this practice ***until everyone in the group can make the cell structures without looking at the handout (i.e. from memory)***.
4. You will be quizzed on the prokaryotic cell model by your instructor. They will initial the checkpoint below when you and your group successfully identify structures on the prokaryotic cell model from memory.

Checkpoint: _____

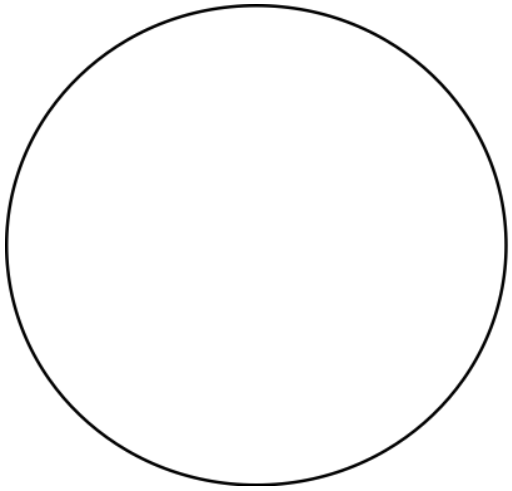
If you are waiting for assistance from your instructor, move on to the next section(s) until they are available.

Prokaryotic Cell Shapes

1. Use a microscope to examine the three different bacterial types on the Bacteria: Three Types slide (there are three different sections to the slide – move the slide around to see all three).

2. Make detailed and clear illustrations of all three types of bacteria in the spaces provided below clearly showing the cell shapes/arrangements.
3. In the spaces provided, indicate the appropriate term or terms that describe the cell shapes (and arrangements if appropriate).

Bacterial Type #1

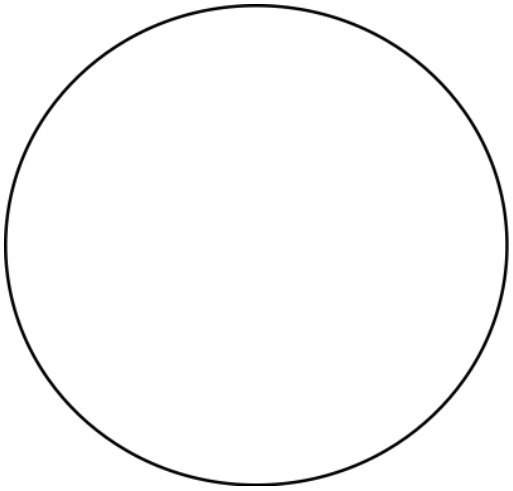


specimen: _____

total magnification: _____

Bacterial shape/arrangement term(s) appropriate for this bacterial species: _____

Bacterial Type #2

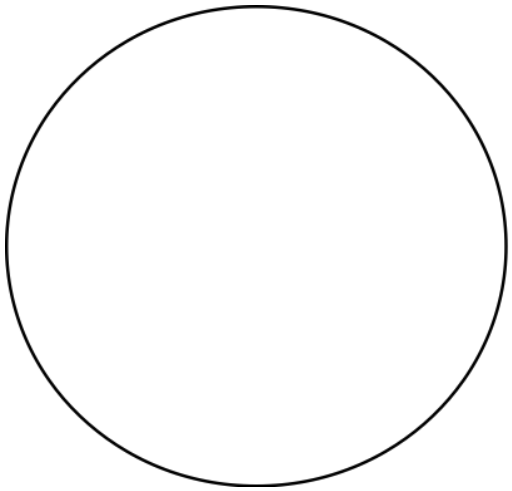


specimen: _____

total magnification: _____

Bacterial shape/arrangement term(s) appropriate for this bacterial species: _____

Bacterial Type #3



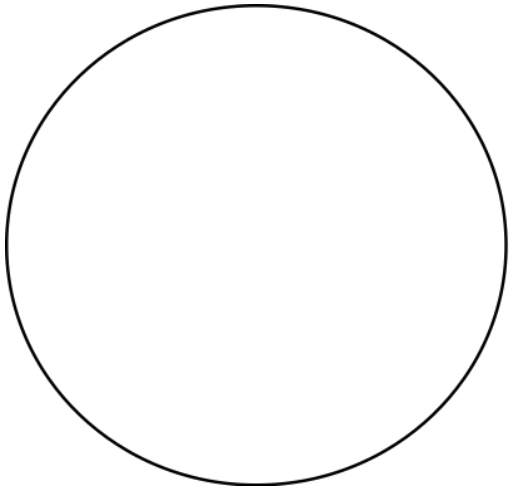
specimen: _____

total magnification: _____

Bacterial shape/arrangement term(s) appropriate for this bacterial species: _____

Endospores

1. Examine a slide that shows bacterial endospores.
2. Make a clear illustration of the sample.
3. In the illustration, label an endospore that is forming inside of a vegetative cell as “endospore forming inside vegetative cell”
4. In the illustration, label a free spore as “endospore.”



specimen: _____

total magnification: _____

Flagellar Arrangements

Some bacterial species do not have any flagella. Those bacterial species that do have flagella exhibit flagella in different arrangements. Make illustrations in each box below to show what the terms describing flagellar arrangements look like. If you are uncertain, use the lab manual for help.

monotrichous	**amphitrichous**
lophotrichous (draw two different types of arrangements)	**peritrichous**

Attributions

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1.12: 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.

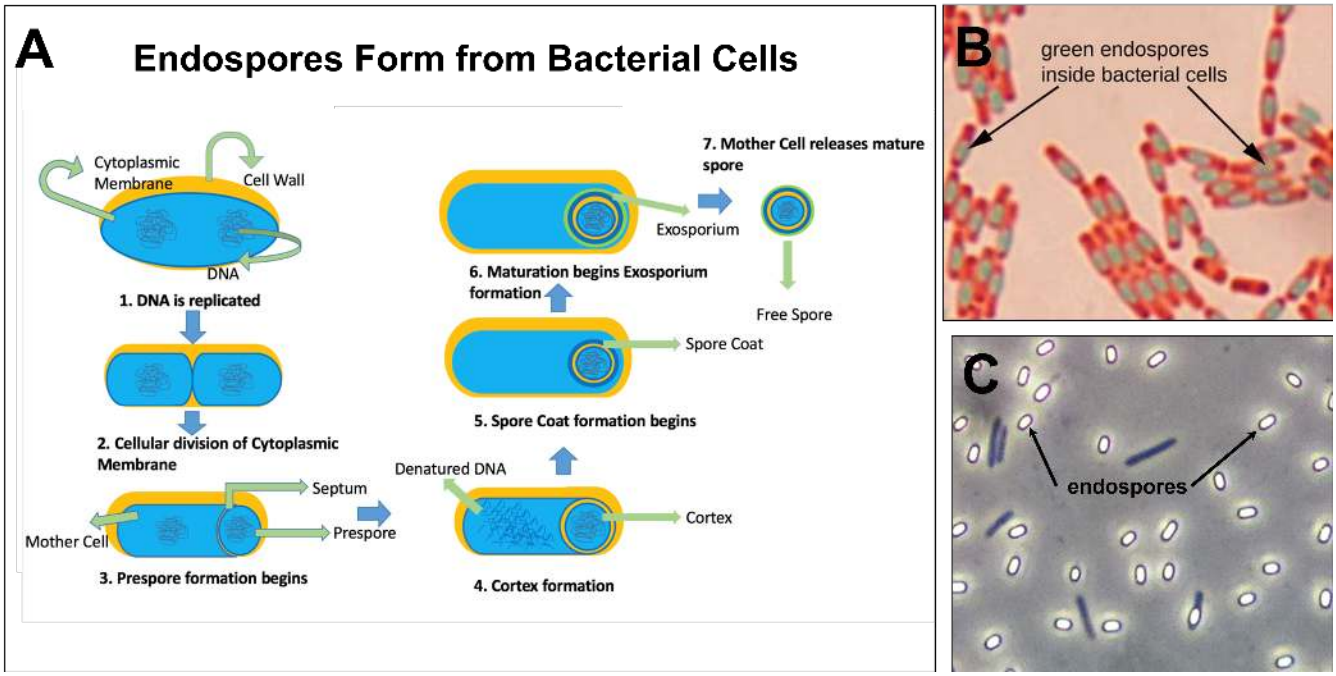


Figure 1: (A) Endospores form from bacterial cells. Diagram shows the process how bacterial cells experiencing poor growth conditions can form endospores in the following steps: 1. DNA is replicated, 2. cellular division of cytoplasmic membrane, 3. prespore formation begins, 4. cortex forms, 5. spore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

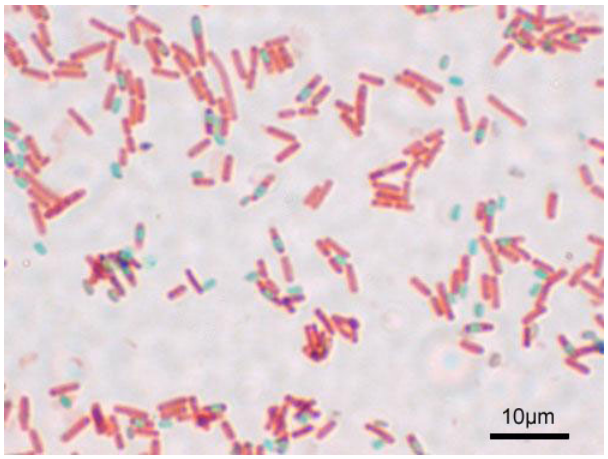


Figure 2: Bacterial cells that have undergone the endospore stain. This species, *Bacillus subtilis*, forms endospores that are visible in green. The vegetative cells are also present and appear pink. This image shows cells magnified by a microscope at 1000x.

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 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.

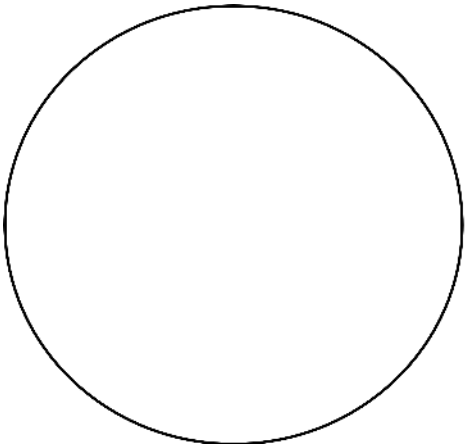
1. Pass the underside of the microscope slide through a flame three times.
2. Allow the slide to cool and then continue with your staining protocol.

> **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.

Results & Questions



specimen: _____

total magnification: _____

1. Draw the cells you observe after the endospore in the space above.
2. Critique your cell drawings:
   - Does the drawing accurately show how the cells appear when you look into the microscope (including cell shape and arrangement)?

- Does the drawing accurately show the cell size you see in the microscope?
- Does the drawing accurately show the color(s) you see in the microscope?

3. 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?
4. If you observed endospores in your stain, were these endospores terminal, subterminal, or central in the vegetative cells? Explain your answer.
5. What color are the endospores/spores in the endospore stain?
6. What color are the vegetative cells in the endospore stain?
7. Fill in the blanks: The _____ are the metabolically active cells that stain _____ colored in the endospore stain. The _____ are mainly metabolically inactive cells that stain _____ colored in the endospore stain.
8. Why might a species of bacteria form endospores? What is the advantage of forming spores for bacteria?
9. What genera of bacteria are known to have pathogenic (disease-causing) species and are endospore-forming genera?
10. 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?
11. What is the purpose of the steam in the endospore stain?
12. Why does there not have to be a decolorizer in this stain?

Attributions

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1.13: 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

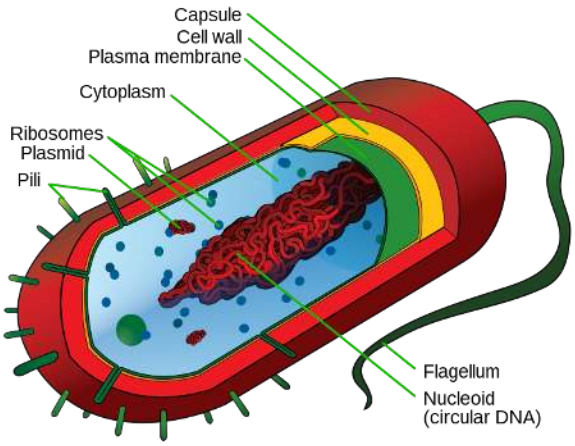


Figure 1: Diagram of a prokaryotic cell shows that capsules are a layer located outside of the cell wall.

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.

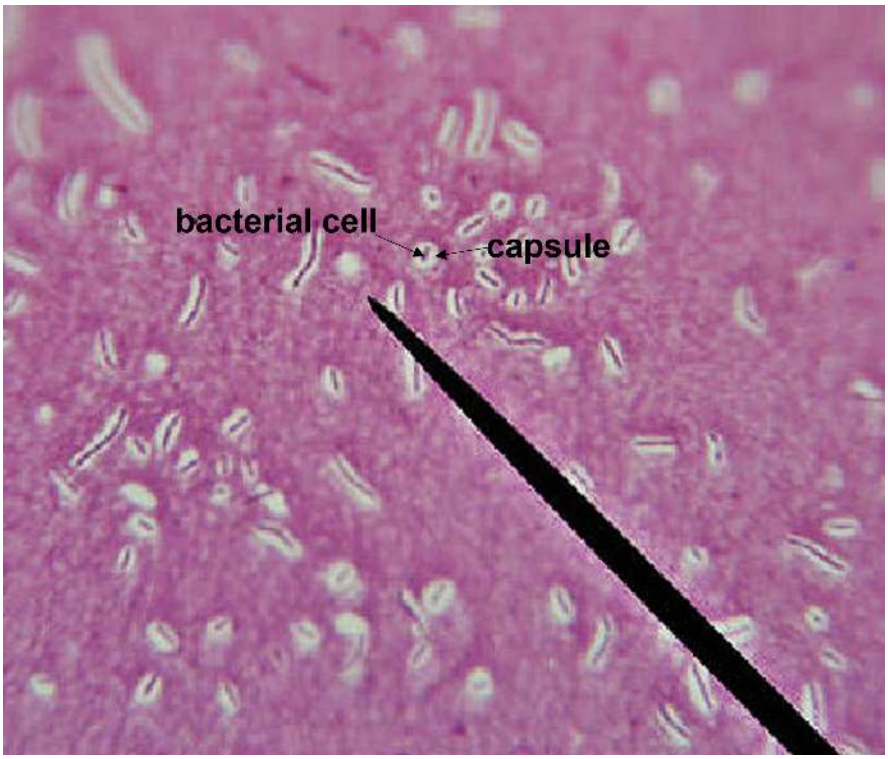


Figure 2: Microscopic image of bacteria stained with the capsule stain. The environment surrounding bacterial cells and the bacterial cells are is stained pink/purple. The unstained region that appears white surrounding each bacterial cell is the capsule.

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.

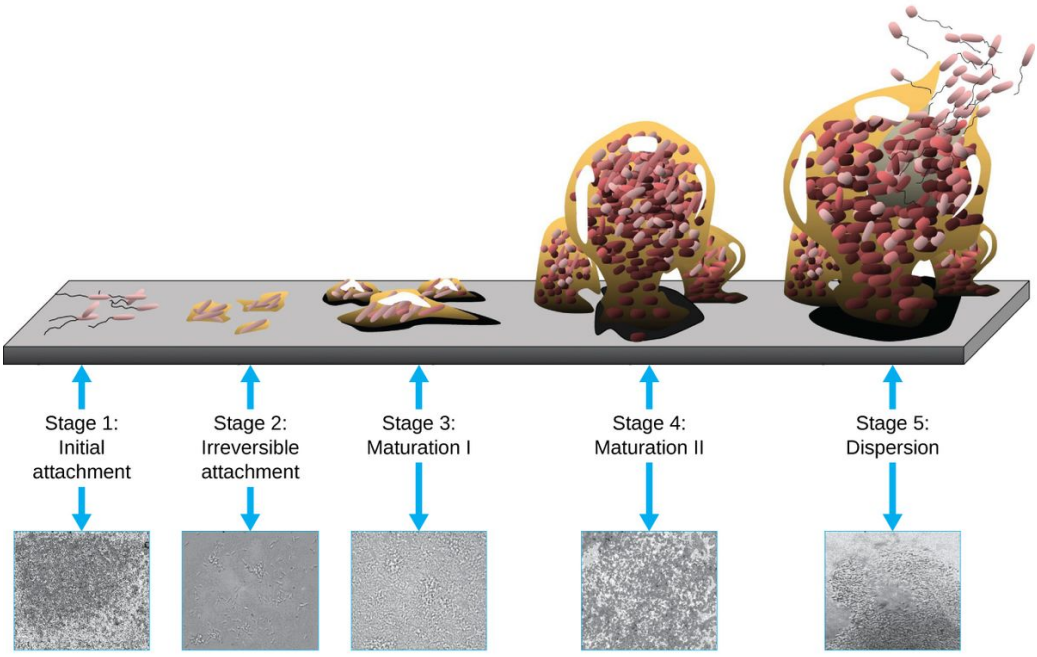


Diagram showing five stages of biofilm development of *Pseudomonas aeruginosa*.
All photomicrographs are shown to same scale.

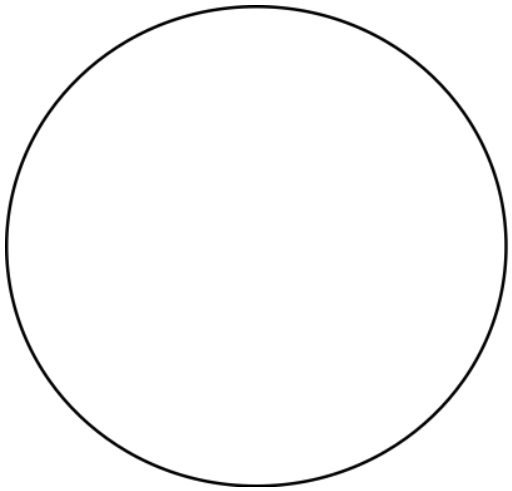
Figure 3: Process of biofilm development. Stage 1: initial attachment of bacteria to a surface. Stage 2: irreversible attachment. Stage 3: maturation I. Stage 4: maturation II. Stage 5: dispersion of cells out of the biofilm.

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.

Results & Questions



specimen: _____

total magnification: _____

1. Illustrate the bacterial cells viewed in the microscope. Label a bacterial cell and a capsule in your illustration.
2. Where are bacterial capsules located in a bacterial cell?
3. What type of molecule are bacterial capsules typically composed of?
4. Why do bacterial capsules appear white/clear after staining?
5. Give three different ways bacterial capsules give species/strains that have them an advantage.
6. Do all bacteria have/need a capsule? Explain your answer.

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1.14: 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.

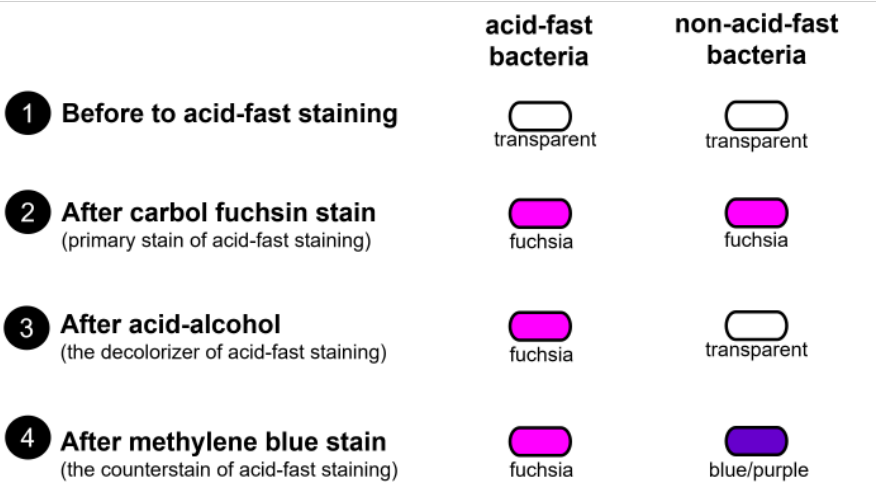


Figure 1: Diagram shows how cells change color during the steps of the acid-fast stain by comparing acid-fast bacteria and non-acid-fast bacteria. Acid-fast bacteria and non-acid-fast bacteria begin transparent. After staining with carbol fuchsin, acid-fast and non-acid-fast bacteria both appear the 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 looses 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.

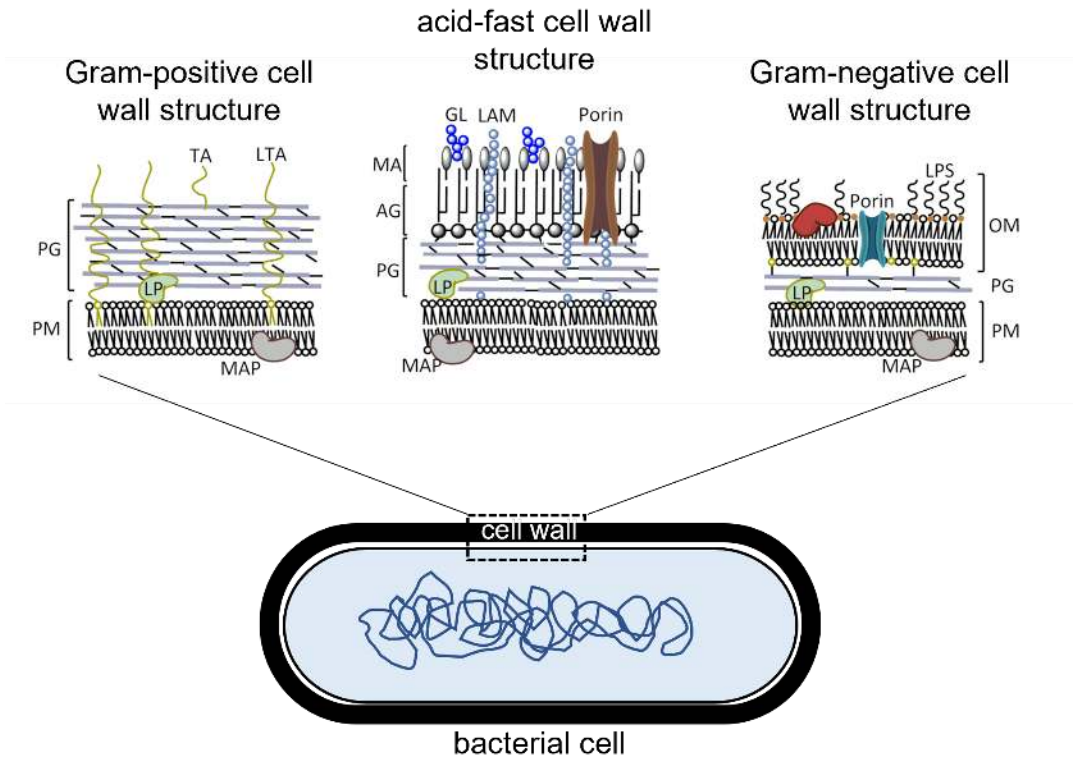


Figure 2: Comparison of the structures of different types of bacterial cell walls. (Left) Gram-positive cell walls consist of a thick layer of peptidoglycan (PG) outside of the cell's plasma membrane (PM) with teichoic acid (TA) and lipoteichoic acid embedded across the peptidoglycan layer. (Right) Gram-negative 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 arabinogalactin (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.

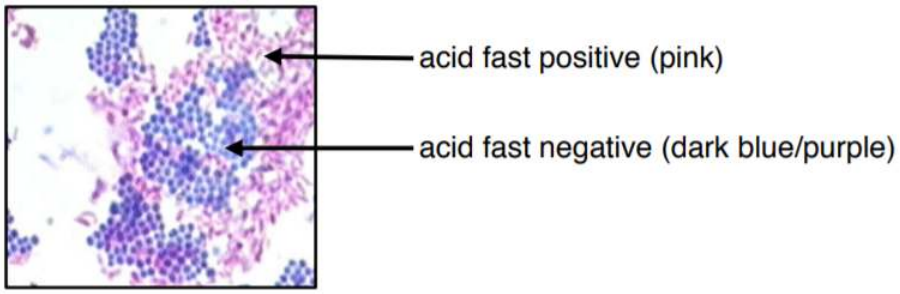


Figure 3: Microscopic image of two different species mixed together with acid fast stain. The acid-fast bacteria appears fuchsia or pinky-purple. The non-acid-fast bacteria appears dark blue or blue-purple. The colors in this image may be slightly off due to printing/copying.

Results & Analysis

1. Take a photo of the cells or make an illustration of them.
2. Was your acid-fast stain successful? How do you know?
3. What color are the acid-fast bacteria?
4. What color are non-acid-fast negative bacteria?
5. Describe the structure of the cell walls of bacteria that appear fuchsia with the acid-fast stain.
6. Describe what you know about the structure of the cell walls of bacteria that appear blue/purple with the acid-fast stain.
7. What is the medical importance of using the acid-fast stain (what diseases/infections can it help to identify and how might it relate to virulence and antibiotic resistance)?

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.

Attributions

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1.15: 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 is 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.
- Successful 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).

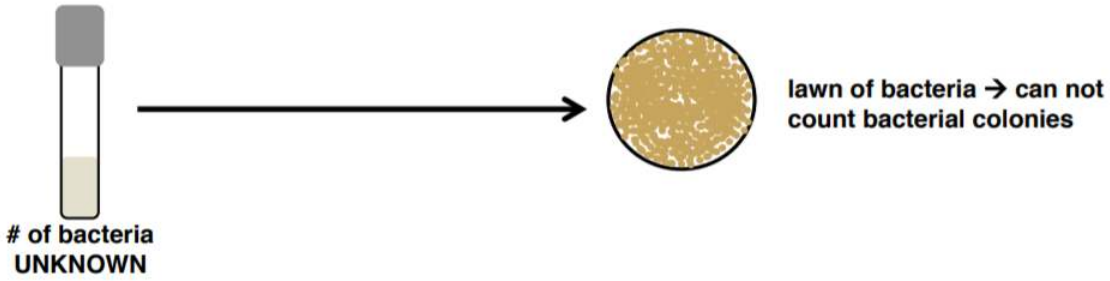


Figure 2: A bacterial culture can contain millions or billions of cells per milliliter! If even a small amount of that culture is plated directly onto a petri plate undiluted, it will form a "lawn" of bacteria where the colonies cannot be distinguished from each other since there are just so many of 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).

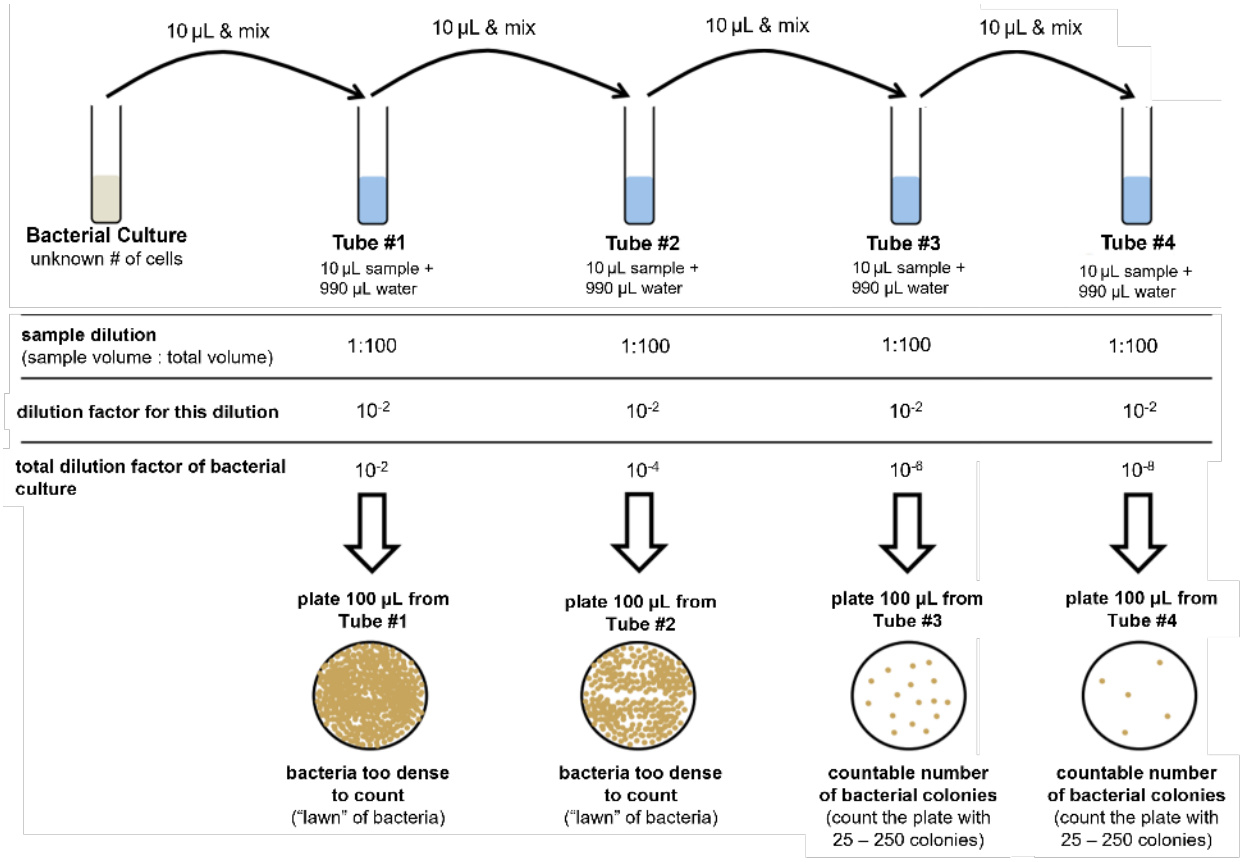


Figure 3: (Top) The process of diluting a bacterial culture in a dilution series is shown. In this process, 10 µL of the culture is diluted in 990 µL of water and mixed. This is a 1:100 dilution, or 10^{-2} dilution. A sample from this first dilution is plated onto a petri plate. The first dilution is 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:

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = 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:

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = 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:

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = CFU in diluted sample (cells/mL)

(129 colonies) ÷ (0.2 mL diluted sample added to petri plate) = 645 cells/mL in the diluted sample

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

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = CFU in original sample (cells/mL)

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

CFU = 645,000,000 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.

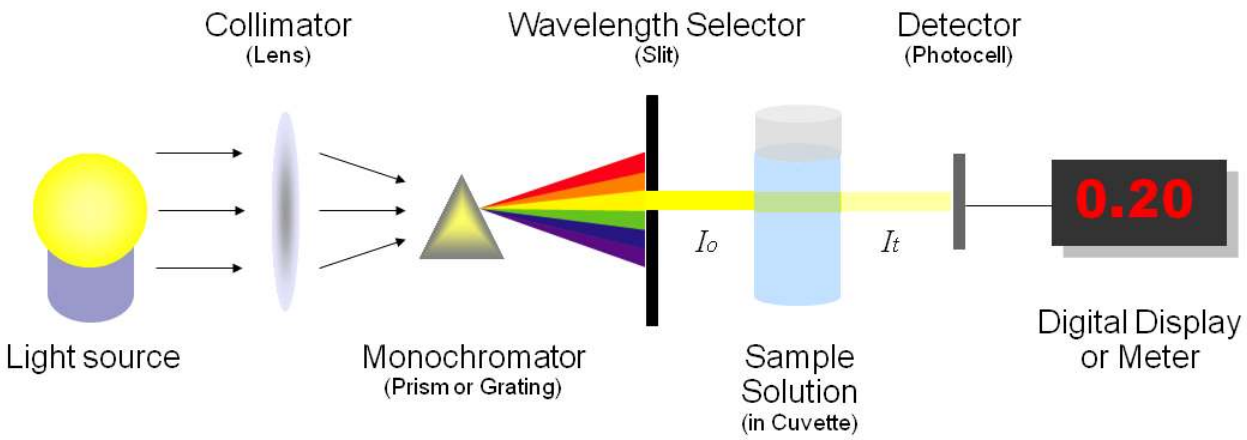


Figure 4: The diagram shows the components of a spectrophotometer. (Left) Light is emitted from a light source. (Second from the left) The wavelength of light is narrowed to a specific wavelength of light (accomplished by the monochromator) that is selected by the person using the spectrophotometer. (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 might 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.

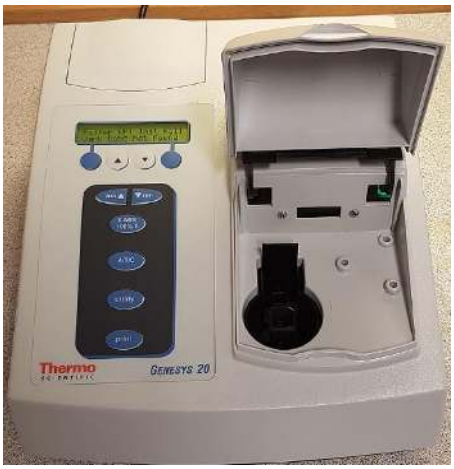


Figure 5: This image shows one type of spectrophotometer. The dark circle toward the right is where a sample is placed inside of the spectrophotometer. The lid is closed to allow light to pass through the sample and collect an absorbance or transmittance reading. The person using the spectrophotometer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

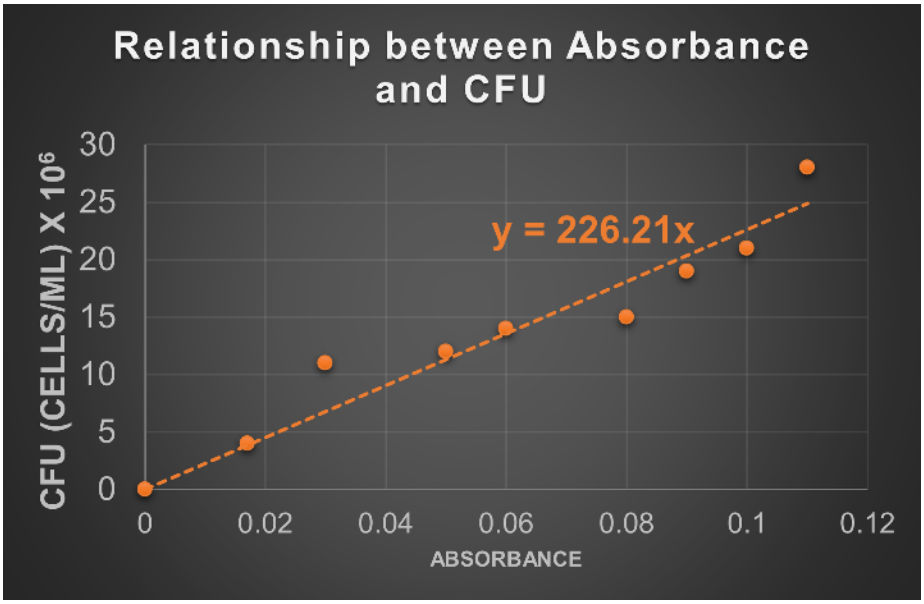


Figure 6: Absorbance data for a sample that has undergone serial dilutions is plotted against the CFU (based on standard plate count data) calculated at those dilutions. The result is a series of data points that can be used to plot a trend line. That trend line may be used to determine the CFU of samples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

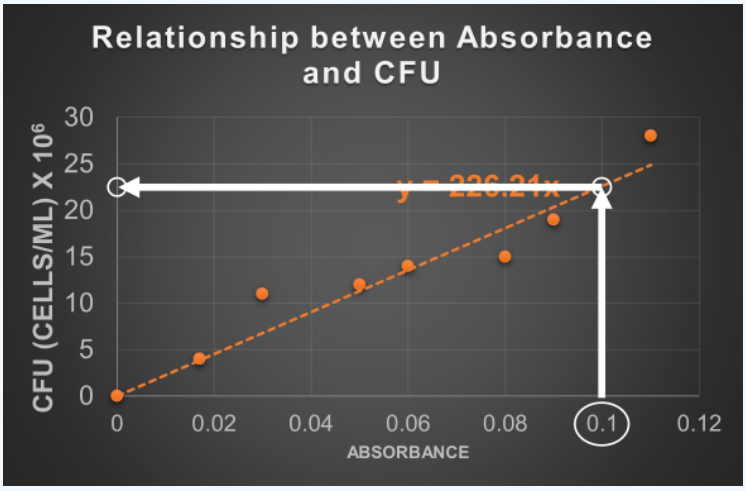
Example of Using Position of Standard Line to Determine CFU of a Sample

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 x 10^6 cells/mL **or** 2.3 x 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 x 10^6 cells/mL was determined as the CFU at this absorbance. We may adjust how we present the number to 2.3 x 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-acis, 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 x 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) x (0.2)
- **Calculate y:** 45.242
- **Recall that the graph axis indicates y is x 10^6, so the final answer is:** 45.242 x 10^6 cells/mL **or** 4.5 x 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

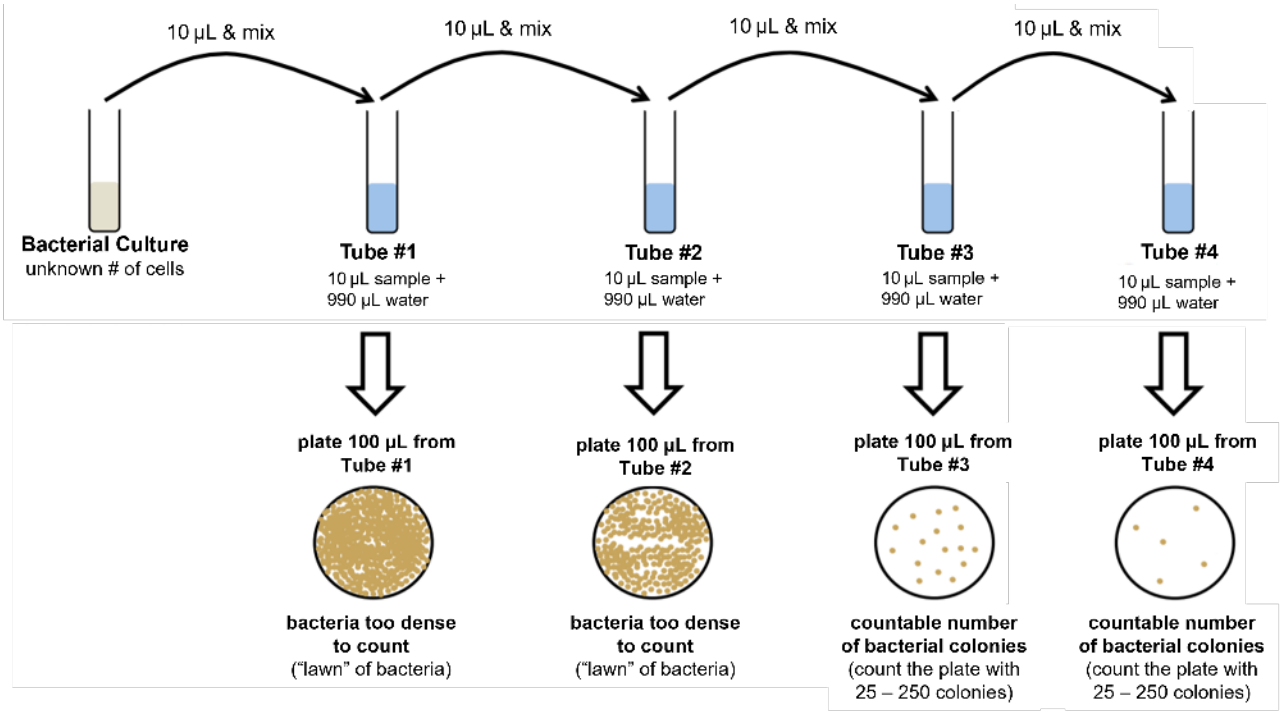


Figure 7: Summary of the serial dilution/dilution series process (top row) and how those dilutions are used to produce multiple petri plates for the standard plate count (center and bottom rows).

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 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.

Results & Questions

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	
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

Culture Dilution	Absorbance (600 nm)
1 (undiluted)	
1/2	
1/4	
1/8	
1/16	

1. Record the absorbance readings in the table above.
2. Examine any trends in the table able. 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

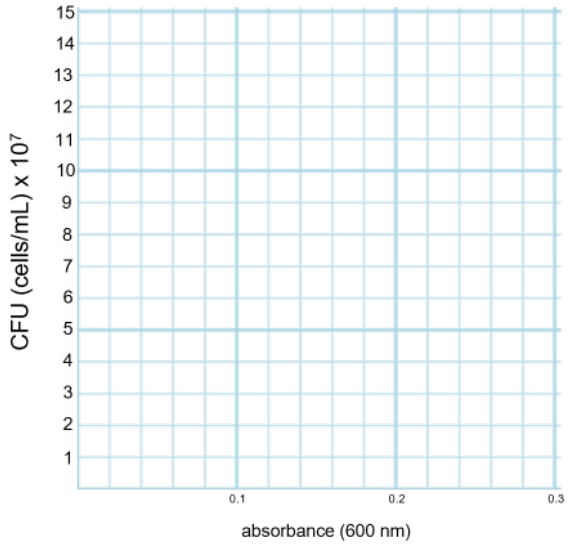
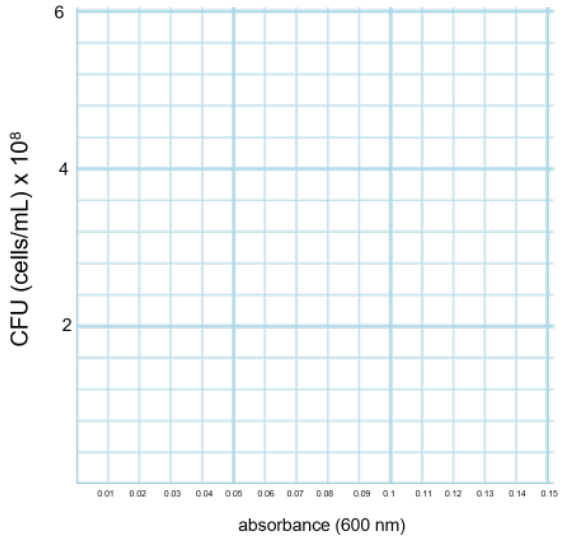
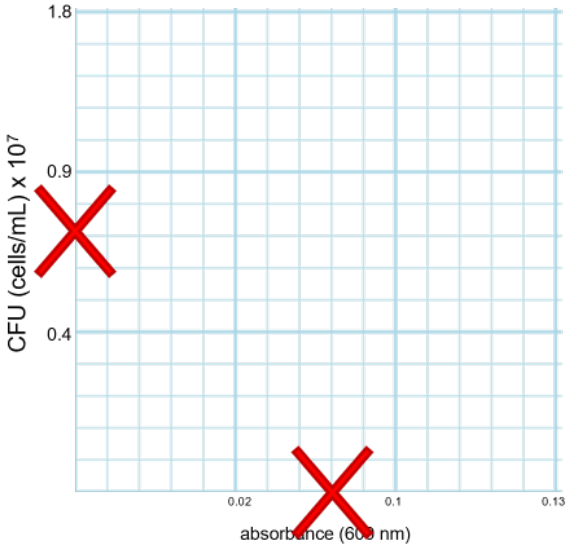
Culture Dilution	Absorbance (600 nm)	CFU (cells/mL)
1 (no dilution)		
1/2		
1/4		
1/8		
1/16		

1. Complete the table above:
    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: (CFU) x (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: (CFU) x (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: (CFU) x (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: (CFU) x (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 x 10^6, x 10^7, or x 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!

1. 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)	Do not Graph This Way! (Boxes on the Axis Do Not have a Defined Value)
Example 1:  Example 2:  	Example of what NOT to do: 

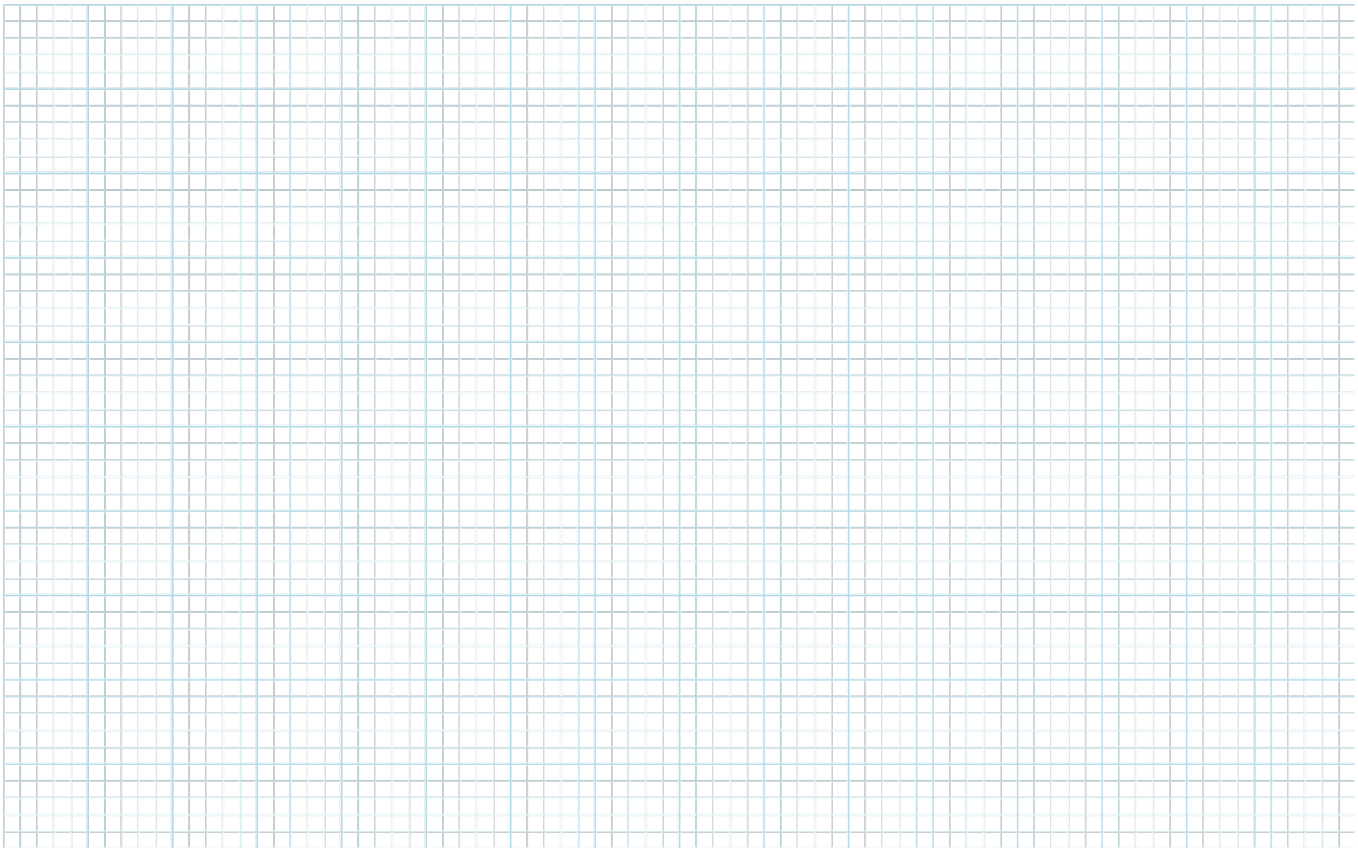
3. If you used graphing software add a trend line to your data and set the y-intercept at zero. Also make sure to have the software place the equation for the line on the graph. If you are using graph paper, use a ruler and it place along the data points making sure the ruler passes through the bottom 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 x 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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1.16: 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.

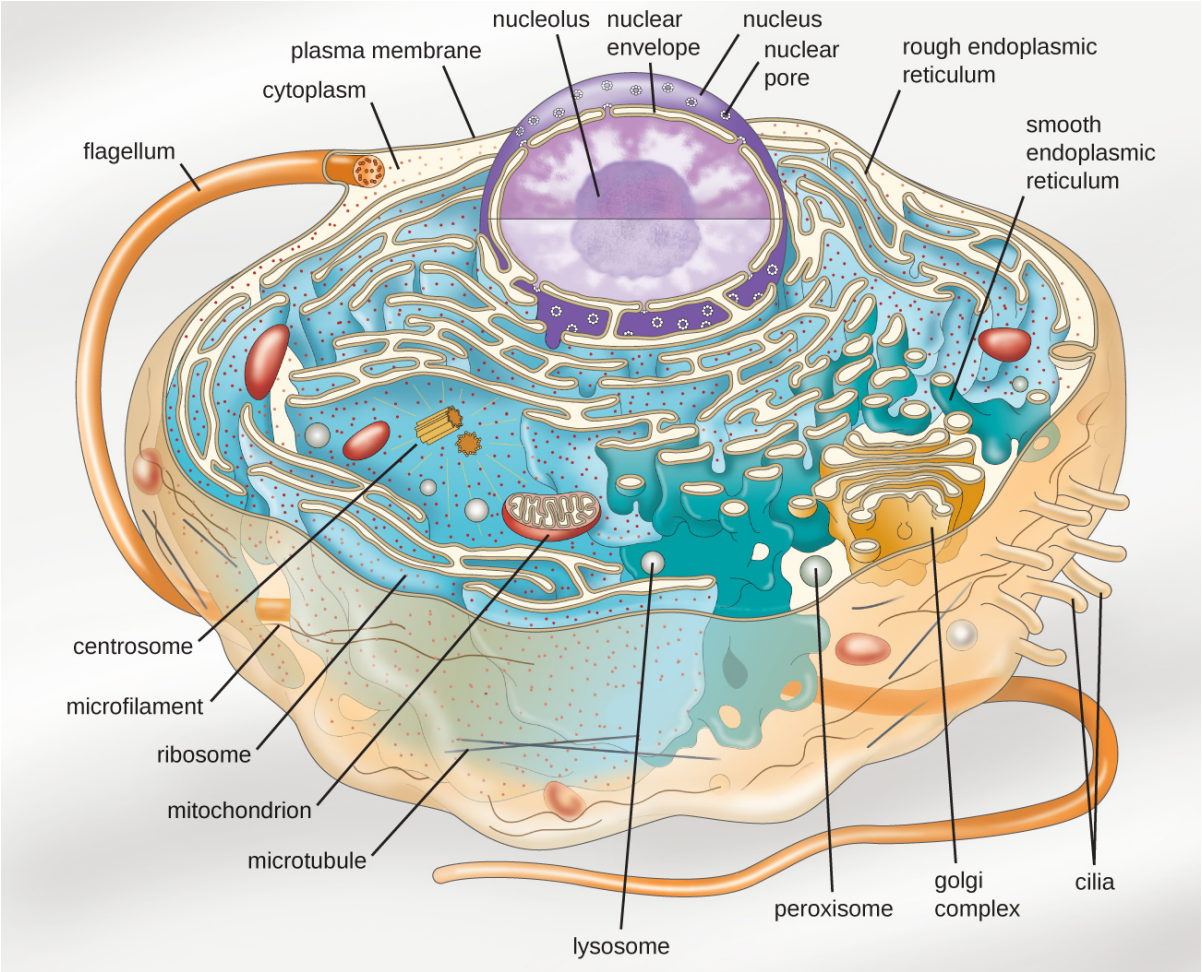


Figure 1: An illustration of a generalized, single-celled eukaryotic organism. Note that cells of eukaryotic organisms vary greatly in terms of structure and function, and a particular cell may not have all of the structures shown here.

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

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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

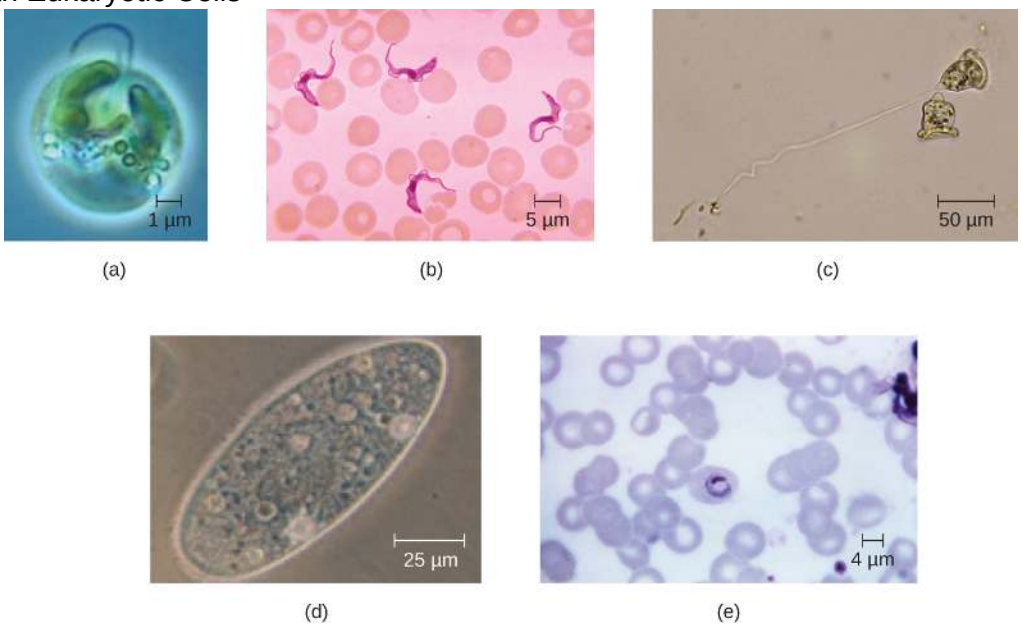


Figure 2: Eukaryotic cells come in a variety of cell shapes. (a) Spheroid *Chromulina* (a species of algae). (b) Fusiform shaped *Trypanosoma* (a parasitic protozoan shown with red blood cells of its host). (c) Bell-shaped *Vorticella* (a free-living protozoan). (d) Ovoid *Paramecium* (a free-living 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**.

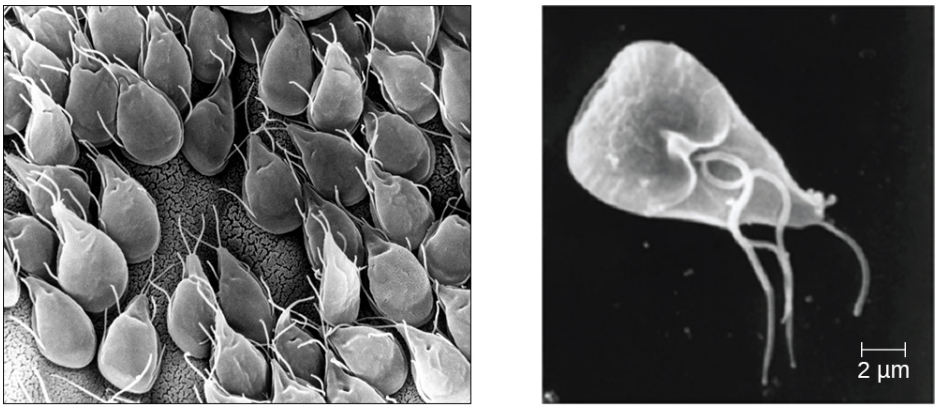


Figure 3: (a) A scanning electron micrograph shows many *Giardia* parasites in the trophozoite, or feeding stage, in a gerbil intestine. (b) An individual trophozoite of *G. lamblia*, visualized here in a scanning electron micrograph. This waterborne protozoan causes severe diarrhea when ingested. (credit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

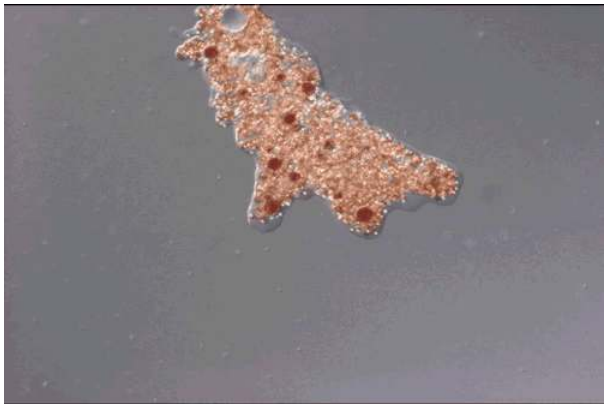


Figure 4: Amoeba motion involves formation of pseudopodia ("false feet"). These extensions enable the cell to creep along in a liquid environmental and also means that the shape of the cell is constantly changing.

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 kinetoplastids (modified mitochondria) or hydrogenosomes.

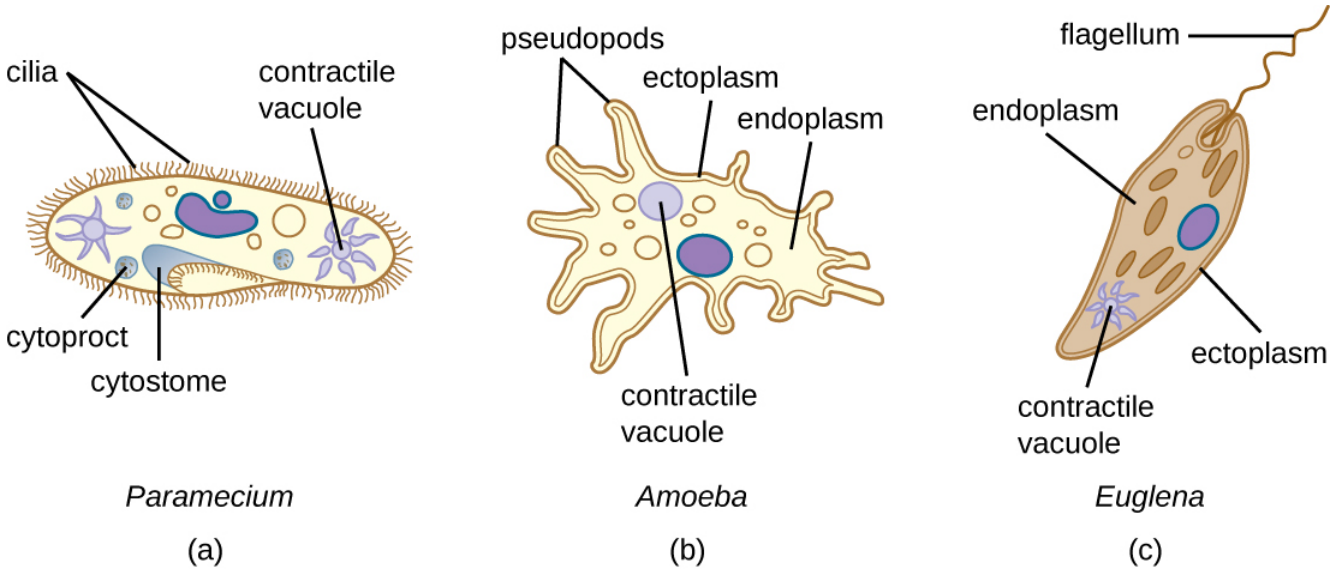
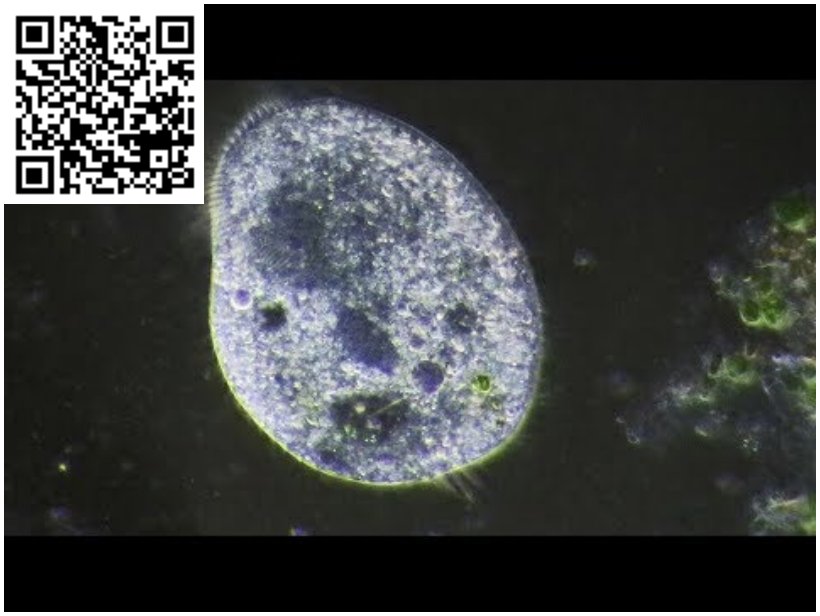


Figure 5: (a) *Paramecium* spp. have hair-like appendages called cilia for locomotion. (b) *Amoeba* spp. use lobe-like pseudopodia to anchor the cell to a solid surface and pull forward. (c) *Euglena* spp. use a whip-like structure called a flagellum to propel the cell.



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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.

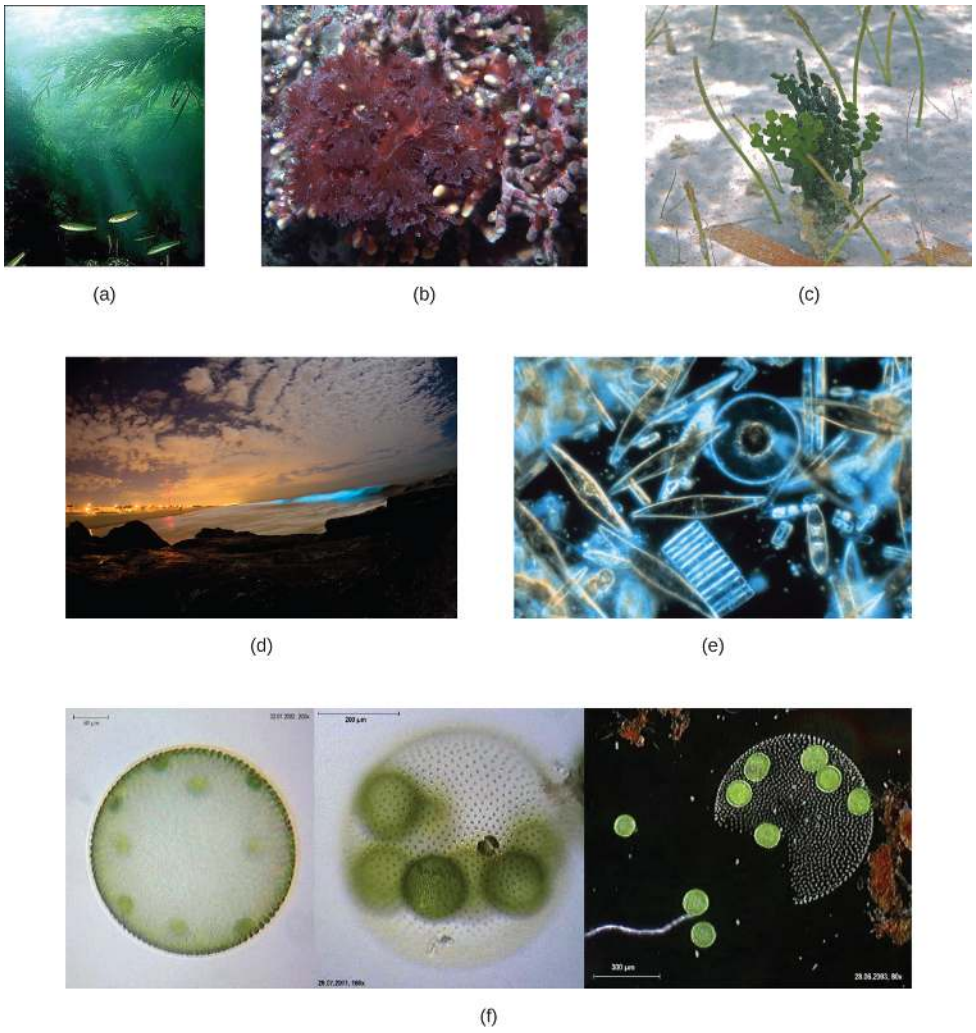


Figure 7: Some of the diversity of algae. (a) These large multicellular kelps are members of the brown algae. Note the "leaves" and "stems" that make them appear similar to green plants. (b) This is a species of red algae that is also multicellular. (c) The green alga *Halimeda incrassata*, shown here growing on the sea floor in shallow water, appears to have plant-like structures, but is not a true plant. (d) Bioluminesence, visible in the cresting wave in this picture, is a phenomenon of certain dinoflagellates. (e) Diatoms (pictured in this micrograph) produce silicaceous 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**).

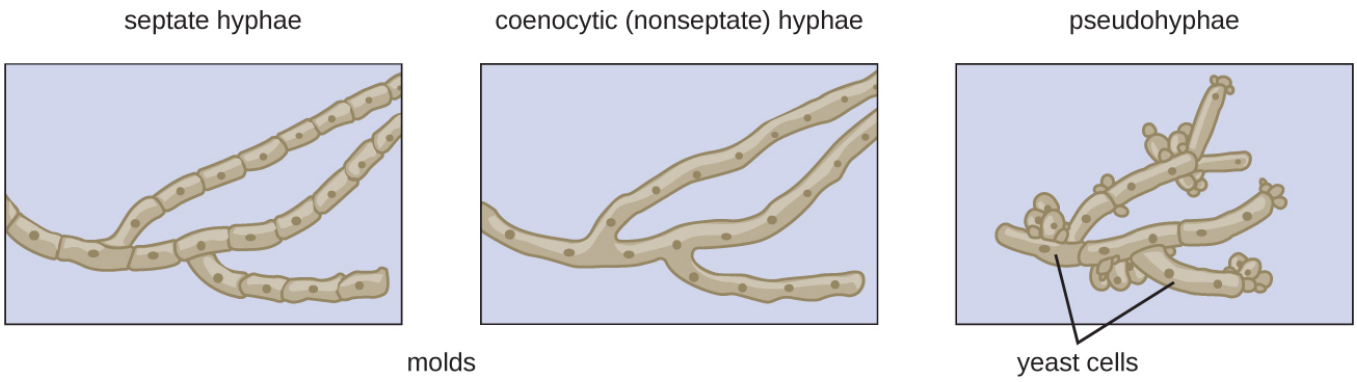


Figure 8: Multicellular fungi (molds) form hyphae, which may be septate or nonseptate. Unicellular fungi (yeasts) cells form pseudohyphae from individual yeast cells.

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.

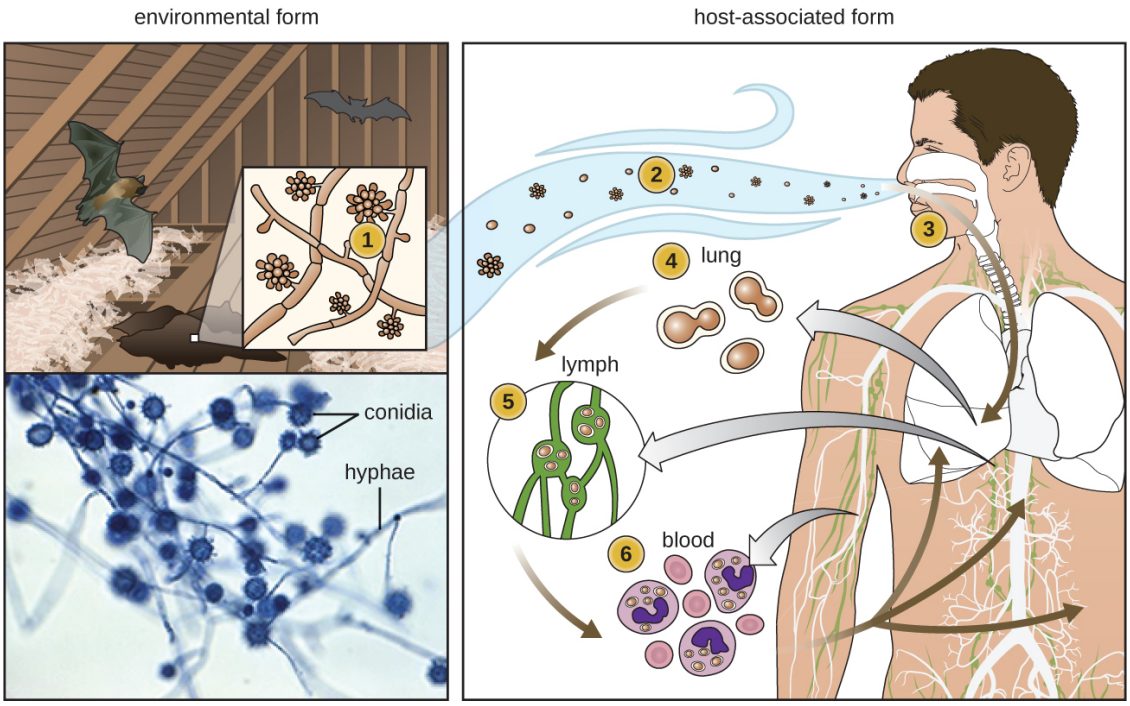


Figure 9: *Histoplasma capsulatum* is a dimorphic fungus that grows in soil exposed to bird feces or bat feces (guano) (top left). It can change forms to survive at different temperatures. In the outdoors, it typically grows as a mycelium (as shown in the micrograph, bottom left), but when the spores 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 cytoplasms 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.

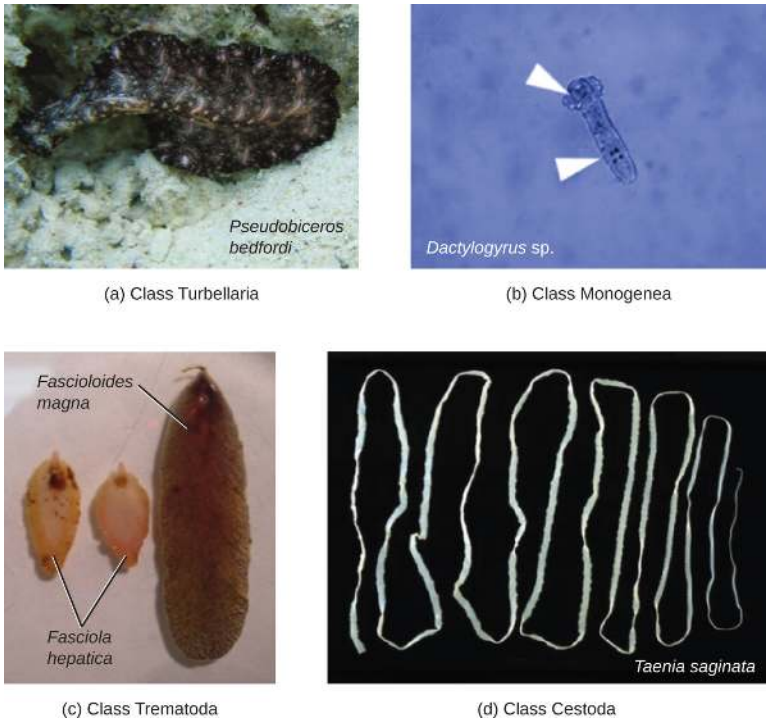


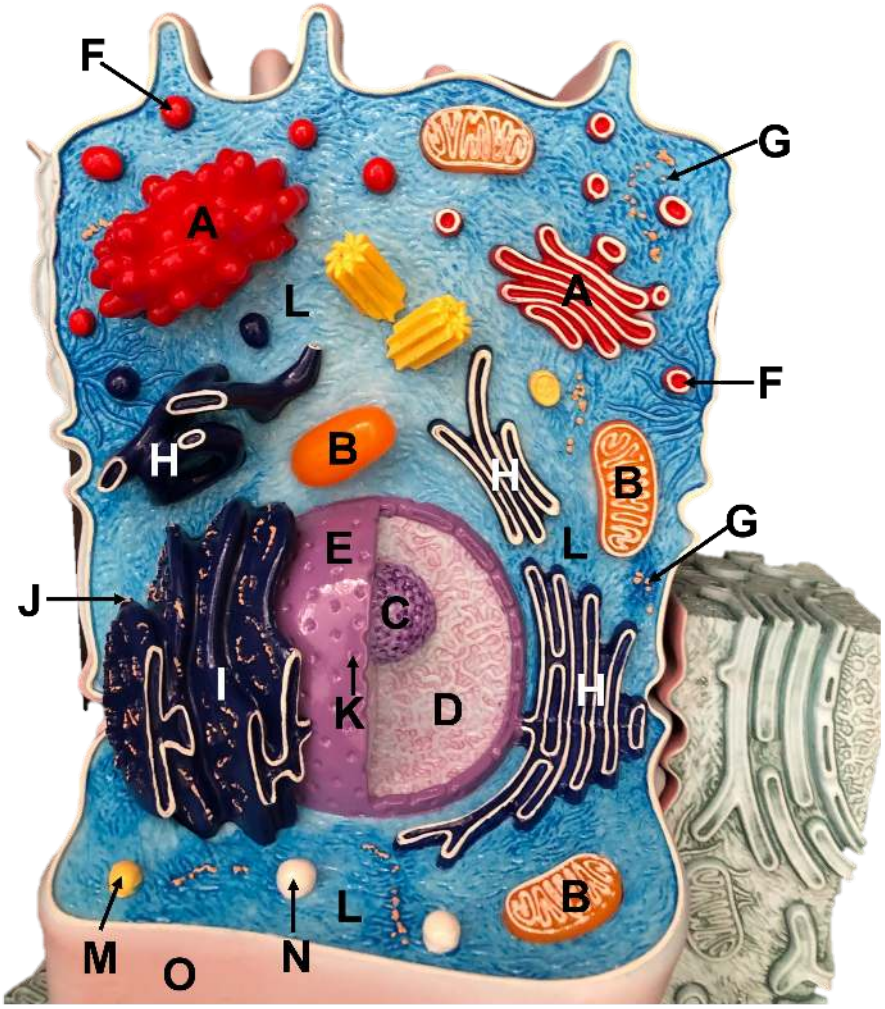
Figure 11: Phylum Platyhelminthes is divided into four classes. (a) Class Turbellaria includes the Bedford's flatworm (*Pseudobiceros bedfordi*), which is about 8–10 cm long. (b) The parasitic class Monogenea includes *Dactylogyrus* spp. Worms in this genus are commonly called gill flukes. The specimen 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. *Diphylobothrium 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.

Laboratory Activities

Eukaryotic Cell Structures



A: _____
B: _____
C: _____
D: _____
E: _____
F: _____
G: _____
H: _____

I: _____
J: _____
K: _____
L: _____
M: _____
N: _____
O: _____

1. Examine the eukaryotic cell model and name the cell structures labeled with letters A-O.

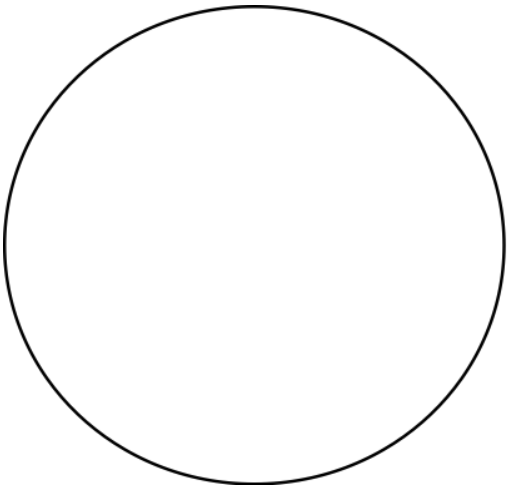
2. Match the descriptions with the corresponding eukaryotic cell structures.

1. _____ rigid structure outside of the plasma membrane that creates cell structure, support and shape	
2. _____ short protein fibers on the outside of the cell that wave to move the cell	
3. _____ dense region inside of the nucleus where ribosomes are put together	
4. _____ membrane sphere where oxygen-containing toxins are destroyed	
5. _____ complex of DNA and protein found in the nucleus	
6. _____ a cell structure where photosynthesis occurs	A. nucleolus
7. _____ a network of different types of protein strands that support the cell structure, organize the cell, can enable cell movement, and help transport materials around the cell	B. cytoplasm C. ribosomes D. nuclear membrane
8. _____ tiny structures that make proteins	E. rough endoplasmic reticulum
9. _____ small passageways in the nuclear membrane where materials can pass in and out of the nucleus	F. plasma membrane G. cytoskeleton
10. _____ a membrane composed of phospholipids and other structures that separates the inside and outside of the cell and controls what materials move in and out of the cell	H. lysosome I. peroxisome J. nucleus
11. _____ a series of membranes where lipids are made and some detoxification activities take place	K. nuclear pore L. Golgi complex
12. _____ location where many cellular molecules are dissolved in a semi-fluid and cellular reactions take place	M. chloroplast N. mitochondria
13. _____ membrane sphere where food and waste materials are broken down	O. cilia P. flagella
14. _____ long and slender external protein filament that move in order to move a cell around its environment	Q. cell wall R. smooth endoplasmic reticulum
15. _____ a membrane that separates the nucleus from the rest of the cell	S. chromatin
16. _____ a large cell structure separated by a membrane where DNA is stored and protected	
17. _____ a series of membranes with ribosomes attached that produce membrane proteins and external proteins	
18. _____ a cell structure where ATP (an energy-rich molecule) is produced using nutrients	
19. _____ a series of membranes where proteins are modified, packaged, and transported to their destinations	

Examine Microbes that have Eukaryotic Cells

Protozoa

Your instructor will provide you with a slide of a species of protozoan. Examine the protozoan with the microscope, make an illustration in the location below, label the nucleus of the protozoan cell, and label any additional cell structures you can identify.

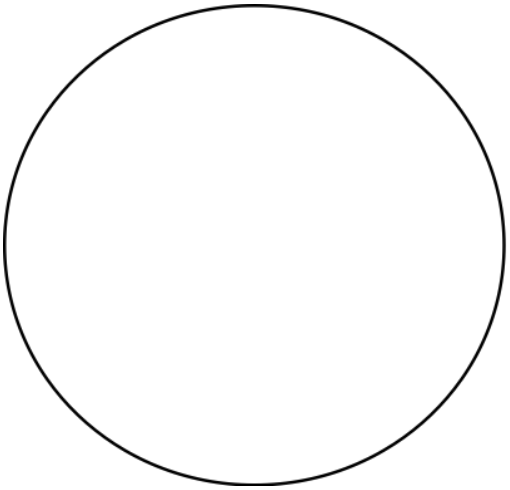


specimen: _____

total magnification: _____

Algae

Your instructor will provide you with a slide of a species of algae. Examine the algae with the microscope, make an illustration in the location below, label the nucleus of the algal cell, and label any additional cell structures you can identify.

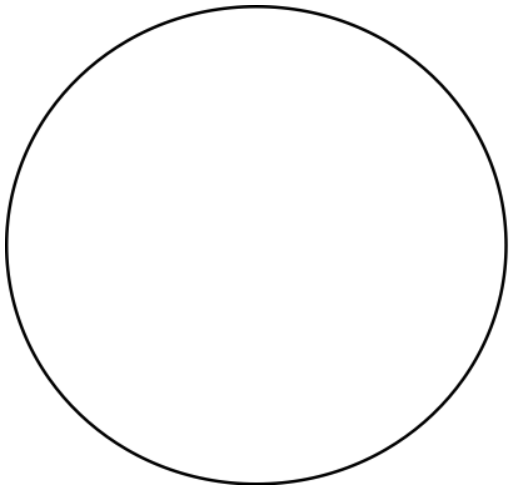


specimen: _____

total magnification: _____

Fungi

Your instructor will provide you with a slide of a species of fungus. Examine the fungus with the microscope, make an illustration in the location below, label the nucleus of the fungal cell, and label any additional cell structures you can identify.

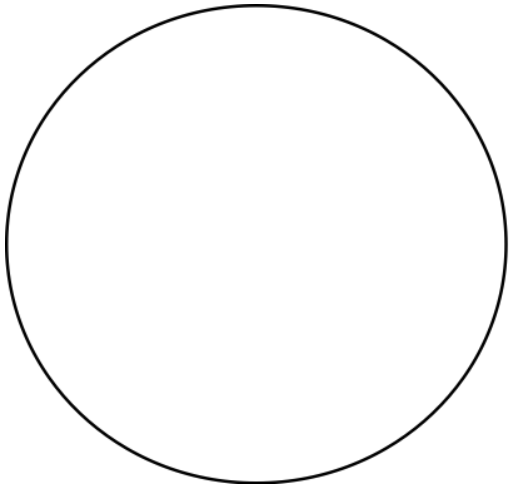


specimen: _____

total magnification: _____

Helminth

Your instructor will provide you with a slide of a species of helminth. Examine the helminth with the microscope, make an illustration in the location below and label any structures you can identify.



specimen: _____

total magnification: _____

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1.17: 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).

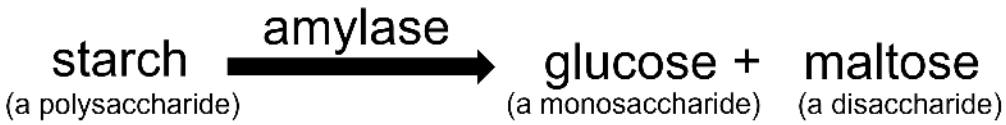


Figure 1: Above is the chemical reaction catalyzed by the enzyme **amylase**. The long polysaccharide starch is broken down (hydrolyzed) by the enzyme amylase. The products are glucose molecules (a monosaccharide) and maltose (a disaccharide). These smaller sugars are small enough that a bacterial cell 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.

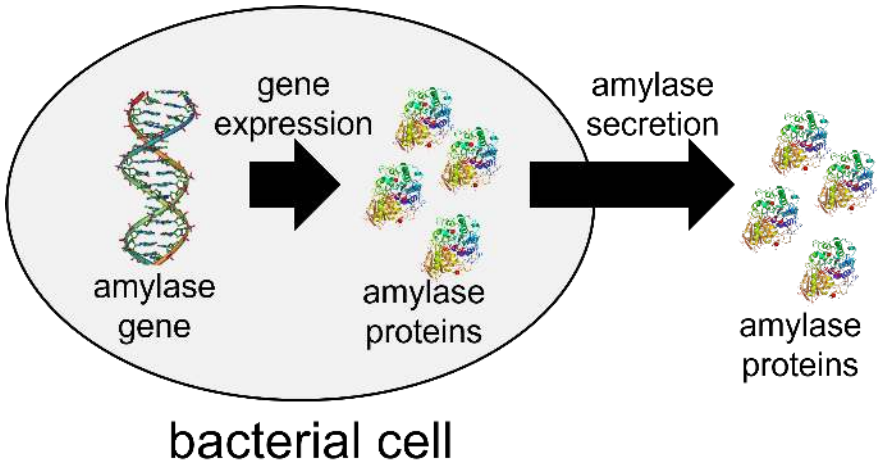


Figure 2: Diagram summarizing how a bacterial cell produces and secretes amylase. The information for building amylase is found in the cell's DNA. The amylase gene is expressed (RNA is created from the information in the gene (the DNA) and from the RNA, ribosomes build the amylase proteins [DNA-->RNA-->protein]). 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 25º C or 37º C for 24-28 hours.

Adding Iodine to Visualize Location of Starch

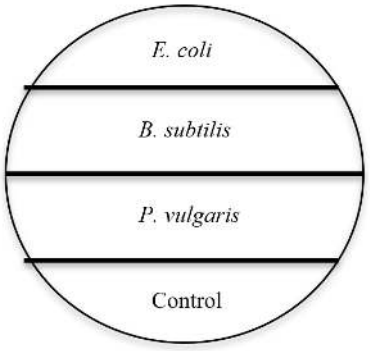


Figure 4: Draw on the bottom of the starch plate the lines and the labels shown above. The bacterial species names have been abbreviated for simplicity. Their full names are *Escherichia coli*, *Bacillus subtilis*, and *Proteus vulgaris*. The control will be used for comparison of how the plate should 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?
Escherichia coli			
Bacillus subtilis			
Proteus vulgaris			

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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1.18: 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.

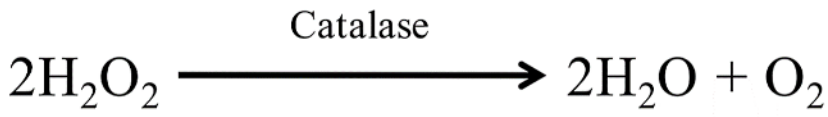


Figure 1: The reaction catalyzed by the enzyme catalase. Two hydrogen peroxide molecules (2 H_2O_2) is converted into two water molecules (2 H_2O) and an oxygen molecule (O_2). "Catalase" is written over the reaction arrow to show that this is the enzyme that enables this chemical reaction to occur.

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-->RNA-->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.

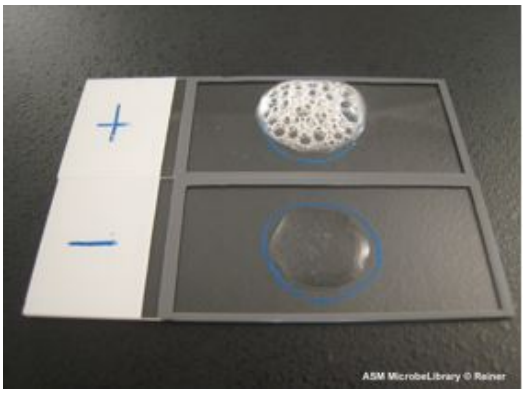


Figure 2: Slide catalase test results. Hydrogen peroxide was added directly to bacteria placed on a microscope slide. A catalase positive reaction is indicated by bubbling (top); a negative reaction is indicated by lack of bubbling (bottom). Image by Karen Reiner, Andrews University, Berrien Springs,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

	Catalase (+/-)
Streptococcus pyogenes	
Staphylococcus aureus	

1. Complete the table above with results from the catalase test.
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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1.19: 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**.

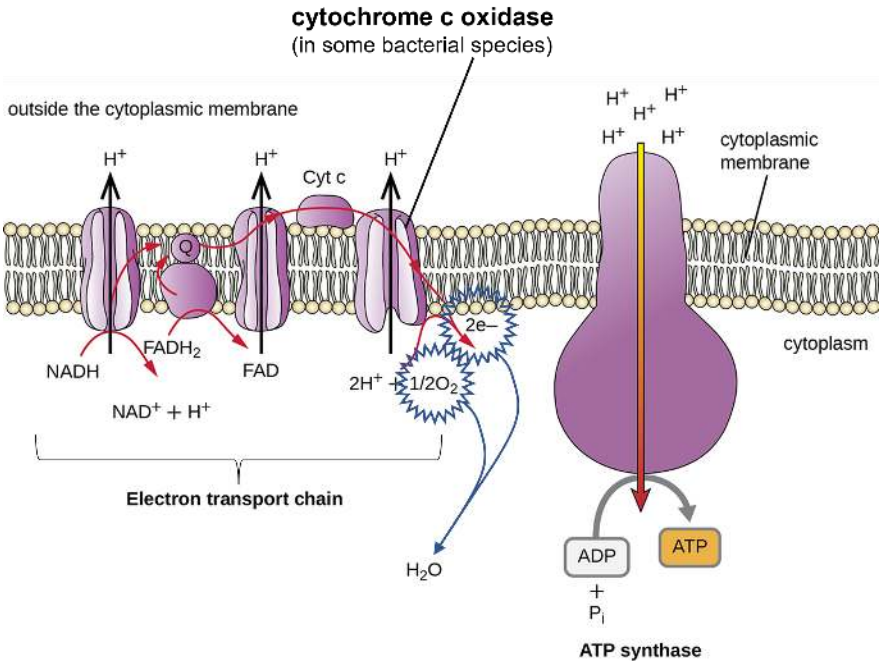


Figure 1: The bacterial electron transport chain is a series of protein complexes, electron carriers, and ion pumps that are used to pump H^+ out of the bacterial cytoplasm into the extracellular space. The last enzyme in the electron transport chain can be (depends on the species) cytochrome c oxidase. 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.

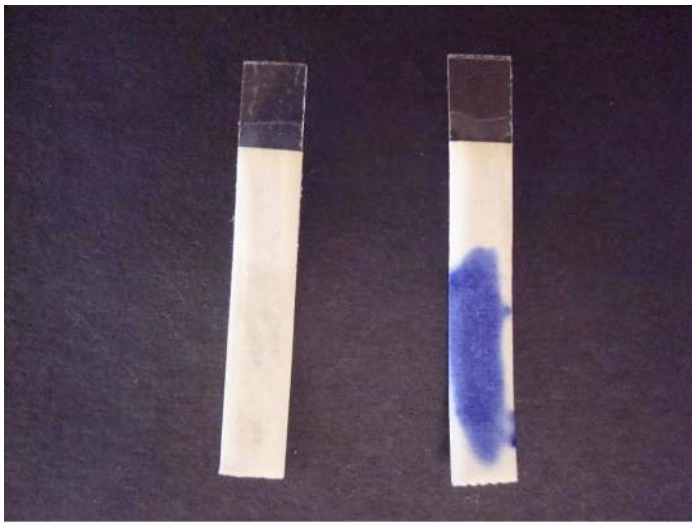


Figure 2: Results from the oxidase test. (Left) An oxidase test strip containing oxidase reagent is still white indicating bacteria is oxidase negative. (Right) The oxidase test strip containing oxidase reagent is purple indicating the bacteria are oxidase positive. The test only indicates oxidase positive 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

	Oxidase (+/-)	**Species produces cytochrome c oxidase (+/-)**
Escherichia coli		
Pseudomonas aeruginosa		

1. Complete the table above with results of the oxidase test.
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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1.20: 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.

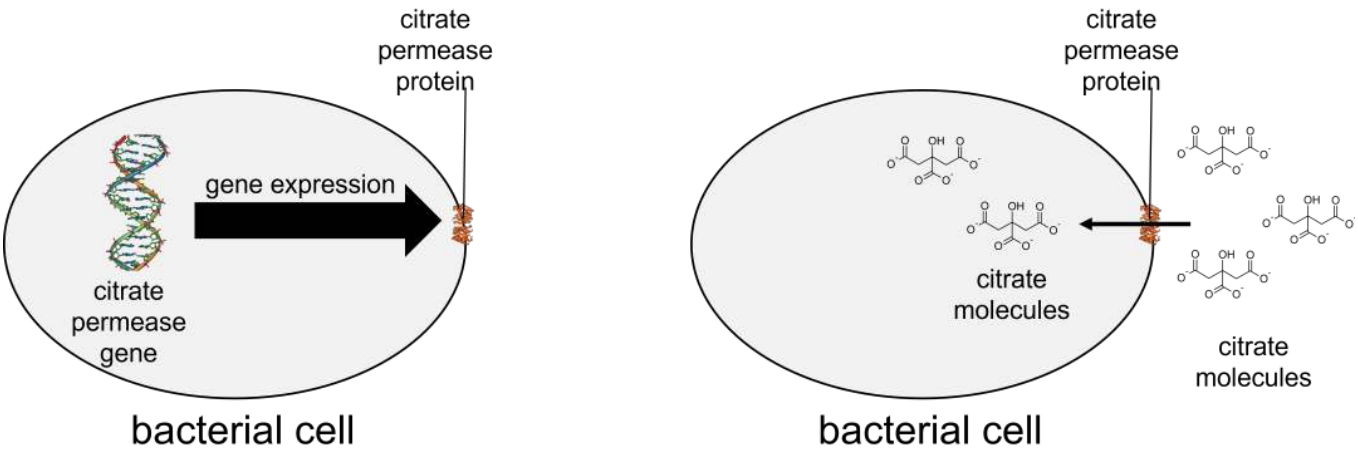


Figure 1: Bacteria with the citrate permease gene can produce the protein citrate permease by gene expression (left). Citrate permease is a membrane protein that enables the transport of citrate into the cell. (Right) When a bacterial cell produces citrate permease, citrate molecules are transported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

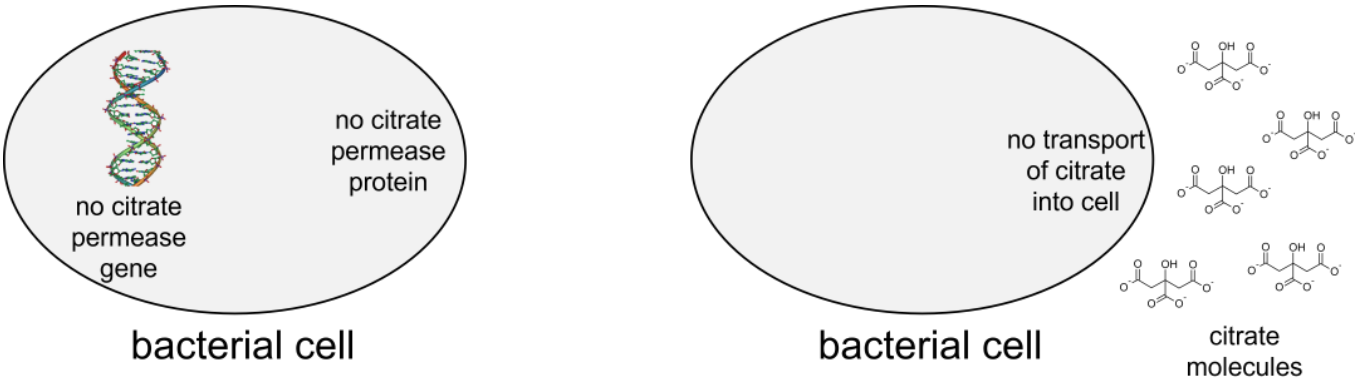


Figure 2: Bacteria without the citrate permease gene cannot produce the protein citrate permease (left). Without the citrate permease protein, citrate is unable to enter the bacterial cell (right). As a result, the bacteria cannot use citrate as a source of carbon.

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.

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 (+/-)
Escherichia coli			
Serratia marcescens			

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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1.21: 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 thyioglycollate cultures to determine oxygen requirements of bacteria.
- Successfully grow bacteria in aerobic and anaerobic conditions and interpet the results.

O_2 and Bacterial Metabolism & Growth

Bacteria can differ dramatically in their ability to utilize oxygen (O_2). 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 (O_2), 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 (NO_3), sulfate (SO_4), and carbonate (CO_3).

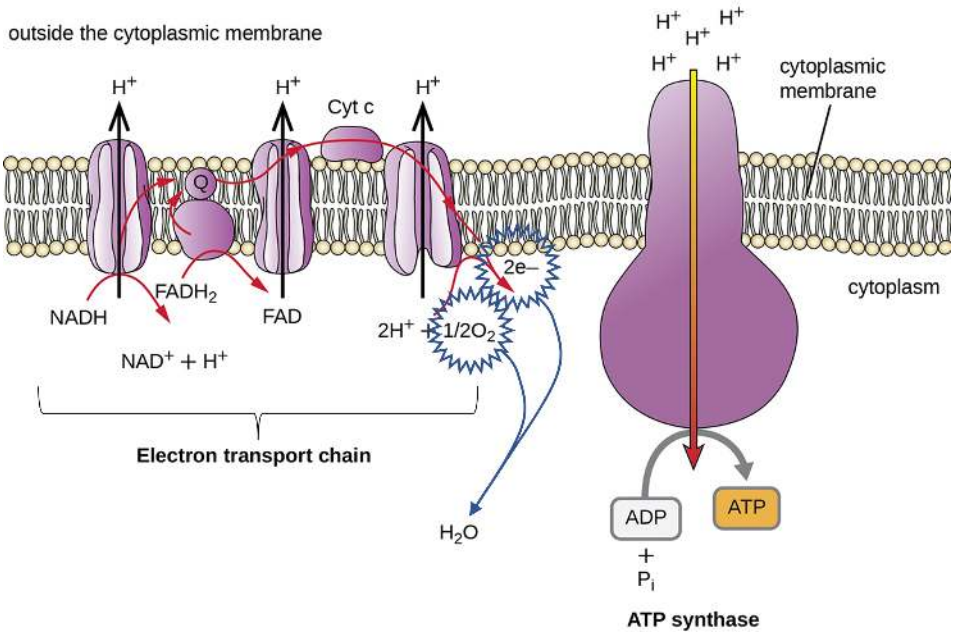


Figure 1: The electron transport chain and oxidative phosphorylation in aerobic respiration. This process is aerobic because oxygen (O_2) is the final electron acceptor and combines with electrons from the electron transport chain to remove them. The electron transport chain and oxidative phosphorylation 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, NO_3, sulfate, SO_4, and carbonate, CO_3).

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 O_2 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.

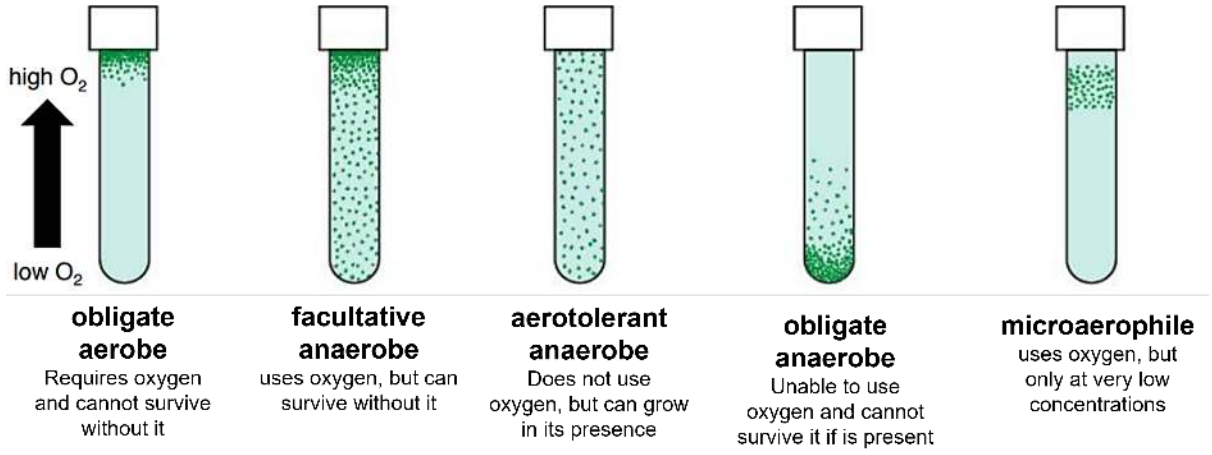


Figure 2: Microbial oxygen requirements can be determined using thioglycollate agar tubes. The green dots in this diagram represent bacterial colonies within in the agar or on its surface. The surface of the agar tube is directly exposed to atmospheric oxygen, and will be aerobic. The oxygen content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



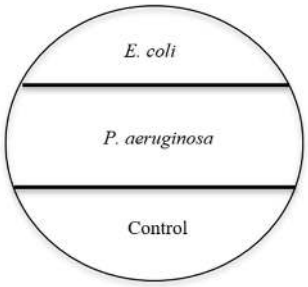
Figure 4: An anaerobic jar for cultivating obligate anaerobic species. Plates and test tubes with cultures to be cultivated in an anaerobic environment are added to the jar with a gas-generating pouch. The jar is then sealed. The gas generator pouch removes O_2 inside the jar. The jar environment then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

Laboratory Instructions

Determining Bacterial Oxygen Requirements with Thioglycollate Medium

1. The thioglycollate broth should be either boiled first before inoculation **OR** recently made so that the oxygen content is very low. (Your instructor will tell you if it needs to be boiled).
2. Use tape to label the test tube with your group name, the bacterial species, and the type of medium.
3. Inoculate a tube of thioglycollate broth with the assigned bacterial species. Make sure that the loop or needle goes down to the very bottom of the broth, but do not get the metal holder region of the loop in the sterile broth since it will contaminate it.
4. Incubate at 25 °C or 37 °C.

Growing Bacteria With & Without O_2



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

Determining Bacterial Oxygen Requirements with Thioglycollate Medium

	growth in the aerobic region of the thioglycollate medium (+/-)	growth in the microaerobic region of the thioglycollate medium (+/-)	growth in the anaerobic region of the thioglycollate medium (+/-)	distribution of growth in the medium (even, uneven); if uneven, where was most of the growth?	classify this species by its oxygen requirement (e.g. obligate aerobe, facultative anaerobe, etc.)
Escherichia coli					
Pseudomonas aeruginosa					
***Clostridium* sp.** (if used)					

1. Examine the thioglycollate medium. Do not shake or stir. Complete the table above to summarize results and determine the oxygen requirement for each species.
2. Explain how these results relate to aerobic respiration and anaerobic respiration?
3. How does the thioglycollate test tube create aerobic, microaerobic, and anaerobic conditions to test oxygen tolerances of bacterial species?
4. True or False. All bacterial species can grow in the presence of oxygen. Explain your answer.
5. True or False. All bacterial species can grow in the absence of oxygen. Explain your answer.
6. Can testing the oxygen tolerances of bacterial species be useful for species identification and characterization? Explain your answer.

Growing Bacteria With & Without O_2

	aerobic growth (+/-)	anaerobic growth (+/-)	classify this species by its oxygen requirement (e.g. obligate aerobe, facultative anaerobe, etc.)
Escherichia coli			
Pseudomonas aeruginosa			
***Clostridium* sp.** (if used)			

1. Examine the aerobic and anaerobic petri plates and fill out the table above with 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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1.22: 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!

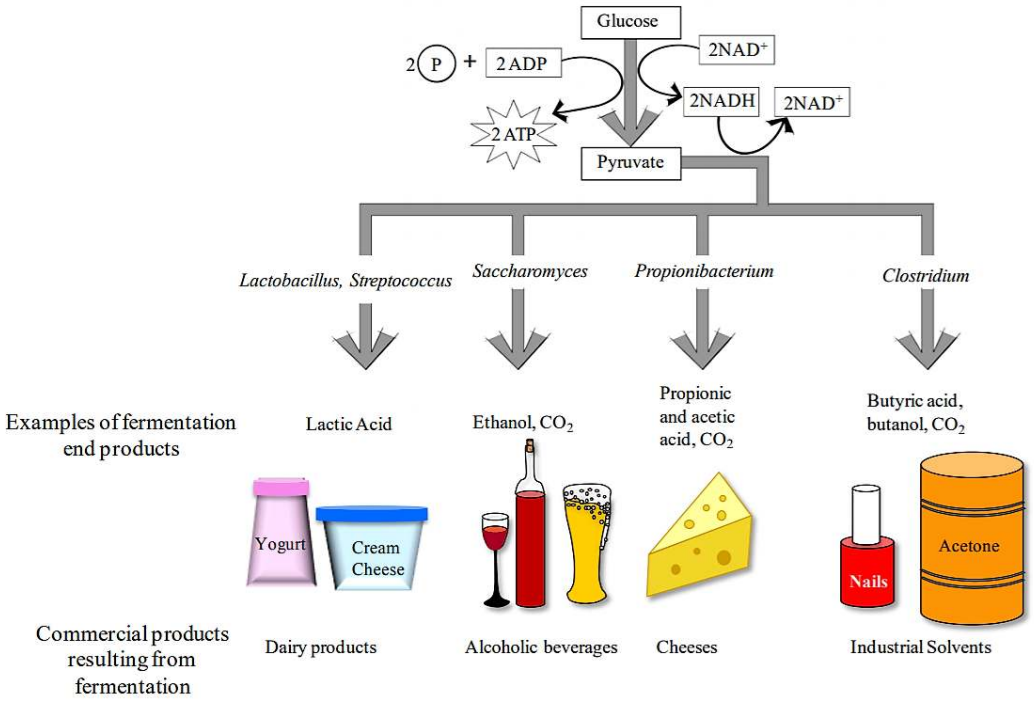


Figure 1: Carbohydrate fermentation. Fermentation is a microbial process utilized to produce a variety of products used by humans including dairy products (yogurt and cream cheese), alcoholic beverages, cheeses, and industrial solvents. Fermentation is a metabolic process the begins with glycolysis to 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 is 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.

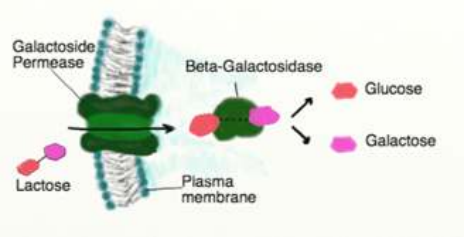


Figure 2: Lactose on the outside of a bacterial cell can be brought into the cell if the cell has the enzyme permease to transport it. Lactose metabolism also requires beta-galactosidase to break down the disaccharide lactose into monosaccharides.

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.

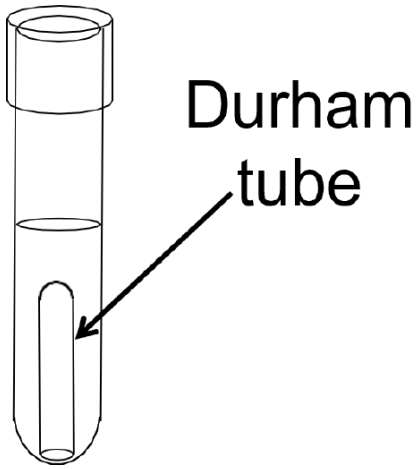


Figure 3: Diagram showing the fermentation test setup with a test tube containing medium and a tiny tube inside of the medium that is upside down to capture gases produced during fermentation. This tiny upside down tube is called a Durham tube. If gas is produced during fermentation, there will be a 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.

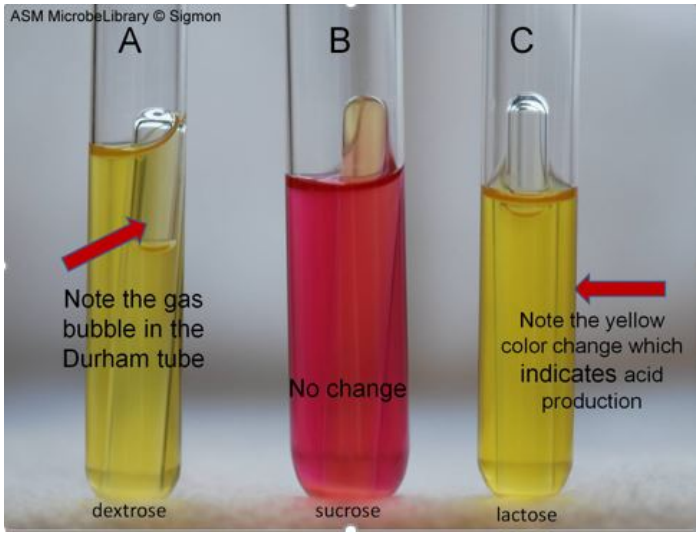


Figure 4: Fermentation reactions produced by *Escherichia coli* in phenol red sugar broths containing dextrose, sucrose, and lactose. Image by Janie Sigmon, York Technical College, Rock Hill, SC.

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

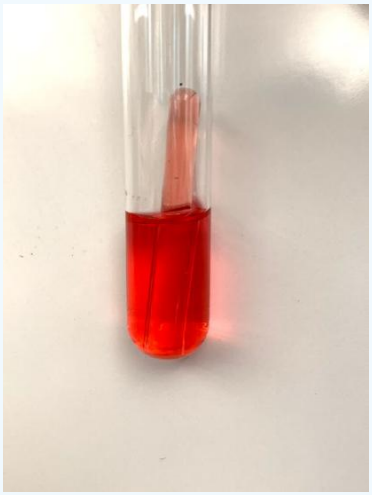


Interpret the result of this fermentation test:

- 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 (+/-)**
Escherichia coli					
Bacillus subtilis					
Proteus vulgaris					

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

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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1.23: 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 is indicates whether or not bacterial species produce flagella for motility.
- Successfully conduct SIM tests and Interpore 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 **s**ulfur reduction, **i**ndole production and **m**otility. 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 $Fe(NH_4)_2(SO_4)$, sodium thiosulfate $Na_2S_2O_3$, agar (3.5 g/L) and distilled or deionized water.

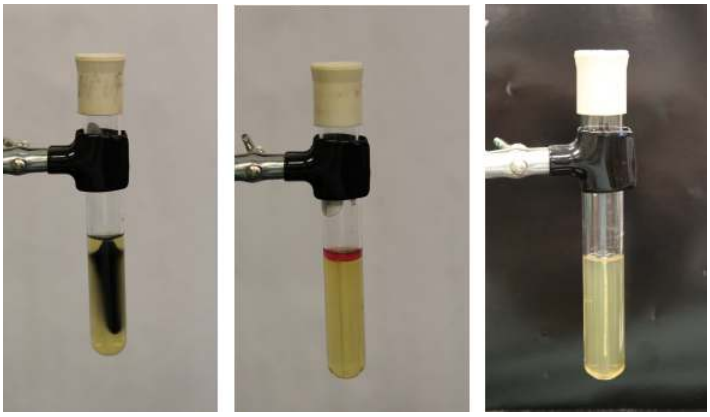


Figure 1: SIM medium is for deep cultures and an inoculation needle is used to inoculate it. After incubation, a black color indicates that the bacteria has produced hydrogen sulfide (H_2S). (Left) This culture turned black and therefore this bacterial species is positive for hydrogen sulfide production. (Right and middle) These cultures did not turn black and is therefore are negative for hydrogen sulfide production. If bacterial 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 ($S_2O_3^{2-}$) 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.

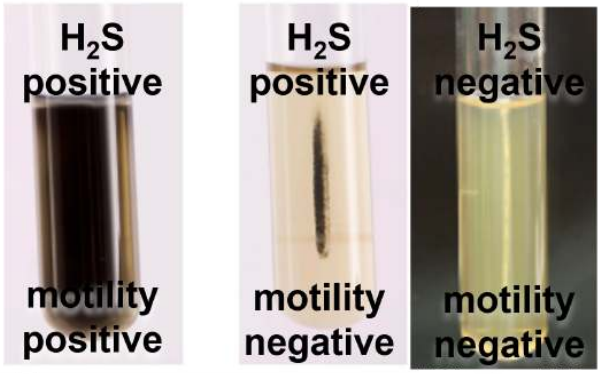


Figure 2: Results of SIM deep cultures (no Kovac's reagent). (Left) The medium is black throughout indicating hydrogen sulfide production and motility. (Middle) The medium is black along the stab line only indicating hydrogen sulfide production and no motility. (Right) The medium is not black and there 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.

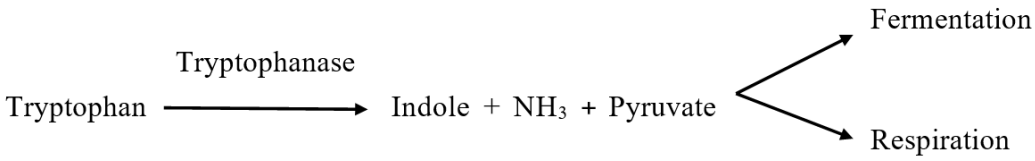


Figure 3: Chemical reaction catalyzed by tryptophanase. Bacteria that have the tryptophan gene can break down tryptophan to produce indole. Indole is not used by the bacteria and therefore will accumulate in the medium. Kovac's reagent can be used to detect the accumulated indole and therefore is an 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.

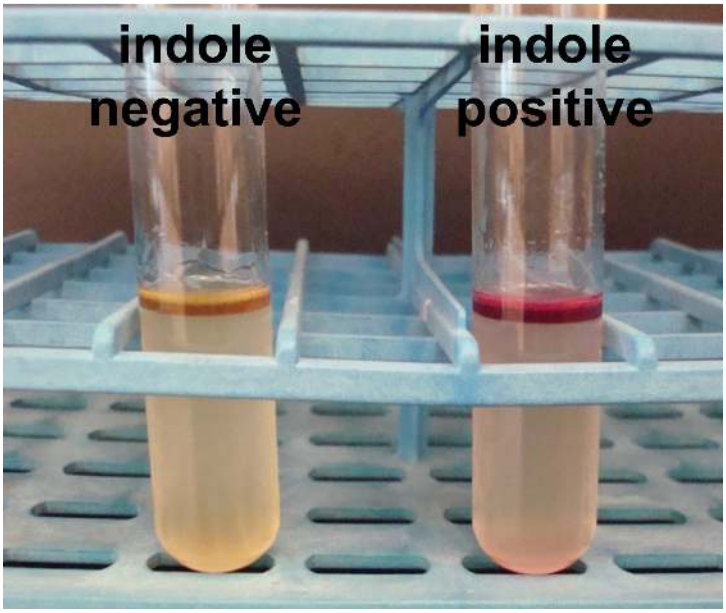


Figure 4: Results from indole test. Kovac's reagent is on top of the SIM agar deep inside of these test tubes. The reagent appears yellow when the bacterial species is indole negative (left) and the reagent appears red when the bacterial species is indole positive (right).

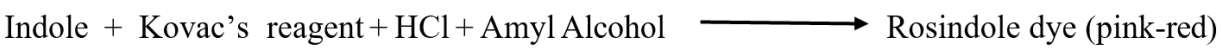


Figure 5: Reaction that occurs between indole and Kovac's reagent. A reaction between indole and Kovac's reagent produces a dye that is pink-red when indole is present.

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.

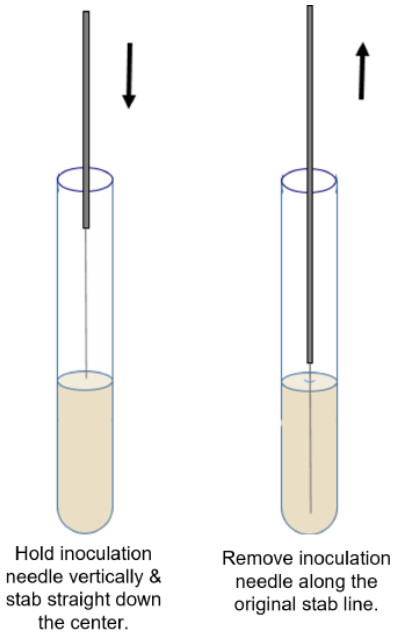


Figure 6: How to inoculate a deep with an inoculation needle. Hold the inoculation needle vertically and stab straight down the center of the deep. Then remove the inoculation needle along the original stab line.

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.

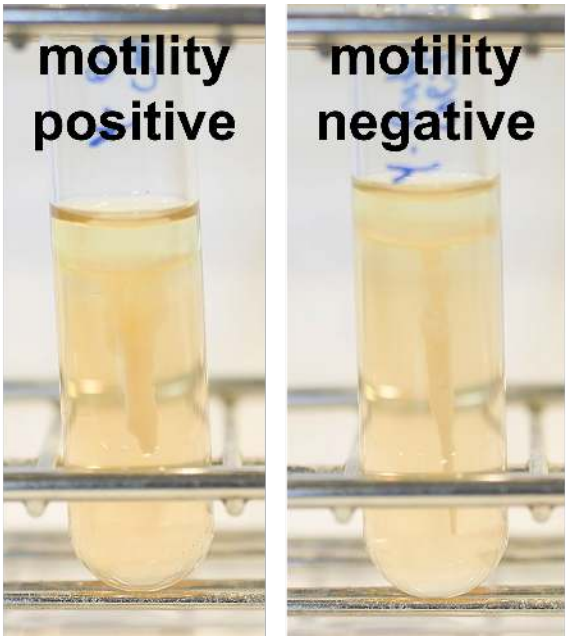


Figure 7: SIM deep results shown with Kovac's reagent present on top of the medium. (Left) Bacterial growth has moved away from the stab and therefore is **motility positive**. The Kovac's reagent is not red and therefore is indole negative. The medium did not produce any black coloration and is therefore hydrogen sulfide reduction negative. (Right) Bacterial growth stayed close to the initial stab and there is 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 meidum 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 (+/-)
Escherichia coli						
Proteus vulgaris						
Staphylococcus aureus						

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 H_2S 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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1.24: 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

Slide Agglutination Test

1. Make a 1 inch diameter circle on a clean glass slide using a wax pencil.
2. Place two drops of thawed rabbit plasma into the circle, using a wooden pick or a clean loop.
3. Add a HEAVY inoculum and emulsify it in the plasma (should be milky-looking).
4. Fibrin threads form between the cells, causing them to agglutinate, or clump.
5. There will a visible clumping of cells within 10-15 seconds.
6. This test is for the bound coagulase enzyme.

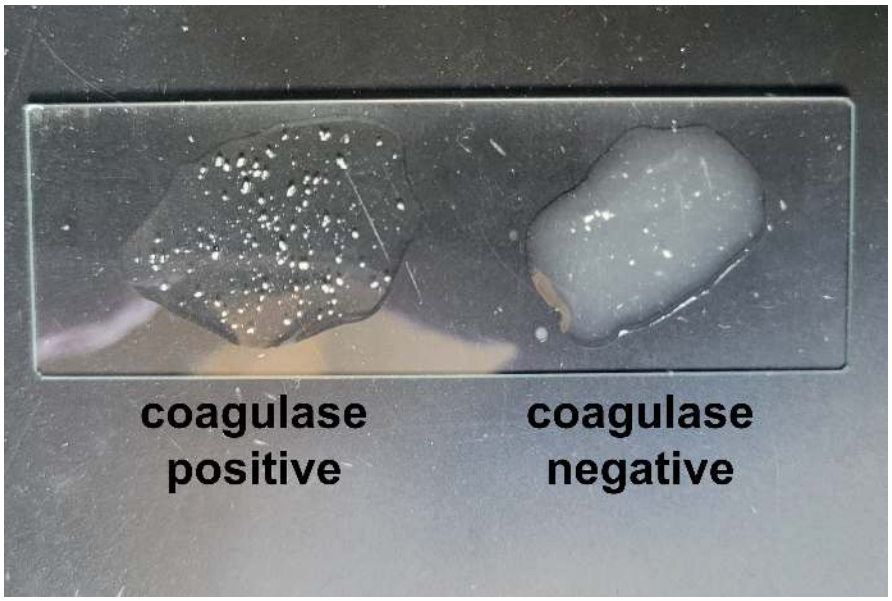


Figure 1: The slide agglutination coagulase test is coagulase positive when clots (clumps) occur with a clear solution in the background (left). The slide coagulase test is coagulase negative when there is a smooth mixture with a milky background (right).

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 37º 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.

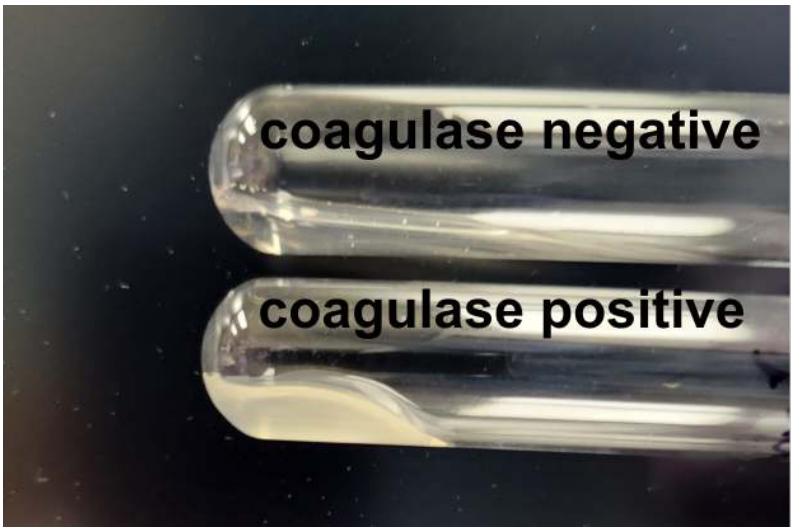


Figure 2: The tube agglutination coagulase test. When the test tube is tilted, if the fluid is not clumped and does not contain clumps (clotting), the result is coagulase negative (top). When the test tube is tilted, the fluid either exhibits a clump or contains clumps in it (clotting), the result is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

Results & Questions

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

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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1.25: 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.

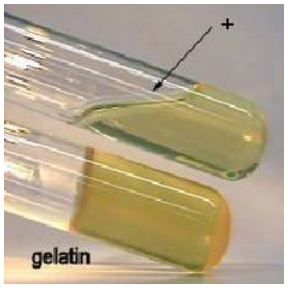


Figure 1: Results from two gelatin hydrolysis tests. The top test tube shows a gelatin medium after chilling that has a more liquid form indicating that gelatin hydrolysis occurred (gelatin hydrolysis positive). The bottom test tube shows a gelatin medium after chilling that has a semi-solid state indicating 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 25º 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

Bacterial Species	Gelatin medium liquid after incubation and chilling (+/-)	Species hydrolyzes gelatin (+/-)	Species has a gelatinase enzyme (+/-)	Species has a gelatinase gene (+/-)

1. Complete the table above with experimental results and interpretations.
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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1.26: 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:

$$\mathbf{NO_3\ (nitrate) \rightarrow NO_2\ (nitrite)}$$

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

$$\mathbf{NO_3\ (nitrate) \rightarrow NO_2\ (nitrite) \rightarrow NH_3\ (ammonia)}$$

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:

$$\mathbf{NO_3\ (nitrate) \rightarrow NO_2\ (nitrite) \rightarrow N_2\ (nitrogen\ gas)}$$

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 produce, 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) → NO_2 (nitrite) → 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 nitrate (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 nitrate (**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) → 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.

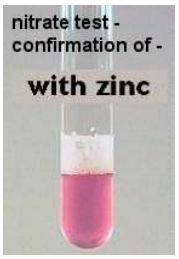


Figure 3: If no bubble is present in the Durham tube and the medium did not turn red, it is still possible that nitrate was reduced to ammonia. Zinc is added to determine whether the nitrate was reduced or not. If the nitrate was not reduced by the microbes in the tube, the zinc will cause the medium 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, 30º C or 37º C, for these bacterial species.
3. After Incubation: Look for N_2 gas first ***before adding reagents*** and record results for whether or not N_2 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 (NO_2) or N_2 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 (NO_3) by zinc takes a couple of minutes. Record results for whether or not ammonia was produced.

Results & Questions

	NO_3 present/absent (+/-)	NO_2 present/absent (+/-)	N_2 present/absent (+/-)	nitrate reduction (+/-)
Alcaligenes faecalis				
Escherichia coli				
Pseudomonas aeruginosa				

1. Complete the table above with experimental 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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1.27: 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 a using 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 O_2 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.

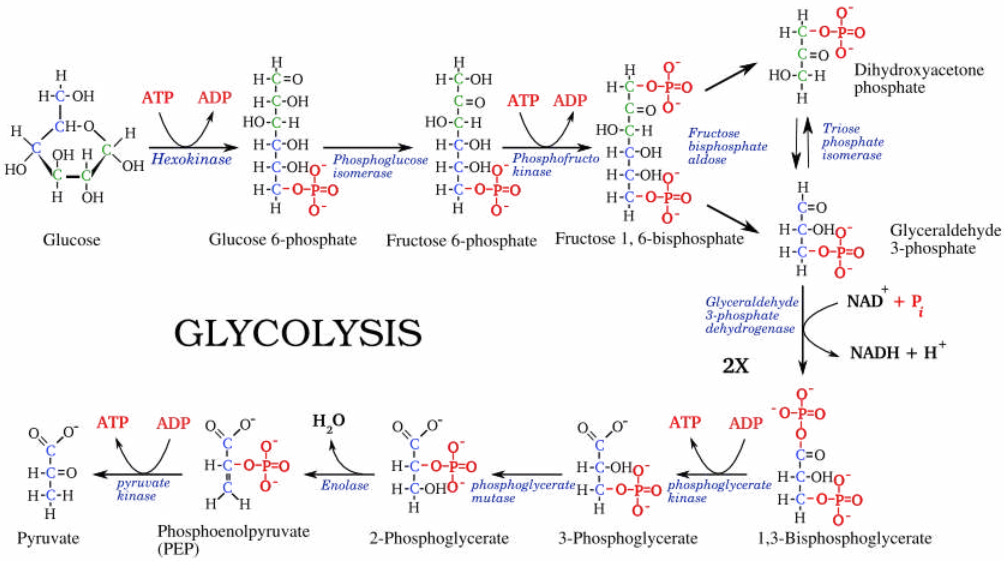


Figure 1: Glycolysis. This chemical pathway begins with a glucose molecule that is converted into two pyruvate molecules. Although two ATP molecules are required at the beginning of this metabolic pathway, in the later reactions, four ATP molecules are produced, leaving a net gain of two ATP molecules 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.

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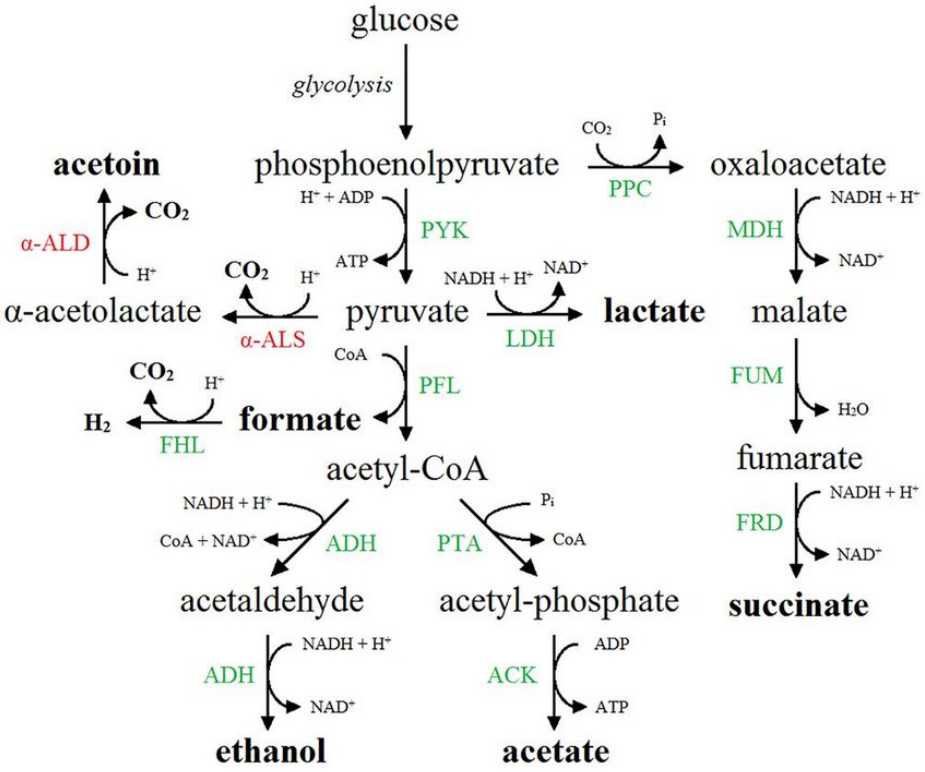


Figure 2: An example of one type of mixed acid fermentation in a strain of *Escherichia coli* that is expressing acetoin synthesis operon (budAB operon). In this example, the fermentation pathway produces acetoin, lactate, formate (which is subsequently broken down to H_2 and CO_2), succinate, acetate, 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)

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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*.

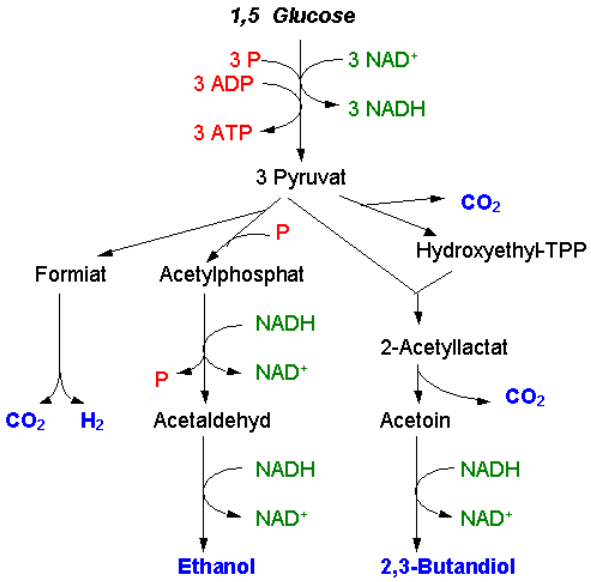


Figure 4: Part of glycolysis is shown with the metabolic reactions occurring in butanediol fermentation (metabolic byproducts are shown in blue).

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 V**oges-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)

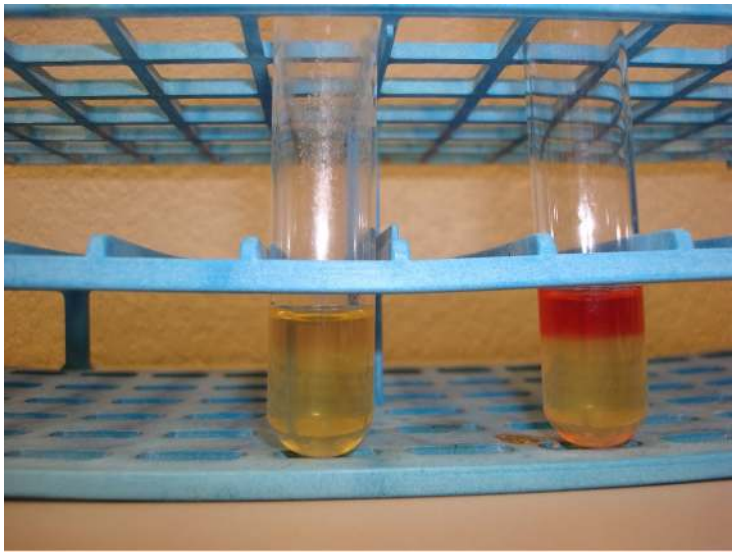


Figure 5: Results of the Voges-Proskauer test. (Left) When the color at the top of the tube is yellow, this is a negative Voges-Proskauer test indicating that the bacterial species tested does not ferment glucose using the butanediol fermentation pathway. (Right) When the color at the top of the tube 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-napththol) 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 (+/-)
Escherichia coli						
Enterobacter aerogenes						

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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1.28: 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 ß-galactosidase gene, and can therefore break down lactose. Coliform bacteria break down lactose to produce acid and gas.

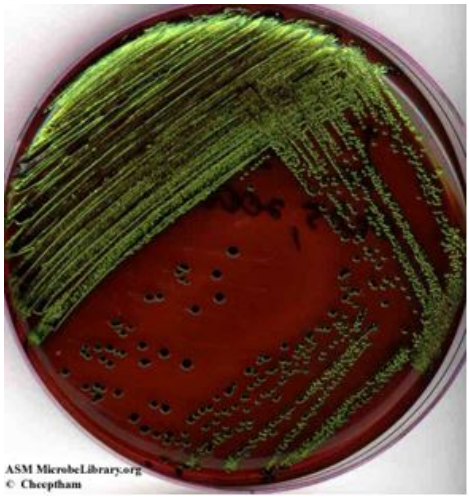


Figure 1: Eosin-methylene blue (EMB) agar plate inoculated with *Escherichia coli* (a Gram-negative coliform bacterium) showing good growth of dark blue-black colonies with metallic green sheen indicating vigorous fermentation of lactose and acid production which precipitates the green metallic pigment. 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 (+/-)
Escherichia coli					
Pseudomonas aeruginosa					
Staphylococcus aureus					

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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1.29: 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 (+/-)
Staphylococcus aureus				
Staphylococcus epidermidis				

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.

Attributions

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1.30: DNA, RNA, and DNA Replication

Learning Objectives

- Describe in detail / identify the structures of DNA from nucleotide components to double helix.
- Describe in detail / identify the structures of RNA from nucleotide components to single strand.
- Compare the structures of DNA and RNA.
- Utilize and identify 5' and 3' directionality of DNA and RNA nucleotides and molecules.
- Explain the process of DNA replication in detail and identify components of the process in diagrams and figures.
- Build DNA and RNA nucleotides using puzzle pieces and compare their structures.
- Build DNA and RNA nucleic acid sequences using puzzle pieces and identify and compare structures of these molecules.
- Replicate the process of DNA replication using a DNA puzzle model.
- Compare the puzzle re-creation of DNA replication with events that occur in the actual process of DNA replication.

Importance of Understanding DNA Structure

Understanding the structure of DNA is fundamental to a better understanding of biology overall, but particularly microbiology, disease, and modern medical approaches. Here are a few ways that having a thorough understanding of DNA structure will impact your ability to understand additional microbiological topics:

- the DNA of a microbe dictates if that microbe is pathogenic or not and the degree of its pathogenicity
- the DNA of a microbe is the basic blueprint for that microbe's metabolism, characteristics, abilities, structure, and survival approaches
- DNA information is used during the process of gene expression
- understanding DNA structure is essential to better understand gene expression
- understanding how gene expression works enables us to understand the link between DNA and a microbe's characteristics
- how pathogens (disease-causing microbes) evolve/change over time (think of the seasonal flu strains or new variants of the virus that causes COVID-19) is related to its DNA (or the closely related molecule RNA in the case of the virus that causes COVID-19) and how that DNA changes over time
- DNA is used to identify microbes (e.g. what microbe is causing an infection) in diagnostic approaches such as PCR testing
- understanding how PCR works for diagnostic techniques requires a firm understanding of DNA structure and DNA replication
- understanding modern biological science is impossible without a firm understanding of DNA structure and DNA replication (and PCR too)

Note

PCR has a huge number of applications beyond medical diagnostics!

DNA Structure

Nucleotides are the Monomers of DNA

The building blocks of DNA are nucleotides. The important components of the nucleotide are a nitrogenous (nitrogen-bearing) base, a 5-carbon sugar (pentose), and a phosphate group. The nucleotide is named depending on the nitrogenous base. The nitrogenous base can be a purine such as adenine (A) and guanine (G), or a pyrimidine such as cytosine (C) and thymine (T).

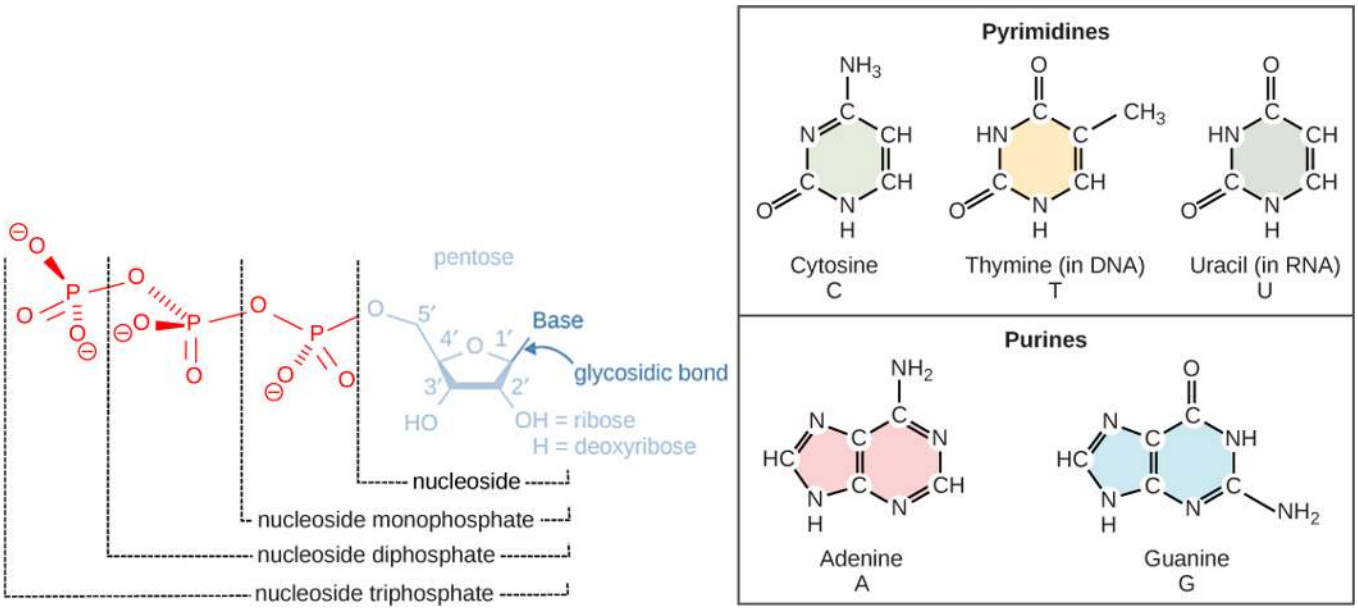


Figure 1: Nucleotides are the building blocks of DNA and RNA. This figure shows the generalized structure of nucleotides and their bases. (Left) A single nucleotide is made of a phosphate group, a pentose sugar (a sugar with five carbons) and a base. The pentose sugar is deoxyribose in DNA and has a hydrogen attached to the 2' carbon atom instead of an OH. The pentose sugar is ribose in RNA and has an OH attached to the 2' carbon atom instead of just a hydrogen. The bases found in the nucleotides can be either classified as purines or pyrimidines. (Right) The purines have a double ring structure with a six-membered ring fused to a five-membered ring. Pyrimidines are smaller in size; they have a single six-membered ring structure. Each base contains at least two nitrogen atoms. It is because of this that the bases are commonly referred to as "nitrogenous bases."

The purines have a double ring structure with a six-membered ring fused to a five-membered ring. Pyrimidines are smaller in size; they have a single six-membered ring structure.

Important

The carbon atoms of the pentose sugar are numbered 1', 2', 3', 4', and 5' (1' is read as “one prime,” 2' is read "two prime," etc.). These prime numbers are used to describe the direction of DNA strands using 5' to designate one side of the DNA molecule and using 3' to designate the other side of the DNA molecule.

When nucleotides are built in processes such as DNA replication and gene expression, these directions are incredibly important since nucleic acids can only form new bonds on the 3' end. As a result, it is often stated that nucleic acids (DNA and RNA) are built from 5' to 3'.

The sugar is deoxyribose in DNA and ribose in RNA. The phosphate, which makes DNA and RNA acidic, is connected to the 5' carbon of the sugar by the formation of an ester linkage between phosphoric acid and the 5'-OH group (an ester is an acid + an alcohol). In DNA nucleotides, the 2' carbon of the sugar deoxyribose is attached to a hydrogen atom. In RNA nucleotides, the 2' carbon of the sugar ribose also contains a hydroxyl group (OH). The base is attached to the 1' carbon of the sugar.

Overall DNA Structure

The nucleotides form covalent bonds with each other to produce **phosphodiester bonds** (a fancy science name for the covalent bonds joining nucleotides together). The phosphate group forms a covalent bond with the hydroxyl group of the 3' carbon of the

sugar of the next nucleotide, thereby forming a 5'-3' phosphodiester bond. In a polynucleotide, one end of the chain has a free 5' phosphate, and the other end has a free 3'-OH. These are called the 5' and 3' ends of the chain.

The result of nucleotides bonding together is a **sugar-phosphate backbone** with the nitrogenous bases hanging off of the side. This structure resembles one half of a ladder. The side support rails of the ladder is analagous to the sugar-phosphate backbone, and the half-rung in the middle of the ladder is analagous to the bases hanging off the side. In order for DNA to be a complete double-stranded molecule, the single DNA strand pairs with another DNA strand. The result is a complete ladder with the side-supports rails being sugar-phosphate backbones and the rungs in the middle are the bases of the two DNA strands interacting with each other in the middle.

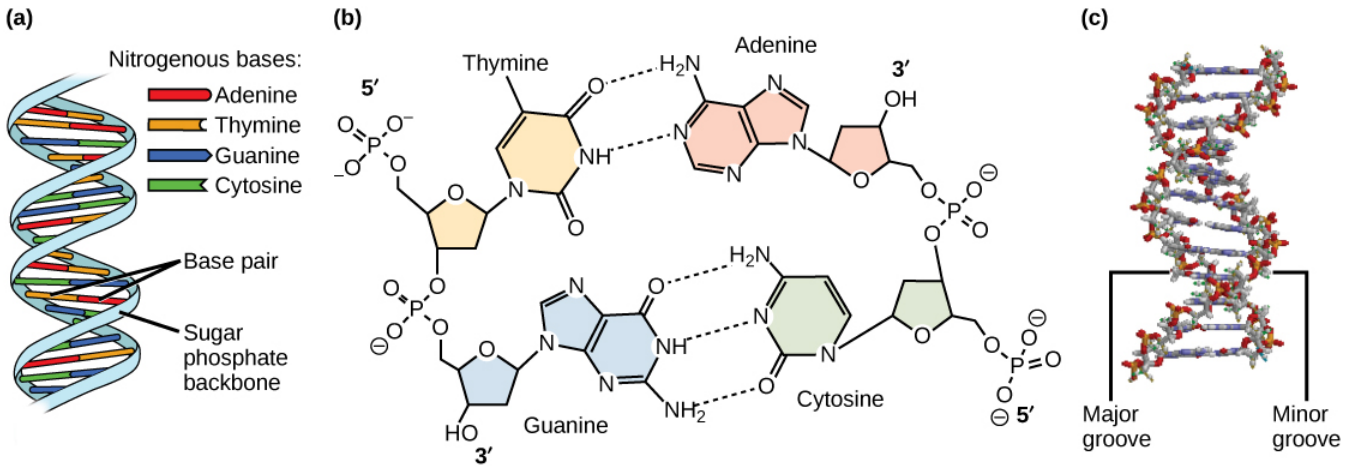


Figure 2: DNA has (a) a double helix structure and (b) phosphodiester bonds; the dotted lines between thymine and adenine and guanine and cytosine represent hydrogen bonds. The (c) major and minor grooves are binding sites for DNA binding proteins during processes such as transcription (the copying of RNA from DNA) and replication.

The two DNA strands can only pair together when the two strands are **antiparallel**, that is, the strands are parallel, but in opposite directions. One DNA strand will be in the 5' to 3' direction, and it will pair with a strand upside-down to it in the 3' to 5' position. This arrangement enables complementary base pairs to hydrogen bond with each other. The DNA bases that meet in the middle of the double-stranded DNA molecule pair such that adenine (A) always pairs with thymine (T) and guanine (G) always pairs with cytosine (C). The A-T pair is held together by two hydrogen bonds and the G-C pair is held together by three hydrogen bonds.

The entire DNA ladder twists in three-dimensions to produce a helical formation to the molecule.

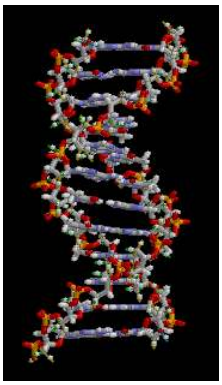


Figure 3: This animation shows the three-dimensional structure of a DNA double helix. Notice how this molecule appears similar to a ladder that has been twisted. The rungs of the ladder are the A-T and G-C bases of the two DNA strands forming hydrogen bonds with each other. The supportive side rails of the ladder are the sugar-phosphate backbones of the two DNA strands.

RNA Structure

DNA structure is very similar to DNA structure. RNA is composed of nucleotides bonded together with phosphodiester bonds forming a sugar-phosphate backbone with bases hanging off of the side. There are three main differences between DNA and RNA:

1. RNA nucleotides contain ribose as the pentose sugar instead of deoxyribose (ribose has an -OH on the 2' carbon whereas deoxyribose has an -H on the 2' carbon)
2. RNA nucleotides will not contain the base thymine, but will instead contain uracil; uracil base-pairs with adenine in the same way thymine does
3. RNA is a single-stranded molecule

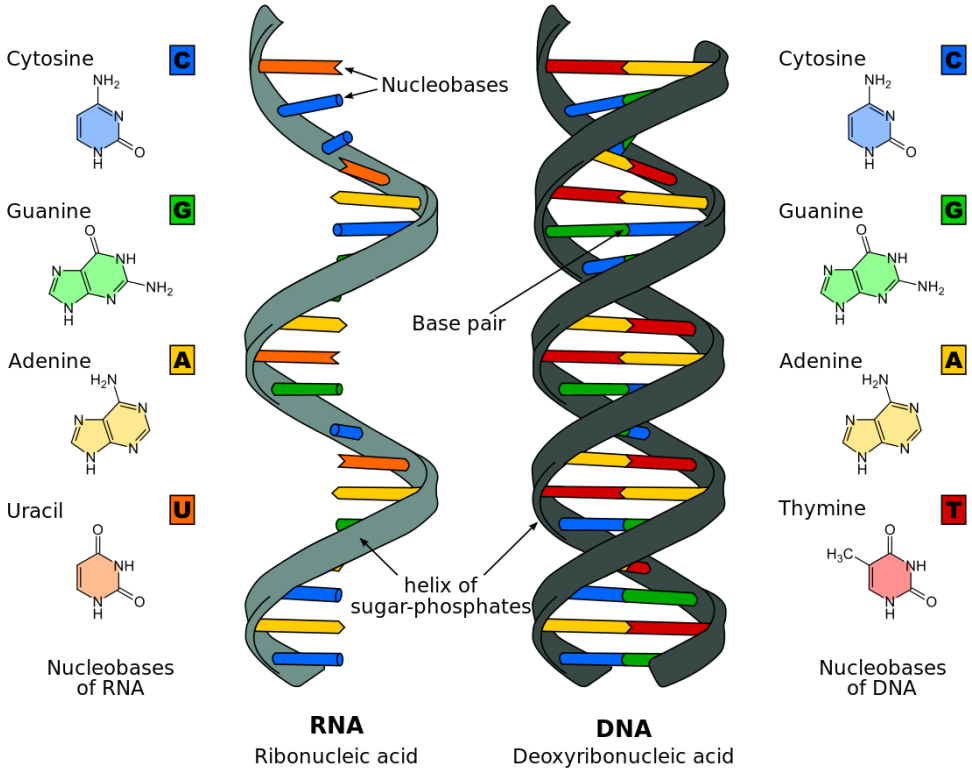


Figure 4: A comparison between the structure of DNA and the structure of RNA. This diagram shows how RNA is single-stranded and DNA is double-stranded, and that RNA contains uracil instead of thymine.

DNA Replication

DNA replication has been well studied in prokaryotes primarily because of the small size of the genome and because of the large variety of mutants that are available. *E. coli* has 4.6 million base pairs in a single circular chromosome and all of it gets replicated in approximately 42 minutes, starting from a single site along the chromosome and proceeding around the circle in both directions. This means that approximately 1000 nucleotides are added per second. Thus, the process is quite rapid and occurs without many mistakes.

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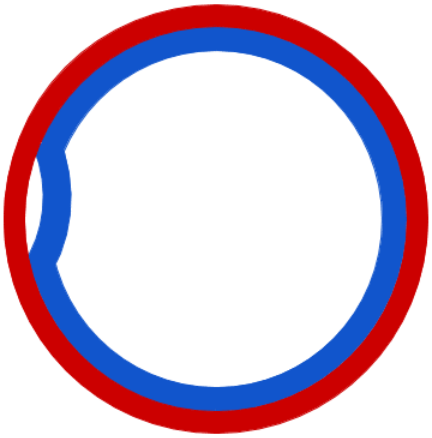


Figure 5: This animation shows how the circular bacterial chromosome is duplicated during DNA replication. The solid red circle and solid blue circle represent the two original DNA strands that are paired together in the bacterial chromosome. DNA replication begins at the origin of replication. DNA is gradually built to complement both the original DNA strands simultaneously (dotted red and dotted blue represent the new DNA being built to complement the original DNA strands). The end result is two bacterial chromosomes, each with one "old" and one "new" DNA strand. This is why the process of DNA replication is called **semiconservative**. One old strand is retained and paired with a new strand in each DNA molecule.

DNA replication employs a large number of structural proteins and enzymes, each of which plays a critical role during the process. One of the key players is the enzyme **DNA polymerase**, also known as DNA pol, which adds nucleotides one-by-one to the growing DNA chain that is complementary to the template strand. The addition of nucleotides requires energy; this energy is obtained from the nucleoside triphosphates ATP, GTP, TTP and CTP. Like ATP, the other **NTPs** (nucleoside triphosphates) are high-energy molecules that can serve both as the source of DNA nucleotides and the source of energy to drive the polymerization. When the bond between the phosphates is “broken,” the energy released is used to form the phosphodiester bond between the incoming nucleotide and the growing chain. In prokaryotes, three main types of polymerases are known: DNA pol I, DNA pol II, and DNA pol III. It is now known that DNA pol III is the enzyme required for DNA synthesis; DNA pol I is an important accessory enzyme in DNA replication, and along with DNA pol II, is primarily required for repair.

How does the replication machinery know where to begin? It turns out that there are specific nucleotide sequences called *origins of replication* where replication begins. In *E. coli*, which has a single origin of replication on its one chromosome (as do most prokaryotes), this origin of replication is approximately 245 base pairs long and is rich in AT sequences. The origin of replication is recognized by certain proteins that bind to this site. An enzyme called *helicase* unwinds the DNA by breaking the hydrogen bonds between the nitrogenous base pairs. ATP hydrolysis is required for this process. As the DNA opens up, Y-shaped structures called *replication forks* are formed. Two replication forks are formed at the origin of replication and these get extended bi-directionally as replication proceeds. **Single-strand binding proteins** coat the single strands of DNA near the replication fork to prevent the single-stranded DNA from winding back into a double helix.

DNA polymerase has two important restrictions: it is able to add nucleotides only in the 5' to 3' direction (a new DNA strand can be only extended in this direction). It also requires a free 3'-OH group to which it can add nucleotides by forming a phosphodiester bond between the 3'-OH end and the 5' phosphate of the next nucleotide. This essentially means that it cannot add nucleotides if a free 3'-OH group is not available. Then how does it add the first nucleotide? The problem is solved with the help of a primer that provides the free 3'-OH end. Another enzyme, RNA **primase**, synthesizes an RNA segment that is about five to ten nucleotides long and complementary to the template DNA. Because this sequence primes the DNA synthesis, it is appropriately called the

primer. DNA polymerase can now extend this RNA primer, adding nucleotides one-by-one that are complementary to the template strand.

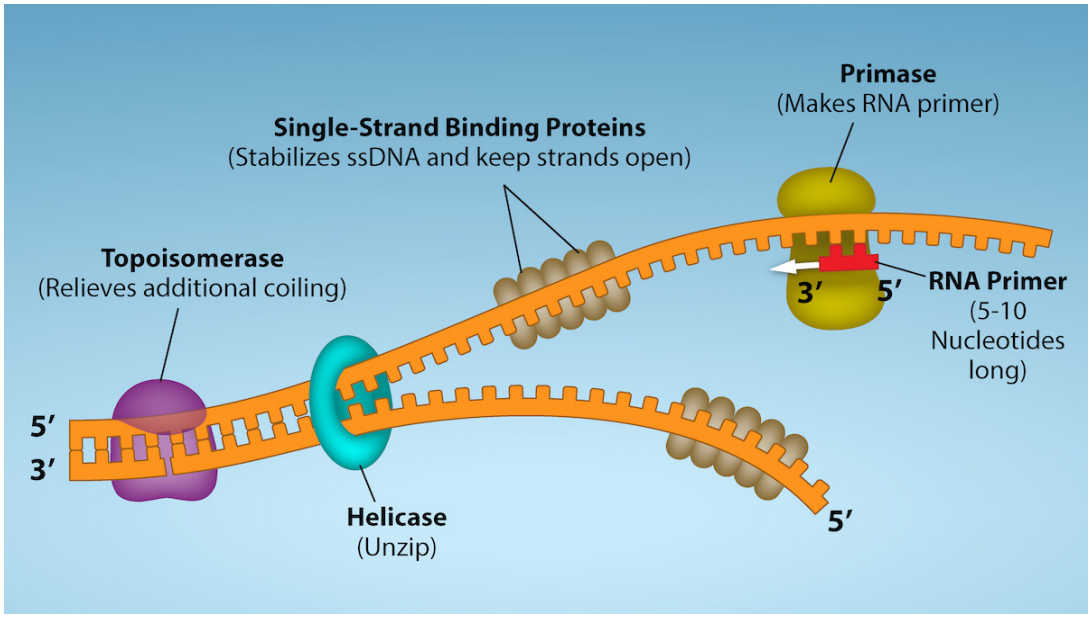


Figure 6: First Components of DNA Replication. As DNA replication begins, DNA Helicase, a large enzyme, separates the two strands of DNA so that they can act as templates for replication. Single-strand binding proteins bind to each strand to stabilize and prevent them from reforming the double helix. Primase, an RNA polymerase, binds to the single stranded DNA and synthesizes a short RNA primer in the 5' to 3' direction that is antiparallel to the parental strand. This RNA primer allows for DNA polymerase to begin replicating the DNA. Topoisomerase binds to the double helix upstream of the replication fork to prevent additional coiling by making small cuts in one of the DNA strands. Credit: Rao, A., Ryan, K. Fletcher, S. and Tag, A. Department of Biology, Texas A&M University.

The replication fork moves at the rate of 1000 nucleotides per second. Topoisomerase prevents the over-winding of the DNA double helix ahead of the replication fork as the DNA is opening up; it does so by causing temporary nicks in the DNA helix and then resealing it. Because DNA polymerase can only extend in the 5' to 3' direction, and because the DNA double helix is *antiparallel*, there is a slight problem at the replication fork. *The two template DNA strands have opposing orientations:* one strand is in the 5' to 3' direction and the other is oriented in the 3' to 5' direction. Only one new DNA strand, the one that is complementary to the 3' to 5' parental DNA strand, can be synthesized continuously towards the replication fork. This continuously synthesized strand is known as the **leading strand**. The other strand, complementary to the 5' to 3' parental DNA, is extended away from the replication fork, in small fragments known as **Okazaki fragments**, each requiring a primer to start the synthesis. New primer segments are laid down in the direction of the replication fork, but each pointing away from it. (Okazaki fragments are named after the Japanese scientist who first discovered them. The strand with the Okazaki fragments is known as the **lagging strand**.)

The leading strand can be extended from a single primer, whereas the lagging strand needs a new primer for each of the short Okazaki fragments. The overall direction of the lagging strand will be 3' to 5', and that of the leading strand 5' to 3'. A protein called the **sliding clamp** holds the DNA polymerase in place as it continues to add nucleotides. The sliding clamp is a ring-shaped protein that binds to the DNA and holds the polymerase in place. As synthesis proceeds, the RNA primers are replaced by DNA. The primers are removed by the exonuclease activity of DNA pol I, which uses DNA behind the RNA as its own primer and fills in the gaps left by removal of the RNA nucleotides by the addition of DNA nucleotides. The nicks that remain between the newly synthesized DNA (that replaced the RNA primer) and the previously synthesized DNA are sealed by the enzyme DNA **ligase**, which catalyzes the formation of phosphodiester linkages between the 3'-OH end of one nucleotide and the 5' phosphate end of the other fragment.

Once the chromosome has been completely replicated, the two DNA copies move into two different cells during cell division.

The process of DNA replication can be summarized as follows:

1. DNA unwinds at the origin of replication.
2. Helicase opens up the DNA-forming replication forks; these are extended bidirectionally.
3. Single-strand binding proteins coat the DNA around the replication fork to prevent rewinding of the DNA.
4. Topoisomerase binds at the region ahead of the replication fork to prevent supercoiling.
5. Primase synthesizes RNA primers complementary to the DNA strand.
6. DNA polymerase III starts adding nucleotides to the 3'-OH end of the primer.
7. Elongation of both the lagging and the leading strand continues.
8. RNA primers are removed by exonuclease activity.
9. Gaps are filled by DNA pol I by adding dNTPs.
10. The gap between the two DNA fragments is sealed by DNA ligase, which helps in the formation of phosphodiester bonds.

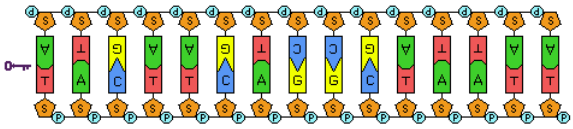


Figure 7: Animation of DNA replication. The double stranded DNA is separated. DNA pol III builds DNA complementary to the template strands (the strands that were separated). In this animation, the new strand on the bottom is the leading strand and is replicated continuously. In this animation, the new strand on the top is the lagging strand and is built in Okazaki fragments. DNA ligase repairs the nicks between the Okazaki fragments on the lagging strand to produce an unbroken DNA strand.

Enzyme/protein	Specific Function
DNA pol I	Removes RNA primer and replaces it with newly synthesized DNA
DNA pol III	Main enzyme that adds nucleotides in the 5'-3' direction
Helicase	Opens the DNA helix by breaking hydrogen bonds between the nitrogenous bases
Ligase	Seals the gaps between the Okazaki fragments to create one continuous DNA strand
Primase	Synthesizes RNA primers needed to start replication
Sliding Clamp	Helps to hold the DNA polymerase in place when nucleotides are being added
Topoisomerase	Helps relieve the strain on DNA when unwinding by causing breaks, and then resealing the DNA
Single-strand binding proteins (SSB)	Binds to single-stranded DNA to prevent DNA from rewinding back.



Laboratory Instructions

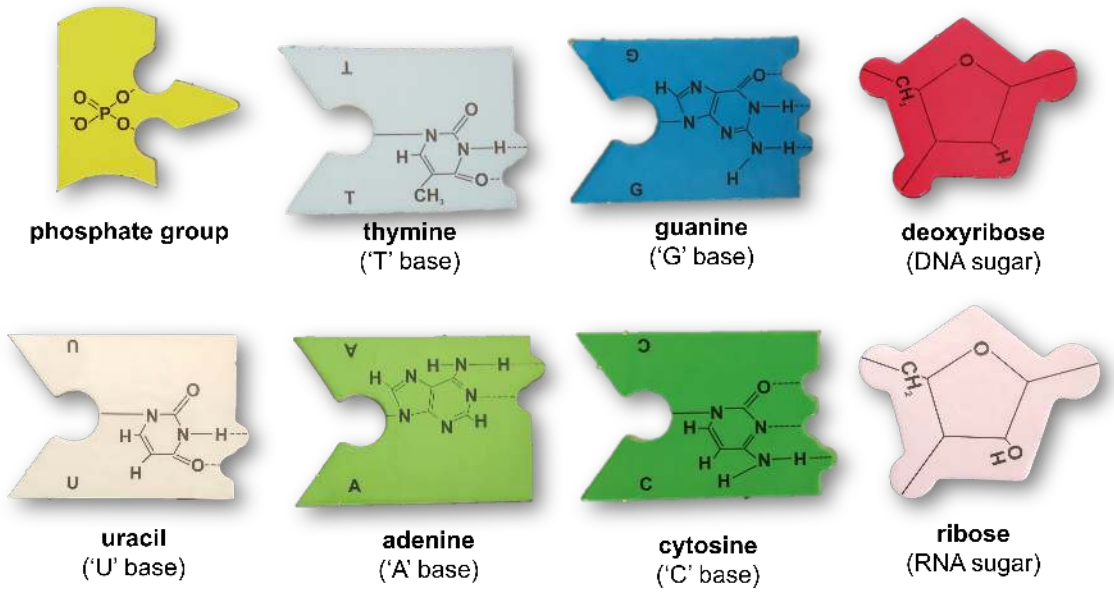


Figure 8: Puzzle pieces that can be used to build DNA and RNA.

DNA Structure & RNA Structure

1. Organize the puzzle pieces so that all of the puzzle pieces and in piles by type (e.g. one pile for phosphate groups, one pile for deoxyribose, one pile for guanine, etc.).
2. Answer questions 1-5 in the "Results & Questions" section under "DNA Structure."
3. Build eight DNA bases: one with adenine, one with thymine, one with guanine, and one with cytosine.
4. Build four RNA bases: one with adenine, one with uracil, one with guanine, and one with cytosine.
5. Answer questions 6-8 in the "Results & Questions" section under "DNA Structure."
6. Connect DNA nucleotides to each other to form the sequence: 5'-GTAC-3'
7. Connect RNA nucleotides to each other to form the sequence: 5'-GUAC-3'
8. Answer questions 9-11 in the "Results & Questions" section under "DNA Structure."
9. Build a complementary strand of DNA to the DNA sequence already built and pair them together.

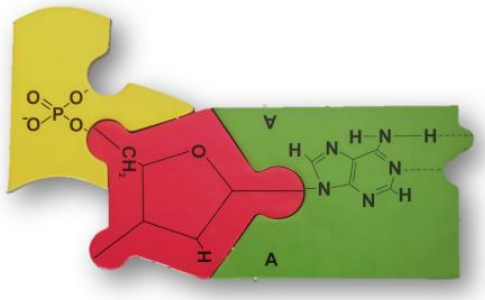
10. Answer questions 12-17 in the "Results & Questions" section under "DNA Structure."

DNA Replication

1. Build the following DNA nucleotides:
    - 4 adenine DNA nucleotides
    - 4 thymine DNA nucleotides
    - 8 guanine DNA nucleotides
    - 8 cytosine DNA nucleotides
2. Build a single strand of DNA with the following structure: 5'-AGCCTG-3'
3. Build the complementary DNA strand to the sequence above and pair it with the DNA molecule you made in step 2.
4. Answer questions 1-6 in the "Results & Questions" section under "DNA Replication."
5. The segment of DNA you built is the origin of replication. ***Presto-change-o!*** You are now the enzyme helicase! Do what helicase would do.
6. Answer questions 7-9 in the "Results & Questions" section under "DNA Replication."
7. ***Presto-change-o!*** You are now the enzyme DNA pol III (a DNA polymerase the elongates growing DNA strands during DNA replication). Build new DNA molecules to complement the template DNA strands starting at the 5' end and ending at the 3' end of the new strands (just like DNA pol III would do).
8. Answer questions 10-13 in the "Results & Questions" section under "DNA Replication."
9. Disassemble all of the puzzle pieces and return to the box/bag where they came from.

Results & Questions

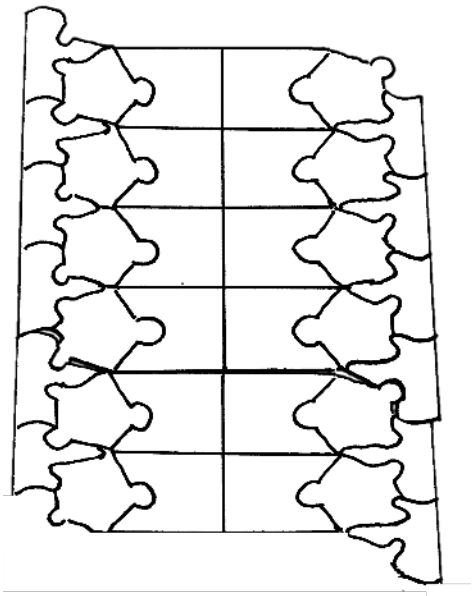
DNA Structure & RNA Structure



1. DNA nucleotides contain deoxyribose as the pentose (5-carbon) sugar and RNA nucleotides contain ribose as the pentose sugar. Compare the chemical structures written on the deoxyribose and ribose puzzle pieces. What is the difference between ribose and deoxyribose?
2. Consider your answer to question 1. What do you suppose "deoxy-" is referring to in the name "deoxyribose?"
3. Identify the different base puzzle pieces (there are 5 types). Examine the chemical structures of the bases written on these puzzle pieces. Why do you suppose that DNA and RNA bases are often referred to as "nitrogenous bases?"
4. Adenine and guanine are classified as "purines" and thymine, cytosine, and uracil are classified "pyrimidines." Looking at the chemical structures written on these puzzle pieces, what pattern do you notice that distinguishes purines from pyrimidines?
5. Adenine pairs with thymine in DNA and with uracil in RNA. Guanine pairs with cytosine in both DNA and RNA. What generalization can you make about base-pairing and whether bases are purines or pyrimidines (e.g. do purines pair with purines, to pyrimidines pair with pyrimidines, or do purines pair with pyrimidines)?
6. Compare the the DNA nucleotides with the RNA nucleotides. What similarities are there?

7. Compare the the DNA nucleotides with the RNA nucleotides. What differences are there?
8. On the image above showing a DNA nucleotide, write in 5' to show the 5' side of the nucleotide and write in 3' to show the 3' side of the nucleotide.
9. Covalent bonds were formed between the nucleotides you joined together. These covalent bonds have a special name. What are these bonds called?
10. Identify the 5' end and the 3' end of the DNA strand. Identify the 5' end of the RNA strand and the 3' end of the DNA strand. What component of the nucleotides do you find at the 5' end of these strands?
11. What is missing from the DNA puzzle?
12. Compare the DNA and RNA structures. What new difference is apparent in their structures?
13. Examine the two DNA strands paired together. Notice that one DNA strand faces 5' to 3' and the other DNA strand is in the reverse direction. What is this arrangement called?
14. The two DNA strands can easily be pulled apart from each other at the bases (unlike other locations in the puzzle, such as between the phosphates and the bases). Why is this? What types of interactions hold the bases of two DNA strands together?
15. What are hydrogen bonds?
16. How many hydrogen bonds are formed in the A-T pair?
17. How many hydrogen bonds are formed in the G-C pair?

DNA Replication



1. Fill in the diagram above with the following:
   - **phos.** = phosphate group
   - **deoxy.** = deoxyribose
   - **A** = adenine
   - **T** = thymine
   - **G** = guanine
   - **C** = cytoskne
   - **5'** - show the 5' end on ***both*** DNA strands
   - **3'** - show the 3' end on ***both*** DNA strands
2. The two DNA strands interact at the bases with hydrogen bonds. Are hydrogen bonds weak or strong attractions?
3. How might it be possible for the two DNA strands to separate at the bases?
4. What is the purpose of DNA replication?

5. Where on a DNA molecule does DNA replication begin?
6. What DNA replication enzyme breaks the hydrogen bonds between the nitrogenous bases to create single-stranded sections of DNA?
7. What did helicase do to the double-stranded DNA puzzle?
8. What enzyme elongates a growing DNA strand during DNA replication?
9. What end of a growing DNA molecule can the enzyme named in question 8 add new nucleotides to (5' or 3')?
10. What features of the actual process of DNA replication are missing from this re-creation of DNA replication? There are at least three.
11. Would DNA pol III be able to create a new DNA molecule such as we did in this puzzle re-creation of DNA replication? Explain your answer.
12. How is DNA replication a semiconservative process and how did we show this in the puzzle re-creation of DNA replication?
13. Compare the newly replicated DNA double-strands in your puzzle pieces. Closely examine the sequences of the DNA and the directions of the strands. Are the two double strands identical? Give the DNA sequences with 5' and 3' directionality to explain.

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1.31: PCR

Learning Objectives

- Define the following: PCR, amplify, *Taq* polymerase, primer, thermal cycler, denaturation, annealing, extension.
- Give at least three applications of PCR in microbiology.
- Tell what PCR does.
- Explain how PCR works including the three steps of PCR and what happens in each of those steps.
- Successfully conduct PCR in the laboratory.
- Successfully interpret results from a PCR experiment.

Introduction to PCR

Polymerase chain reaction (**PCR**) is a technique that scientists use to **amplify** (make many copies of) specific DNA regions for further analysis. PCR has a huge variety of applications including:

- diagnosis of a microbial infection
- studying disease-causing organisms
- determining the presence of difficult to culture, or unculturable, microorganisms in humans or environmental samples
- amplifying a target region of DNA for cloning into a plasmid vector
- detecting genetic diseases
- cloning gene fragments to analyze genetic diseases
- identifying contaminant foreign DNA in a sample
- preparing DNA for sequencing
- determining paternity
- identifying the source of a DNA sample left at a crime scene
- comparing samples of ancient DNA with modern organisms

Most methods of DNA analysis, such as restriction enzyme digestion and agarose gel electrophoresis, or DNA sequencing require large amounts of a specific DNA fragment. In the past, large amounts of DNA were produced by growing the host cells of a genomic library. However, libraries take time and effort to prepare and DNA samples of interest often come in minute quantities. The **polymerase chain reaction (PCR)** permits rapid amplification in the number of copies of specific DNA sequences for further analysis. One of the most powerful techniques in molecular biology, PCR was developed in 1983 by Kary Mullis while at Cetus Corporation.

Taq Polymerase: An Enzyme Making PCR Possible

PCR is an *in vitro* laboratory technique (done outside of cells - in this case in a small centrifuge tube) that takes advantage of the natural process of DNA replication. Recall that DNA replication requires a **DNA polymerase** enzyme to build a DNA molecule complementary to a template DNA molecule. In PCR, A heat-stable DNA polymerase enzyme is used since PCR requires high temperatures to **denature DNA** (separate double-stranded DNA to make the DNA single-stranded). The heat-stable DNA polymerase does not denature in these conditions since it is derived from a hyperthermophilic bacterial species ("loves" hot temperatures) called *Thermus aquaticus*. ***Taq polymerase*** is a DNA polymerase from *T. aquaticus* (taking "*T*" from the genus and "*aq*" from the first two letters of the specific epithet). *T. aquaticus was first* isolated from a hot spring in Yellowstone National Park and thrives in very hot temperatures.

DNA Replication (and PCR) Require Primers

DNA replication requires the use of primers for the initiation of replication. Recall that DNA polymerases can only elongate a DNA molecule and cannot build a new strand from the start. In living cells, DNA replication uses the enzyme primase to build primers composed of RNA that DNA polymerase can elongate to build new DNA strands. In the laboratory setting, RNA is not very stable stable, and therefore, DNA primers are used for PCR. **Primers** not only are necessary for a DNA polymerase to elongate and produce DNA copies, primers are used in PCR to target a specific DNA sequence that we want to copy. Primer sequences are specifically designed and engineered with a specific sequence in order to target a specific DNA region. This insures that only the DNA sequence we want to copy gets copied and not other places in the DNA template molecule(s).

For example, if PCR is used for diagnosis of a microbial infection in a human, primers would be designed with sequences that match a specific region of DNA in that microbe, and does not match DNA in other microbes or in humans (human DNA will be mixed with any samples taken from a human). If the DNA is successfully copied (amplified), and matches positive controls (samples that contain the microbe the test is looking for), that microbe is causing infection in that human.

PCR Uses Temperature Cycles to Amplify (Copy) DNA

PCR occurs over multiple cycles. Each cycle containing three steps: **denaturation**, **annealing**, and **extension**. Machines called **thermal cyclers** are used for PCR; these machines can be programmed to automatically cycle through the temperatures required at each of the denaturation, annealing, and extension steps.

1. **denaturation:** First, double-stranded template DNA containing the target sequence is denatured at approximately 95 °C. The high temperature required to physically (rather than enzymatically) separate the DNA strands is the reason the heat-stable DNA polymerase is required.
2. **annealing:** Next, the temperature is lowered to approximately 50 °C (although this can vary based on the PCR protocol and primers). This allows the DNA primers complementary to the ends of the target sequence to anneal (stick) to the template strands, with one primer annealing to each strand.
3. **extension:** Finally, the temperature is raised to 72 °C, the optimal temperature for the activity of *Taq* polymerase, allowing for the addition of nucleotides to the primer using the single-stranded target as a template.

Each cycle (denaturation, annealing, and extension) doubles the number of double-stranded target DNA copies. Typically, PCR protocols include 25–40 cycles, allowing for the amplification of a single target sequence by tens of millions to over a trillion.

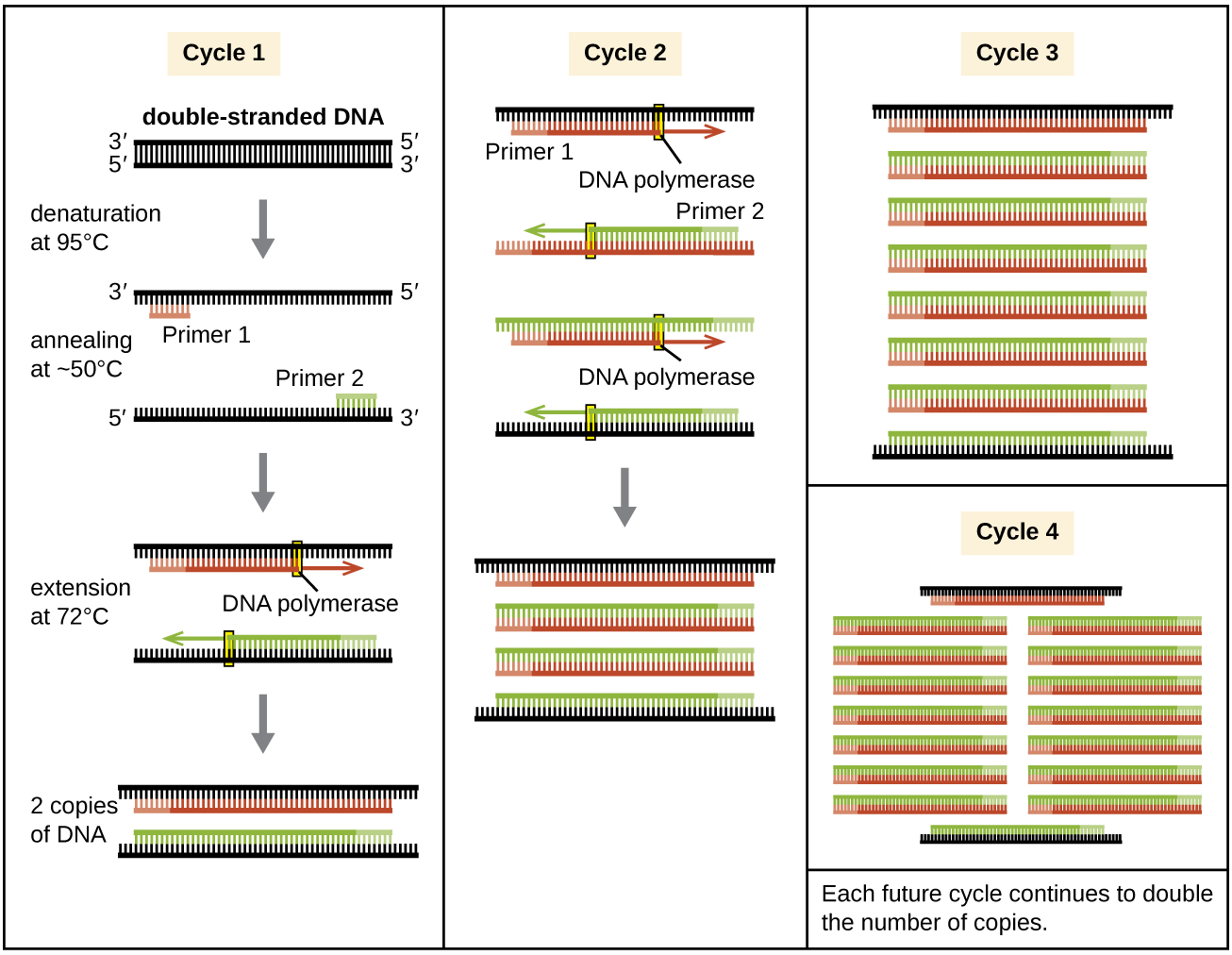


Figure 1: The polymerase chain reaction (PCR) is used to produce many copies of a specific sequence of DNA.



Video 1: Animation showing what occurs inside of a PCR tube during the PCR reaction. Pay careful attention to the thermometer in the top left corner since it is showing how the temperature changes created by the thermal cycler stimulates each of the PCR steps to occur.

Analysis of PCR Products

In order to analyze the PCR products after the DNA region of interest has been amplified, it is common for gel electrophoresis to be used to separate DNA based on size. The PCR products can then be compared with controls and other samples to make conclusions the sample. See the chapter on DNA Fingerprinting to see how gel electrophoresis works.



Figure 2: A gel (produced by gel electrophoresis) of samples amplified by PCR. The first lane (left) contains a **DNA ladder** with DNA fragments of known lengths. The DNA ladder is purchased from a supplier and contains a variety of DNA fragment lengths to enable comparison of the DNA fragment sizes of samples with unknown lengths. Notice that there is mainly one band in each of the other four wells. This is because the DNA in the other samples has been specifically targeted by PCR creating millions to trillions of DNA copies of the same section of DNA. If a sample did not contain the DNA region targeted by the primers, no band would be present.

Laboratory Instructions

Laboratory instructions will vary based on the protocol your instructor is following. Your instructor will provide specific instructions for this laboratory.

Questions

1. What does PCR stand for?
2. Give three examples of applications of PCR.
3. What does PCR do?
4. What cellular process does PCR mimic inside of a laboratory tube?
5. What is *Taq* polymerase and where does it come from?
6. How is *Taq* polymerase different from other DNA polymerases?
7. Why does PCR require a thermostable DNA polymerase?
8. Give two reasons why primers are used in PCR.
9. Explain how primers target a specific DNA sequence.
10. Why are DNA primers used in PCR when RNA primers are used inside cells?
11. What instrument is necessary for PCR and what does it do?
12. List the three steps of PCR and what happens in each step.
13. The three steps you listed in the previous question, how many times are they repeated during the PCR process?
14. How many DNA copies are produced of the primer-targeted DNA during PCR?

Attributions

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1.32: DNA Fingerprinting

Learning Objectives

- Give at least three applications for DNA fingerprinting.
- Explain/apply how restriction enzymes work, including be able to identify recognition sites/sequences and predict DNA fragment sizes from examples.
- Define and use the following terms: restriction enzyme, recognition site/sequence, sticky ends, blunt ends, restriction fragment length polymorphism (RFLP), gel electrophoresis.
- Explain/apply how gel electrophoresis works.
- Successfully load and run an electrophoresis gel.
- Analyze and interpret results from an electrophoresis gel.
- Analyze a viral outbreak scenario and determine the best course of action.

Applications of DNA Fingerprinting

DNA fingerprinting is a way to identify using DNA. Some applications of DNA fingerprinting include:

- identifying a microbe causing an infection (diagnostic test)
- identifying microbes for scientific research
- paternity testing
- forensic DNA analysis to match DNA to criminal suspects
- a wide variety of genetic research

DNA fingerprinting involves multiple biotechnologies, including PCR (see the chapter on PCR), but here this laboratory focuses on creating DNA fingerprints using restriction enzymes and visualizing the DNA fingerprints using gel electrophoresis.

Creating a DNA Fingerprint

Restriction Enzymes

DNA fingerprints are created by first isolating DNA from an unknown sample to be identified and compared with known samples. If the samples match, it enables identification. The isolated DNA (i.e. DNA that has been removed from cells and other cell components) is mixed with a **restriction enzyme** to create a fingerprint. The restriction enzyme will cut the DNA in a pattern that will differ from DNA from other sources, unless the identify of the DNA is the same (matching known and unknown samples enables identification).

The DNA fragments produced by the restriction enzyme are separated by size using an approach called gel electrophoresis (see the Gel Electrophoresis section below). The result is a pattern of bands that can be compared with other patterns from known samples. If fingerprints match, it likely means that the DNA originated from the same organism. For paternity testing, half of the fingerprint will originate from the biological mother and half of the fingerprint will originate from the biological father.

Restriction enzymes are found in some bacteria and have been isolated to use for a variety of biotechnologies such as DNA fingerprinting. These enzymes cut DNA at a characteristic **recognition site.** Recognition sites are different for each restriction enzyme. Typically, recognition sites are palindromic, that is they read the same backwards and forwards. Ordinary words that are palindromic include "mom," "dad," "wow," and "racecar." With DNA, a palindrome is based on reading one DNA strand 5' to 3' and comparing it with its complement DNA strand as read 5' to 3'. For example:

5'-GAATTC-3'

3'-CTTAAG-5'

Notice that the complementary DNA strands above, if reading from the 5' end, have the same sequence: 5'-GAATTC-3'. This example is a recognition sequence from the restriction enzyme known as *Eco*RI. The "*Eco*" part of the enzyme name comes from the fact that this enzyme originates from *Escherichia coli* (*E. coli*). The rest of the restriction enzyme name comes from the strain of the organism (in this case the R strain) and the order in which the enzyme was discovered (in this case it is the first restriction enzyme discovered from the R strain of *E. coli* - I is the Roman numeral for 1).

In the example of EcoRI, this enzyme cuts the DNA between the "G" and the "A" on the 5' side. As a result, both the top and bottom DNA strands are cut and only held together by the few hydrogen bonds between the 5'-AATT-3' on both strands. Because of this (and that hydrogen bonds are rather weak attractions), the two DNA strands fully separate leaving the 5'-AATT-3' overhangs on both broken strands. These overhangs are called **sticky ends**.

Restriction enzymes ***will identify every location on a DNA molecule with the recognition sequence and cut the DNA there***. This means the a restriction enzyme will likely make multiple cuts in the DNA. This will produce DNA fragments of different numbers of fragments with different sizes based on the base sequence of the DNA. The fragment sizes and number of fragments produces the DNA fingerprint used for identification purposes in DNA fingerprinting.

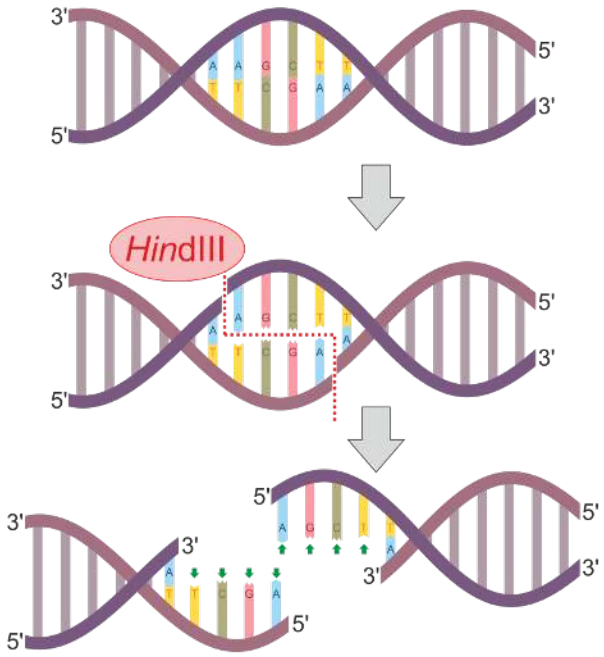


Figure 1: *Hin*dIII is an example of a restriction enzyme. This enzyme has the recognition sequence of 5'-AAGCTT-3'. Notice that this sequence is also a palindrome since its complementary strand will also have the 5'-AAGCTT-3' sequence (just antiparallel). This enzyme scans the entire DNA molecule for this specific sequence. When this sequence is found, it will cut between the "A" and "A" bases. This happens on both DNA strands to produce sticky ends (overhangs). In this case, the overhangs have the sequence 5'-AGCT-3'.



DNA Fingerprinting is also called Restriction Fragment Length Polymorphisms (RFLPs) Analysis

Restriction enzyme recognition sites are short (only a few nucleotides long), sequence-specific palindromes, and may be found throughout the genome. Thus, differences in DNA sequences in the genomes of individuals will lead to differences in distribution of restriction enzyme recognition sites that can be visualized as distinct fingerprints using a technique called gel electrophoresis (see the seciton on Gel Electrophoresis below). **Restriction fragment length polymorphism (RFLP)** analysis compares DNA banding patterns of different DNA samples after restriction digestion.

RFLP analysis has many practical applications in both medicine and forensic science. For example, epidemiologists use RFLP analysis to track and identify the source of specific microorganisms implicated in outbreaks of food poisoning or certain infectious diseases. RFLP analysis can also be used on human DNA to determine inheritance patterns of chromosomes with variant genes, including those associated with heritable diseases or to establish paternity.

Forensic scientists use RFLP analysis as a form of DNA fingerprinting, which is useful for analyzing DNA obtained from crime scenes, suspects, and victims. DNA samples are collected, the numbers of copies of the sample DNA molecules are increased using PCR, and then subjected to restriction enzyme digestion and agarose gel electrophoresis to generate specific banding patterns. By comparing the banding patterns of samples collected from the crime scene against those collected from suspects or victims, investigators can definitively determine whether DNA evidence collected at the scene was left behind by suspects or victims.

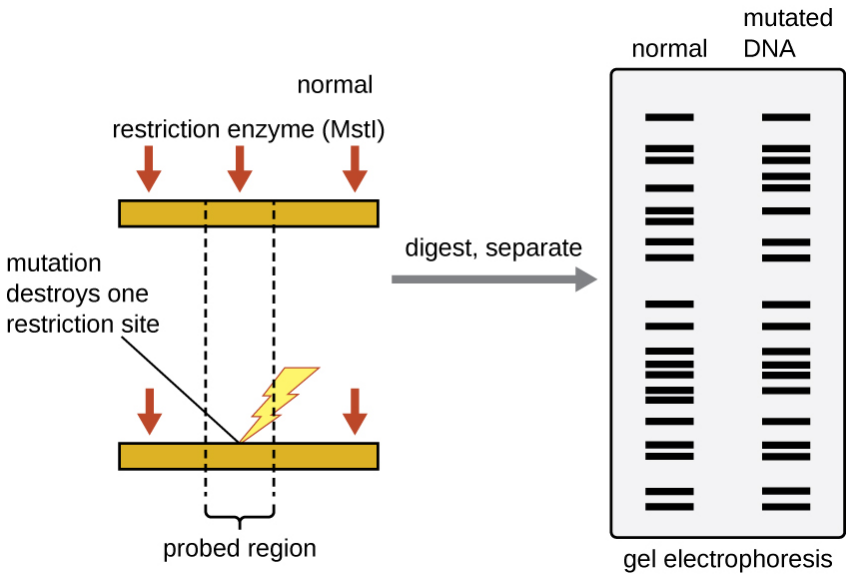


Figure 2: RFLP analysis can be used to differentiate DNA sequences. In this example, a normal chromosome is digested into two fragments, whereas digestion of a mutated chromosome produces only one fragment. The small red arrows pointing to the two different chromosome segments show the locations of the restriction enzyme recognition sites. After digestion and agarose gel electrophoresis, the banding patterns reflect the change by showing the loss of two shorter bands and the gain of a longer band. Each band produced by gel electrophoresis is a DNA fragment of different size. (credit: modification of work by National Center for Biotechnology Information)

Gel Electrophoresis

Gel electrophoresis is a technique commonly used to separate biological molecules based on size and biochemical characteristics, such as charge and polarity. **Agarose gel electrophoresis** is widely used to separate DNA (or RNA) of varying sizes that may be generated by restriction enzyme digestion (such as DNA fingerprinting / RFLP analysis) or by other means, such as the PCR.

Important

DNA molecules have an overall negative charge. This is due to negative charges on the phosphate groups of its nucleotides. As a result, DNA can be pulled toward a positive charge. This is how gel electrophoresis pulls DNA through an agarose gel.

Due to its negatively charged backbone, DNA is strongly attracted to a positive electrode. In agarose gel electrophoresis, the gel is oriented horizontally in a buffer solution. Samples are loaded into sample wells on the side of the gel closest to the negative electrode, then drawn through the molecular sieve of the agarose matrix toward the positive electrode. The agarose matrix impedes the movement of larger molecules through the gel, whereas smaller molecules pass through more readily. Thus, the distance of migration is inversely correlated to the size of the DNA fragment, with smaller fragments traveling a longer distance through the gel. Sizes of DNA fragments within a sample can be estimated by comparison to fragments of known size in a DNA ladder also run on the same gel.

Important

Small DNA fragments travel farther through the electrophoresis gel than larger DNA fragments.

You can think about this as being analagous to rocks in a river. Large boulders do not move very far, even if the current is swift, but small pebbles are capable of moving great distances in river's current. Similarly, large DNA cannot move well through the gel and will remain closer to the wells and smaller DNA can move easily through the gel and will move farther away from the wells.

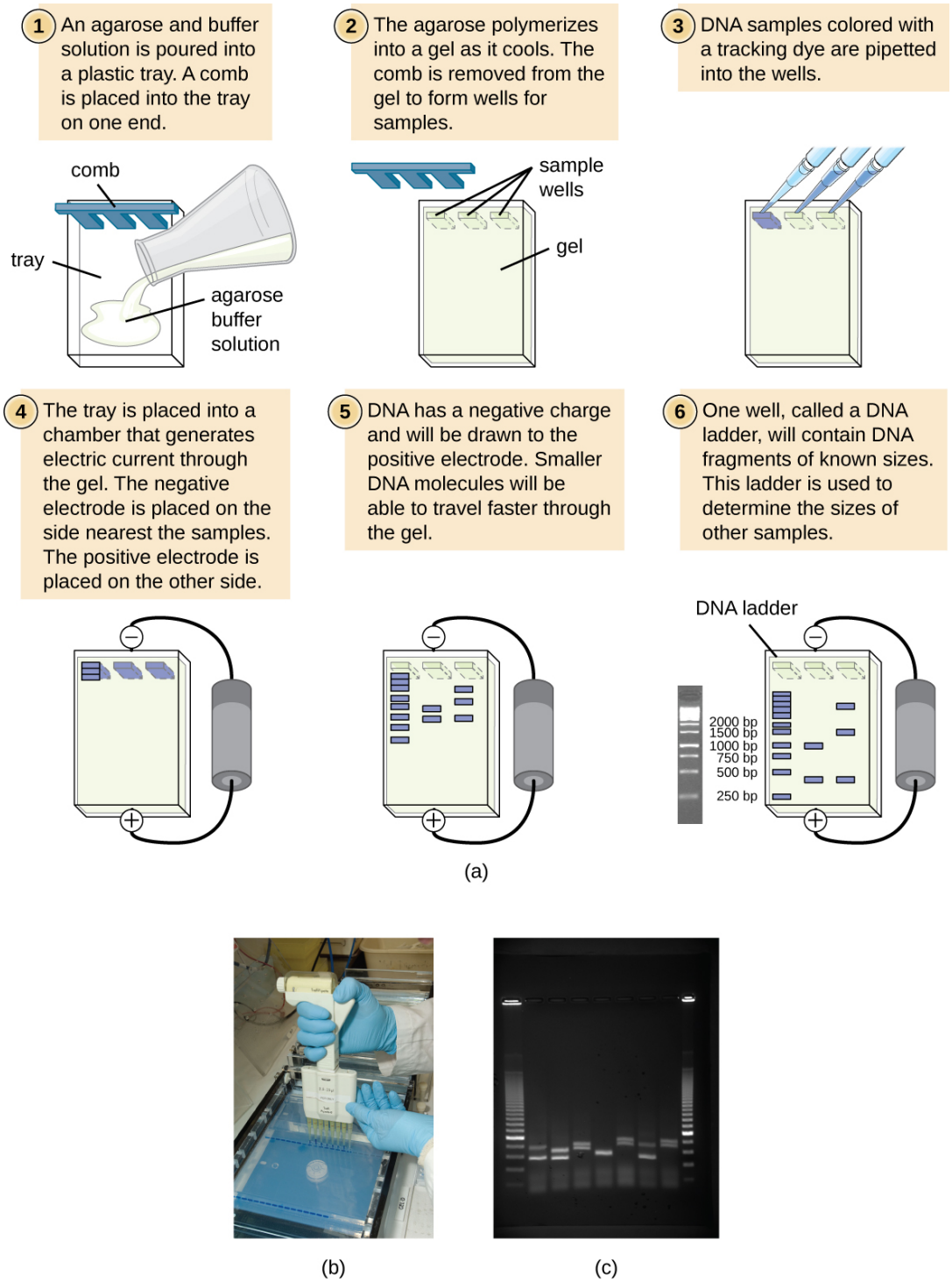


Figure 3: (a) The process of agarose gel electrophoresis. (b) A researcher loading samples into a gel. (c) This photograph shows a completed electrophoresis run on an agarose gel. The DNA ladder is located in lanes 1 and 9. Seven samples are located in lanes 2 through 8. The gel was stained with ethidium bromide and photographed under ultraviolet light. (credit a: modification of work by Magnus Manske; credit b: modification of work by U.S. Department of Agriculture; credit c: modification of work by James Jacob)



Laboratory Instructions

DNA Fingerprinting Scenario

You are an epidemiologist responsible for tracking outbreaks of potentially deadly disease and coordinating responses to help contain the spread of these diseases. It has been reported that a new viral infection is spreading in the Midwestern United States. This infection has symptoms very similar to two pervious viral outbreaks:

1. the East Coast viral outbreak produced disease the progressed to multiple organ failure and death
2. the West Coast viral outbreak produced disease with manageable symptoms and did not progress to organ failure or death

With this new outbreak in the Midwest, you believe that the virus causing the infections is either the a virus similar to the East Coast outbreak or the West Coast outbreak. How you organize the response to this outbreak is going to be dependent on whether the virus in the Midwest is the more severe strain or the less severe strain.

To determine if the virus is the East Coast virus or the West Coast virus, you conduct RFLP analysis (aka DNA fingerprinting) by extracting the viral DNA and digesting it with a restriction enzyme. To visualize the DNA fingerprint and compare it with the DNA fingerprint of the East Coast and West Coast viruses, you must use gel electrophoresis and then analyze the results.

Load and Run an Electrophoresis Gel

1. If the agarose gel has been prepared for you, place it into an electrophoresis chamber, making sure that the wells are positioned on the negative side (black-colored electrode).
2. Cover the gel completely with running buffer.
3. Use a micropipette and sterile tip to carefully load 20 µl of DNA with loading dye into the wells of the gel as instructed by your instructor. Here are some important things to consider:
    - Make sure the micropipette tip is inside the well before pushing down the plunger to put the DNA in the well.
    - Careful that the micropipette tip does not go too far into the well since it can break the bottom of the well open and release your DNA into the buffer rather than into the well.
    - When you are ready, push down the plunger slowly so the DNA does not get pushed out of the well from the force of it being expelled out of the micropipette.
    - Careful not to release the plunger while still inside of the well since this will suck the DNA back up into the micropipette tip.
4. When the gel is loaded, close the electrophoresis chamber and plug it into a power source, making sure that black is plugged into black and red is plugged into red.
5. Set the voltage of the power source as instructed by your instructor and turn the power on.

6. Watch as bubbles are generated in the buffer as the electrical current passes through the buffer.
7. Check back in 10-15 minutes to see how the loading dye has moved across the gel.
8. At the time instructed by your instructor, turn off the power supply.
9. Unplug the leads from the power supply.
10. Remove the top of the electrophoresis chamber.
11. Carefully remove the gel in its plastic holder and follow your instructor's instructions for staining (if there is time / if you are proceeding to staining in class).

Results & Questions

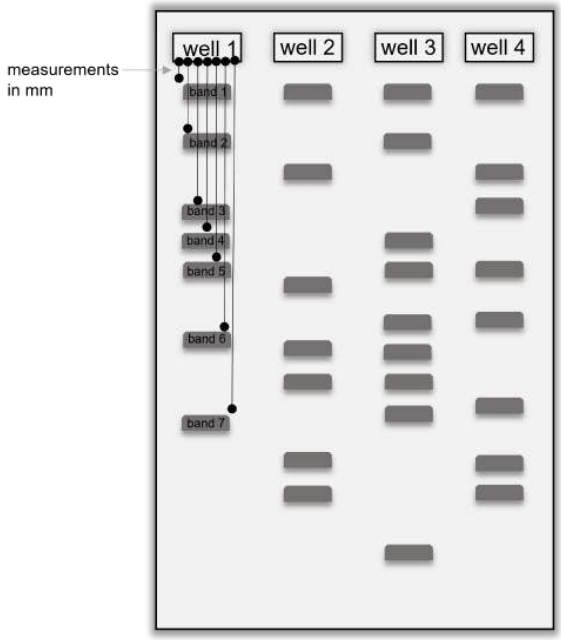


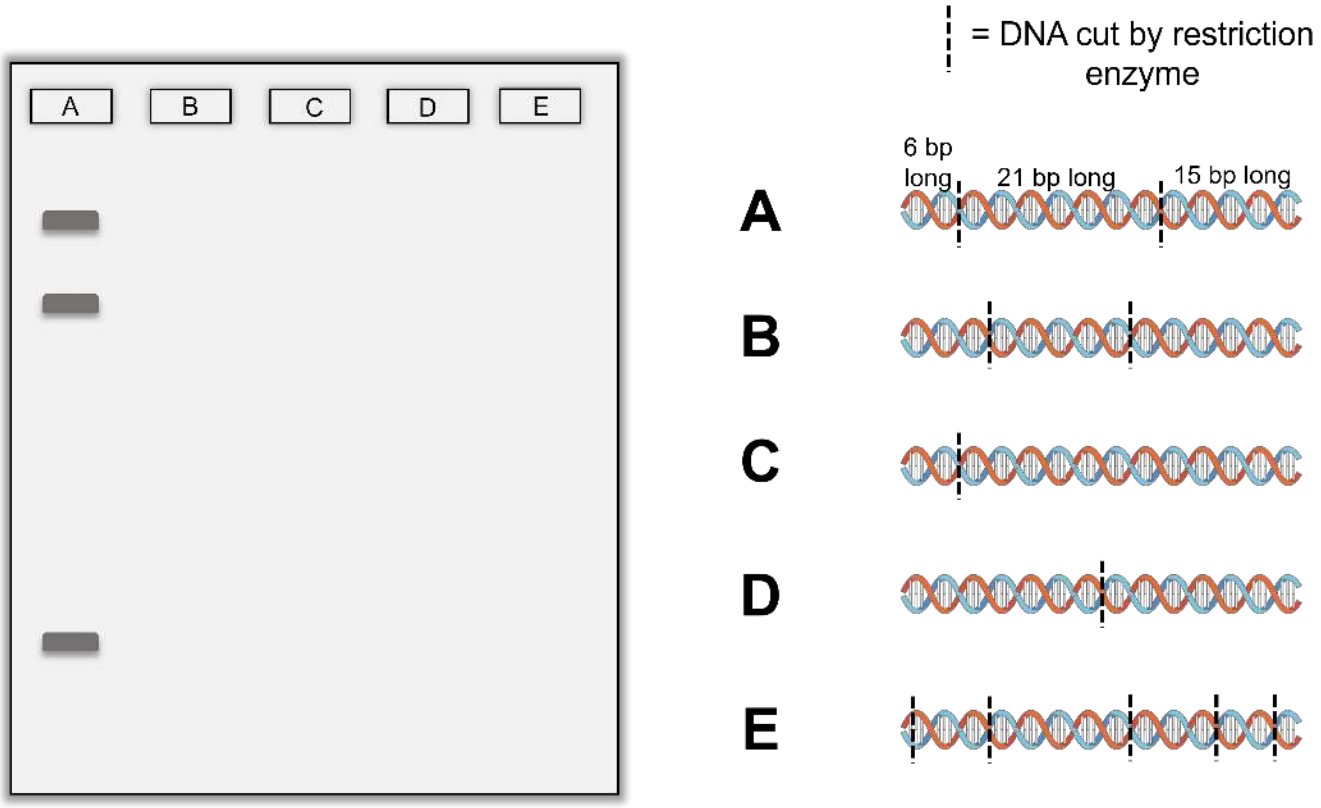
Figure 4: Diagram showing a gel with DNA banding patterns. The wells are numbered to show how to fill out the table below. Black lines show how to measure the migration distance of each band in a single well and how to number the bands, starting with the band closest to the well. Measure band migration distances in millimeters (mm) and be sure to measure each band migration from the edge of the well.

Table 1: Results from gel electrophoresis of DNA fingerprints of East coast virus, West coast virus, and Midwest virus. Some of the columns and rows may not be used in the table below, depending on how your gel was set up and the number of bands in each lane.

	well 1	well 2	well 3	well 4	well 5	well 6
sample in this well:						
band 1 migration (mm)						
band 2 migration (mm)						
band 3 migration (mm)						
band 4 migration (mm)						
band 5 migration (mm)						
band 6 migration (mm)						
band 7 migration (mm)						
band 8 migration (mm)						
band 9 migration (mm)						
band 10 migration (mm)						

1. Analyze the banding pattern on the gel by measuring the distance each band traveled from the well in millimeters (mm). See the diagram above to assist with how to make these measurements. ***Always measure the migration distance from the well, not from the previous band.*** Fill in the table above with your results.
2. Carefully examine the banding pattern in each well. Are there any banding patterns that are similar or the same? If so, which ones?
3. What does it indicate when a known and unknown DNA fingerprint have the same banding pattern?
4. Based on the results you analyzed on the gel, what conclusions can you make about the Midwest virus?
5. Based on the conclusion you made above about the Midwest virus, do you think a lock-down quarantine is necessary to contain the outbreak? Explain your answer.
6. Why is a restriction enzyme is used during DNA fingerprinting?
7. Why is it necessary to compare a DNA fingerprint of an unknown sample to DNA fingerprints of known samples?
8. Why is it that DNA from different sources will produce different banding patterns from each other when you use the same restriction enzyme? Use the following in your answer: restriction enzyme, base sequence, recognition site, fragment size, fragment number
9. How does gel electrophoresis separate DNA based on size? In your answer, be sure to mention: DNA's charge, electrophoresis current, agarose gel
10. Fill in the blank: Larger DNA fragments will produce bands _____ to/from the wells.
11. Fill in the blank: Smaller DNA fragments will produce bands _____ to/from the wells.
12. Below are illustrations of DNA that has been digested by a restriction enzyme in the locations shown with the dotted lines. The bands produced by sample A has been placed on the gel for you. Draw in bands in lanes B through E the correct positions we

would expect to see the bands based on the DNA fragment sizes and fragment numbers shown and relative to the sizes and positions of the bands already in the first lane for sample A.



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1.33: Bacterial Transformation

Learning Objectives

- Describe and explain Griffith's experiment originating bacterial transformation.
- Define and properly use the following terms: transformation, recombinant DNA, transgenic, competent cells, biotechnology, vector, genetic engineering, plasmid, horizontal gene transfer, selectable marker, GFP.
- Tell at least two applications for bacterial transformation.
- Explain how bacterial transformation works.
- Successfully conduct a bacterial transformation.
- Interpret results from bacterial transformation.

The Origins of Bacterial Transformation

British bacteriologist Frederick Griffith reported the first demonstration of bacterial **transformation**—a process in which external DNA is taken up by a cell, thereby changing its morphology and physiology. Griffith conducted his experiments with *Streptococcus pneumoniae*, a bacterium that causes pneumonia. Griffith worked with two strains of this bacterium called rough (R) and smooth (S). The two cell types were called “rough” (R) and “smooth” (S) after the appearance of their colonies grown on a nutrient agar plate.

The R strain is non-pathogenic (does not cause disease). The S strain is pathogenic (disease-causing), and has a capsule outside its cell wall. The capsule allows the cell to escape the immune responses of the host mouse.

When Griffith injected the living S strain into mice, they died from pneumonia. In contrast, when Griffith injected the live R strain into mice, they survived. In another experiment, when he injected mice with the heat-killed S strain, they also survived. This experiment showed that the capsule alone was not the cause of death. In a third set of experiments, a mixture of live R strain and heat-killed S strain were injected into mice, and—to his surprise—the mice died. Upon isolating the live bacteria from the dead mouse, only the S strain of bacteria was recovered. When this isolated S strain was injected into fresh mice, the mice died. Griffith concluded that something had passed from the heat-killed S strain into the live R strain and transformed it into the pathogenic S strain. He called this the *transforming principle*. These experiments are now known as Griffith's transformation experiments.

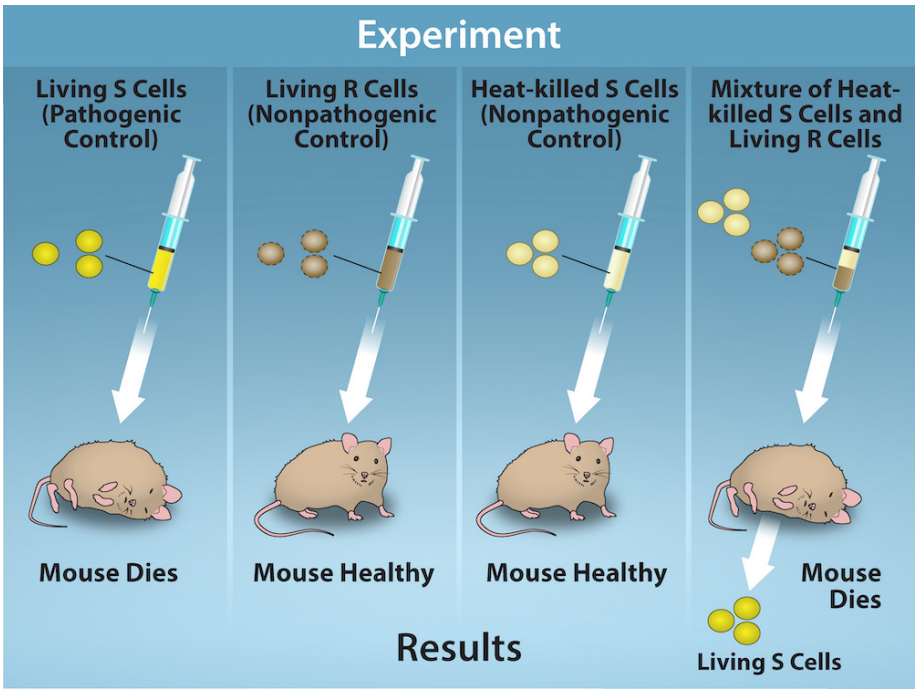


Figure 1: Two strains of *S. pneumoniae* were used in Griffith's transformation experiments. The R strain is non-pathogenic, whereas the S strain is pathogenic and causes death. When Griffith injected a mouse with the heat-killed S strain and a live R strain, the mouse died. The S strain was recovered from the dead mouse. Griffith concluded that something had passed from the heat-killed S strain to the R strain, transforming the R strain into the S strain in the process. Credit: Rao, A., Ryan, K. and Tag, A. Department of Biology, Texas A&M University.

What occurred in Griffith's experiments in 1928 could not be fully explained at that time. Scientists then were unaware of the role of DNA blueprints for a living thing. Further, with such limited knowledge about DNA and what it does, scientists then were also unaware that bacteria are capable of "picking up" DNA from other bacterial cells or picking up DNA from their environment.

In Griffith's experiment, DNA from the dead S strain were transferred into the live R strain. This DNA provided the R strain with instructions for building the S strain's capsule. As a result, the live R strain cells began to produce the S strain's capsule and thereby developed the ability to evade the host's immune system. The result is the R strain acted in the same way as the S strain, became pathogenic, and caused death in the mice. In essence, what happened in these circumstances is **bacterial transformation**. Bacterial transformation is a process where bacteria absorb DNA from their environment resulting in new characteristics in the bacteria. The new characteristics that transformed bacteria (also called **transgenic** bacteria) exhibit is dependent on what types of genes are present in the DNA that they took up from their environment.

Definition

transgenic: describes a living thing that has DNA introduced into it through artificial means; the DNA added to the transgenic organism originates from a different species



Why Transform Bacteria?

Using living systems to benefit humankind is called **biotechnology**. Technically speaking, the domestication of plants and animals through farming and breeding practices is a type of biotechnology, however in a contemporary sense, we associate biotechnology with the direct alteration of an organism's genetics to achieve desirable traits through the process of **genetic engineering**.

Genetic engineering involves the use of **recombinant DNA technology**, the process by which a DNA sequence is manipulated *in vitro*, thus creating **recombinant DNA molecules** that have new combinations of genetic material. The recombinant DNA is then introduced into a host organism. If the DNA that is introduced comes from a different species, the host organism is considered to be **transgenic**.

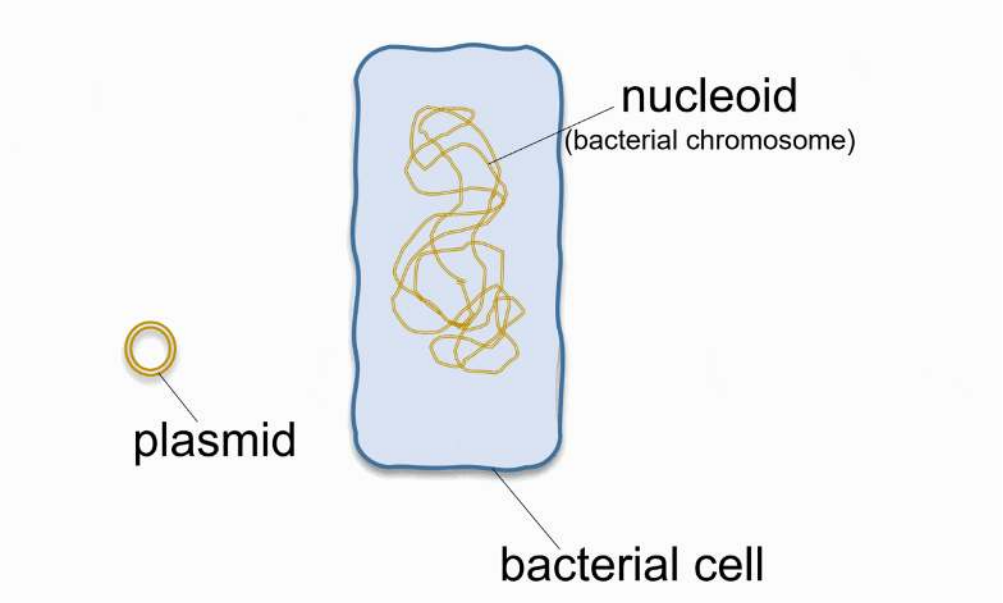


Figure 2: Bacterial transformation commonly uses a plasmid to carry a gene of interest into a bacterial cell. During transformation, a bacterial cell takes up the plasmid. Afterward, that bacterial cell will contain the genes in that plasmid and can express those genes.

One example of a transgenic microorganism is the bacterial strain that produces human insulin. The insulin gene from humans was inserted into a plasmid. This recombinant DNA plasmid was then inserted into bacteria. As a result, these transgenic microbes have the DNA necessary to produce and secrete human insulin.

Many prokaryotes have the ability to acquire foreign DNA and incorporate functional genes into their own genome through "mating" with other cells (conjugation), viral infection (transduction), and taking up DNA from the environment (transformation). These mechanisms of DNA transfers are examples of **horizontal gene transfer**—the transfer of genetic material between cells of the same generation.

Beyond applications that generate useful products for humans, transformation is incredibly useful in studying genetics. Transformation has enabled scientists to expand understanding of the functions of specific genes and regulation of gene expression (which genes are turned on/off in what circumstances, and how those genes are regulated).

How does Bacterial Transformation Work?

Plasmids

A gene of interest (a segment of DNA that codes for a gene to be inserted into a living thing) are commonly inserted into plasmids. Plasmids are small pieces of typically circular, double-stranded DNA that replicate independently of the bacterial chromosome. In recombinant DNA technology, plasmids are often used as **vectors** - they are used for transferring DNA. Plasmids can be used to carry DNA fragments from one organism to another.

Plasmids used as vectors can be genetically engineered by researchers and scientific supply companies to have specialized properties. Some plasmid vectors contain genes that confer a **selectable marker** to help researchers separate bacteria carrying the plasmid from bacteria not carrying the plasmid. One of the most common selectable markers used is antibiotic resistance genes. For this, the plasmid is engineered with an antibiotic resistance gene so that all bacteria carrying the plasmid are resistant to that antibiotic. If the bacteria are then plated on petri plates containing the antibiotic in the medium, then only those bacteria that carry the plasmid with its antibiotic resistance gene will grow. Bacteria without the plasmid will be killed by the presence of the

antibiotic in the medium since they lack the antibiotic resistance gene. The colonies that grow in the presence of the antibiotic can therefore be used in the ongoing research knowing that they carry the plasmid.

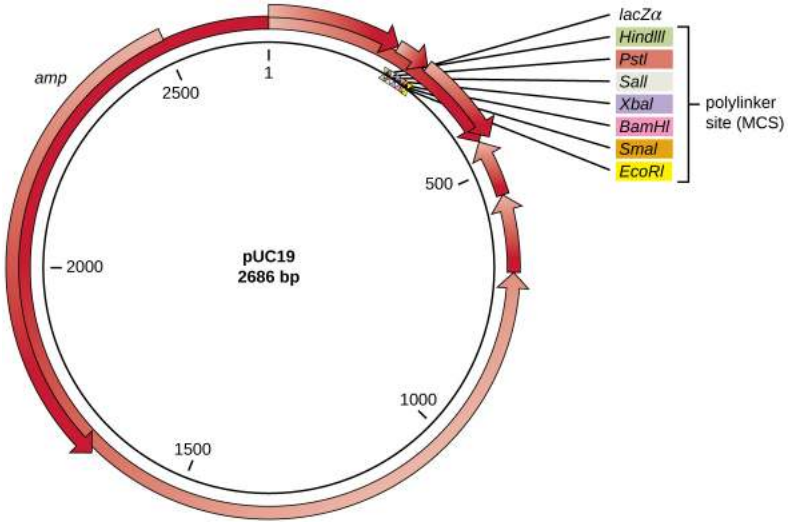


Figure 3: Diagram of an example of an artificially constructed plasmid vector commonly used for cloning foreign DNA. This plasmid's name is pUC19 and it is 2,686 base pairs long. The red and pink arrows represent the regions of DNA in this plasmid. The arrows indicate the directions in which the genes are transcribed. Note the ampicillin resistance gene (*amp*) encoded on the plasmid is used as the selectable marker. Plasmids are also engineered with a polylinker site, containing multiple unique restriction enzyme recognition sites (these recognition sites are locations where enzymes called restriction enzymes can be used to cut the DNA of the plasmid to insert a gene of interest into the plasmid). The polylinker site in this example is positioned within the *lacZ* gene. The *lacZ* gene can be used as a reporter gene in order to determine if the gene of interest was successfully inserted into the plasmid. For more about this, see this page about blue-white screening.

Bacterial Transformation

The most commonly used mechanism for introducing engineered plasmids into a bacterial cell is transformation, a process in which bacteria take up free DNA from their surroundings. In nature, free DNA typically comes from other lysed bacterial cells. In the laboratory, free DNA in the form of recombinant plasmids is introduced to the cell's surroundings.

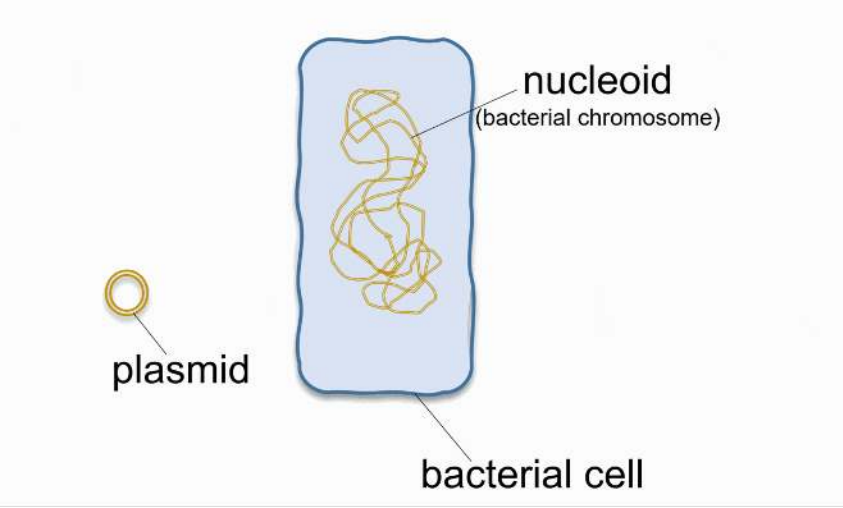


Figure 4: Bacterial transformation commonly uses a plasmid to carry a gene of interest into a bacterial cell. During transformation, a bacterial cell takes up the plasmid. Afterward, that bacterial cell will contain the genes in that plasmid and can express those genes.

Some bacteria, such as *Bacillus* spp., are naturally competent, meaning they are able to take up foreign DNA. However, not all bacteria are naturally competent. In most cases, bacteria must be made artificially competent in the laboratory by increasing the permeability of the cell membrane. This can be achieved through chemical treatments that neutralize charges on the cell membrane or by exposing the bacteria to an electric field that creates microscopic pores in the cell membrane. These methods yield chemically competent or electrocompetent bacteria, respectively.

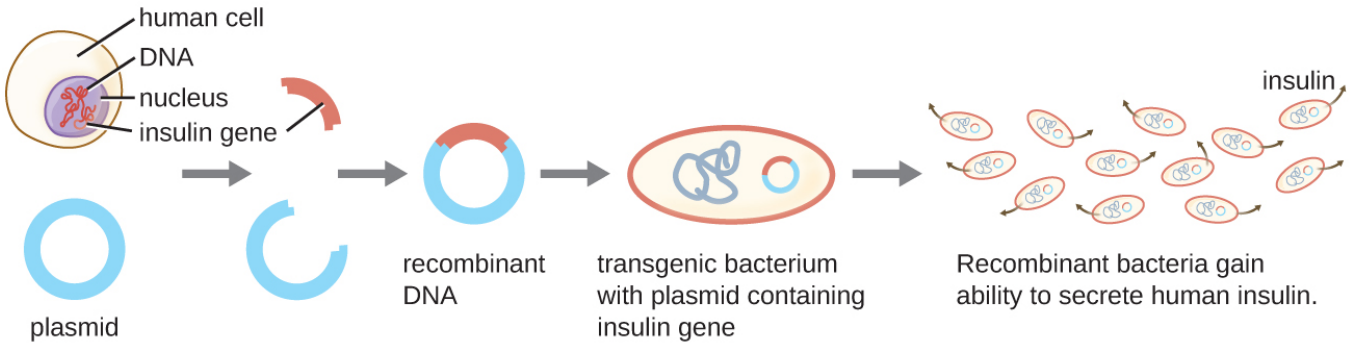


Figure 5: Bacterial transformation process shown with the example of the insulin gene. Recombinant DNA technology is the artificial recombination of DNA from two organisms. In this example, the human insulin gene is inserted into a bacterial plasmid. This recombinant plasmid can then be used to transform bacteria, which gain the ability to produce the insulin protein.



GFP

GFP stands for **green fluorescent protein**. This protein is coded in a gene that originated in the jellyfish species *Aequorea victoria*. GFP is commonly used in genetics research to visualize expression of a gene. GFP glows fluorescent green under ultraviolet light.

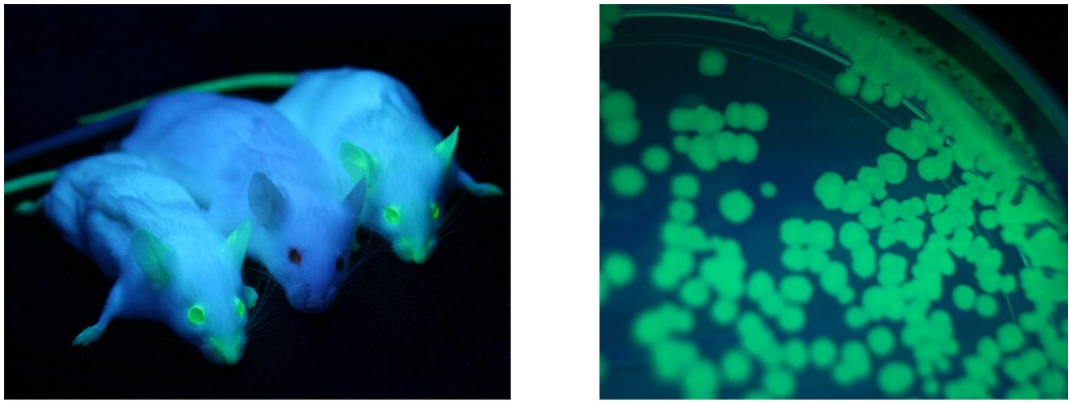


Figure 6: (a) The gene encoding green fluorescence protein is a commonly used reporter gene for monitoring gene expression patterns in organisms. Under ultraviolet light, GFP fluoresces. Here, two mice are expressing GFP, while the middle mouse is not. (b) GFP can be used as a reporter gene in bacteria as well. Here, a plate containing bacterial colonies expressing GFP are shown. (credit a: modification of work by Ingrid Moen, Charlotte Jevne, Jian Wang, Karl-Henning Kalland, Martha Chekenya, Lars A Akslen, Linda Sleire, Per Ø Enger, Rolf K Reed, Anne M Øyan, Linda EB Stuhr; credit b: modification of work by "2.5JIGEN.com"/Flickr)

Laboratory Instructions

Your instructor will provide instructions for the transformation protocol you will use in this laboratory.

Questions

1. Define the following:
   - transformation
   - Recombinant DNA
   - Genetic engineering

- Transgenic
- Biotechnology
- Vector
- Selectable marker
- Plasmid
- GFP
- Competent cells

2. Cells are made competent using one of two different approaches. What are these?
3. Explain how antibiotic resistance can be used as a selectable marker in genetic engineering.
4. Why are selectable markers used in genetic engineering?
5. Give at least two reasons it is useful to transform bacteria with certain genes of interest.
6. What is the purpose of the heat-shock step during bacterial transformation?
7. What would happen if you forgot to add the plasmid before the heat shock step? Would this change the results from this experiment? Explain.
8. What was added into the medium in the petri plates to insure that only bacteria carrying the plasmid would grow?

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1.34: Protozoan Parasites

Learning Objectives

- Identify and name the following protozoan parasites in microscopic samples: *Plasmodium* sp., *Trypanosoma cruzi*, and *Trichomonas vaginalis*
- Name the diseases caused by the following protozoan parasites: *Plasmodium* sp., *Trypanosoma cruzi*, and *Trichomonas vaginalis*
- Explain how the following protozoan parasites are transmitted to humans: *Plasmodium* sp., *Trypanosoma cruzi*, and *Trichomonas vaginalis*
- Interpret the life cycles of the following protozoan parasites: *Plasmodium* sp., *Trypanosoma cruzi*, and *Trichomonas vaginalis*
- Examine the following protozoan parasites using a microscope and illustrate and label the specimens: *Plasmodium* sp., *Trypanosoma cruzi*, and *Trichomonas vaginalis*

Introduction to Protozoan Parasites

All protozoans are found in a life stage called **trophozoites**. The “troph” stage is actively feeding, mostly motile and responsible for symptoms in a host. Some protozoans can form a **cyst** stage – a resting, inactive stage that protects the organism because of a thick wall that is produced. These cysts enable the protozoan to survive harsh environmental conditions outside of a host and ensure the organism will be passed to other hosts. Ingestion of the cyst in contaminated food or water is a common method of acquiring many protozoan infections.

Many protozoan parasites require **vectors** for transmission to occur. A vector is a transport mechanism. With parasites requiring a vector, the vector is a living thing, often an arthropod species (e.g. mosquito).

The **life cycles** of parasites are very specific. Parasites require transmission to specific species of organisms (the **hosts**) in a specific order to continue spreading and reproducing. At times, a parasite may be transmitted to a host that is not in their life cycle. This **accidental host** can experience symptoms of parasite infection, but the parasite is unable to continue its life cycle and continue reproduction.

In additional to an accidental host, host species can also be classified as either the definitive host or the intermediate host:

- **definitive host:** a host species that can harbor either the adult form of a parasite or the sexual stage of a parasite
- **intermediate host:** a host species that can harbor an immature form of a parasite in a non-sexual stage

Protozoan parasites can undergo **sexual reproduction**, **asexual reproduction**, or both sexual and asexual reproduction depending on the species. Asexual reproduction will typically involve cell divisions to produce new individuals, whereas sexual reproduction will involve production and fusion of **gametes** ("male" and "female" cells that have half the DNA of the other cells and can fuse to produce genetically unique individuals).

Plasmodium sp. (Cause of Malaria)

Introduction to *Plasmodium* sp.

Plasmodium (plaz-mo’dee-um) causes malaria, one of the common causes of hemolytic anemia worldwide. There are four species of *Plasmodium* – *P. vivax* (most common), P. *ovale*, *P. malariae*, and *P. falciparum* (most lethal). Typical symptoms are fever, chills, headache, muscle pain, and sweating. According to WHO, there were an estimated 229 million cases of malaria per year worldwide, mostly (94%) in the African Region. Cases of malaria diagnosed in the United States were mostly seen in travelers and immigrants returning to the U.S. from countries where malaria is endemic.

Malaria is the number one cause of death by parasites in the world. To continue existing, it must alternate sexual and asexual cycles. Interruption of either life cycle will control the disease. Measures taken to interrupt the life cycle include attempts to eliminate the *Anopheles* mosquito, to protect the host from being bitten using chemical repellants and mosquito netting, to prophylactically treating travelers in high risk areas, to cure active cases with various antiparasitic drugs. The occurrence of drug resistant strains worldwide has dramatically increased in recent years. Contact with CDC will aid in determining the best prophylactic drug and drug of choice for treatment depending on the patient's health and the area in which malaria was acquired.

Clinical Presentation of Malaria

The symptoms of uncomplicated malaria can be rather non-specific and the diagnosis can be missed if health providers are not alert to the possibility of this disease. Since untreated malaria can progress to severe forms that may be rapidly (<24 hours) fatal, malaria should always be considered in patients who have a history of exposure (mostly: past travel or residence in disease-endemic areas). The most frequent symptoms include fever and chills, which can be accompanied by headache, myalgias, arthralgias, weakness, vomiting, and diarrhea. Other clinical features include splenomegaly, anemia, thrombocytopenia, hypoglycemia, pulmonary or renal dysfunction, and neurologic changes. The clinical presentation can vary substantially depending on the infecting species, the level of parasitemia, and the immune status of the patient. Infections caused by *P. falciparum* are the most likely to progress to severe, potentially fatal forms with central nervous system involvement (cerebral malaria), acute renal failure, severe anemia, or acute respiratory distress syndrome. Other species can also have severe manifestations. Complications of *P. vivax* malaria include splenomegaly (with, rarely, splenic rupture), and those of *P. malariae* include nephrotic syndrome.

Asexual Stage of *Plasmodium* sp. in Humans

One becomes infected with *Plasmodium* through the bite of the infected female *Anopheles* mosquito. The mosquito transmits *Plasmodium* sporozoites via her saliva when she inserts her proboscis into human skin to obtain a blood meal. The blood provides nourishment for the eggs she will lay. Sporozoites injected into the blood stream leave the blood vascular system within a period of forty minutes and invade the parenchymal cells of the liver. In liver cells, the sporozoites undergo asexual multiplication. They are then liberated and invade red blood cells, initiating the blood stream phase of the infection.

An asexual cycle, known as schizogony, takes place within the red cells of the infected host. This process results in the formation of four to thirty-six new parasites in each red cell. Immature trophozoites, called a "ring" forms, develop which then enlarge to become mature trophozoites, filling most of the parasitized red blood cells. Asexual multiplication occurs when the trophozoites' nuclear material and cytoplasm split. At the end of the schizogonic cycle the infected blood cells rupture, liberating merozoites, which, in turn, infect new red blood cells. Lysis of the red cells liberates products of metabolism of the parasites and the red cells. These toxic materials cause the symptoms of malaria – chills, fever, nausea, vomiting and headache. The fever spikes occur at varying intervals of 24, 48, or 72 hours depending on the species of *Plasmodium* present. The febrile period may last several hours, ending with a profuse sweating stage. This cycle is repeated many times.

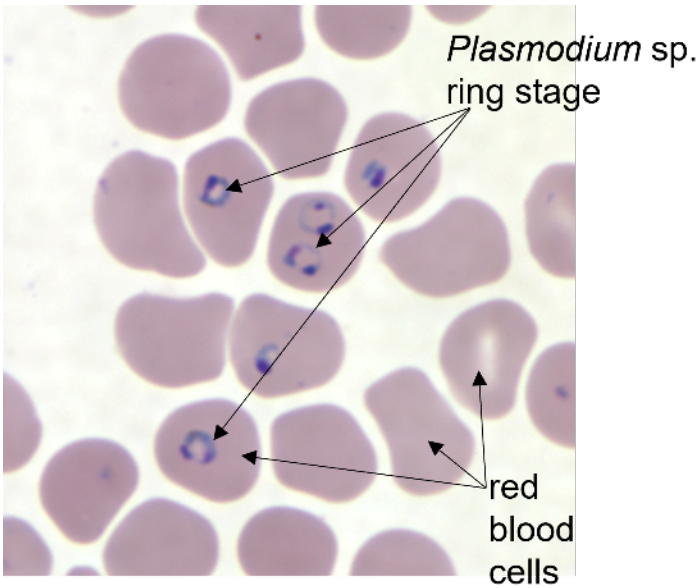


Figure 1: *Plasmodium* sp. infecting a human blood sample. The larger pink round-ish structures are all red blood cells from human blood. The purple-stained round structures found inside some of the red blood cells are the ring stage of *Plasmodium*. The ring stage is an intracellular stage, meaning the parasite lives inside of the host's cells. This phase is a common way malaria is diagnosed. Diagnosis involves identification of the *Plasmodium* sp. ring stage within red blood cells.

Sexual Stage of *Plasmodium* sp. in Mosquitos

Sexual stage male and female gametocytes may also appear in the red blood cells. When the mosquito bites an infected person, she draws blood into her stomach which may contain male and female gametocytes. In the mosquito's gut, the male gametocytes form spermatozoa and the female forms an ovum. Fertilization takes place. The resulting zygote can invade the gut wall and produce numerous sporozoites. The sporozoites migrate through the tissue of the mosquito to the salivary glands where they will be injected into the next human host when the mosquito takes another blood meal. The asexual cycle then proceeds in the new host.

Plasmodium sp. Life Cycle

Blood parasites of the genus *Plasmodium*. There are approximately 156 named species of *Plasmodium* which infect various species of vertebrates. Four species are considered true parasites of humans, as they utilize humans almost exclusively as a natural intermediate host: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*.

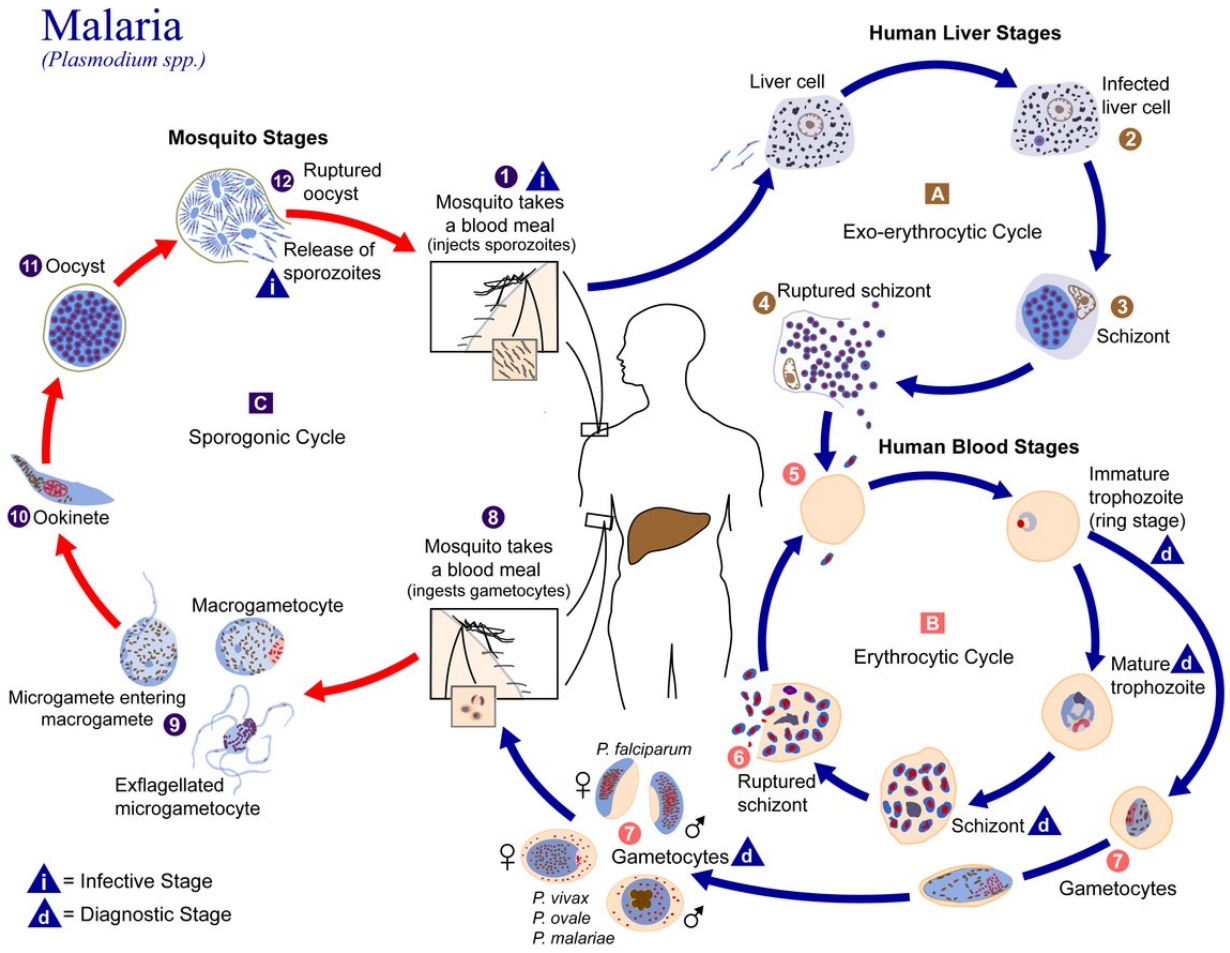


Figure 2: *Plasmodium* sp. life cycle for species that infect humans. See the table below for details about the letters and numbers shown on this diagram.

1	During a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host.
A	**Human Liver Stages** (exo-erythrocytic schizogony)
2	**Sporozoites** infect liver cells
3	…and mature into **schizonts**…
4	… which rupture and release **merozoites**.
	(Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.)
B	**Human Blood Stages** (erythrocytic schizogony) The parasites undergo asexual multiplication in the erythrocytes (red blood cells).
5	Merozoites infect red blood cells.
6	The **ring stage** trophozoites mature into schizonts, which rupture releasing merozoites.

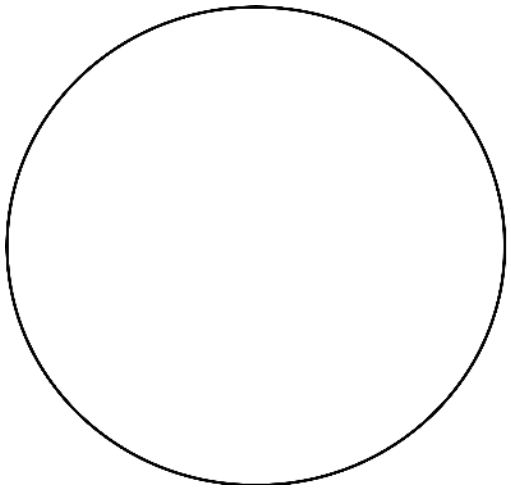
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7	Some parasites differentiate into sexual erythrocytic stages (gametocytes).
	Blood stage parasites are responsible for the clinical manifestations of the disease.
8	The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal.
C	**Mosquito Stages**. The parasites' multiplication in the mosquito is known as the **sporogonic cycle**.
9	While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes.
10	The zygotes in turn become motile and elongated (**ookinetes**)…
11	… which invade the midgut wall of the mosquito where they develop into **oocysts**.
12	The oocysts grow, rupture, and release sporozoites…
1	…which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

Laboratory Instructions: *Plasmodium* sp.

1. Examine a blood smear infected with *Plasmodium* sp. and identify the ring-like immature trophozoites (will require 400x or 1000x magnification).
2. Carefully illustrate the sample as you see it in the microscope. Label "red blood cells" and "*Plasmodium* sp. ring stage" in your illustration.

Results & Questions: *Plasmodium* sp.



specimen: _____

total magnification: _____

1. Carefully illustrate the sample as you see it in the microscope. Label "red blood cells" and "*Plasmodium* sp. ring stage" in your illustration.
2. What stage of the *Plasmodium* sp. life cycle did you examine (human liver stage, human blood stage, or mosquito stage)?

3. What disease does *Plasmodium* sp. cause in humans?
4. What human tissues are inhabited by the *Plasmodium sp.* parasite in humans?
5. How is *Plasmodium sp.* transmitted to humans?
6. What species is the definitive host of *Plasmodium sp.?* How do you know?
7. What species is the intermediate host of *Plasmodium sp.?* How do you know?
8. What are the four *Plasmodium* species that cause malaria in humans?
9. Where in the world is malaria present?
10. How can people in the United States present with a malaria infection?
11. Give at least four symptoms of malaria.

Trypanosoma cruzi (Cause of Chagas Disease)

Introduction to *Trypanosoma cruzi*

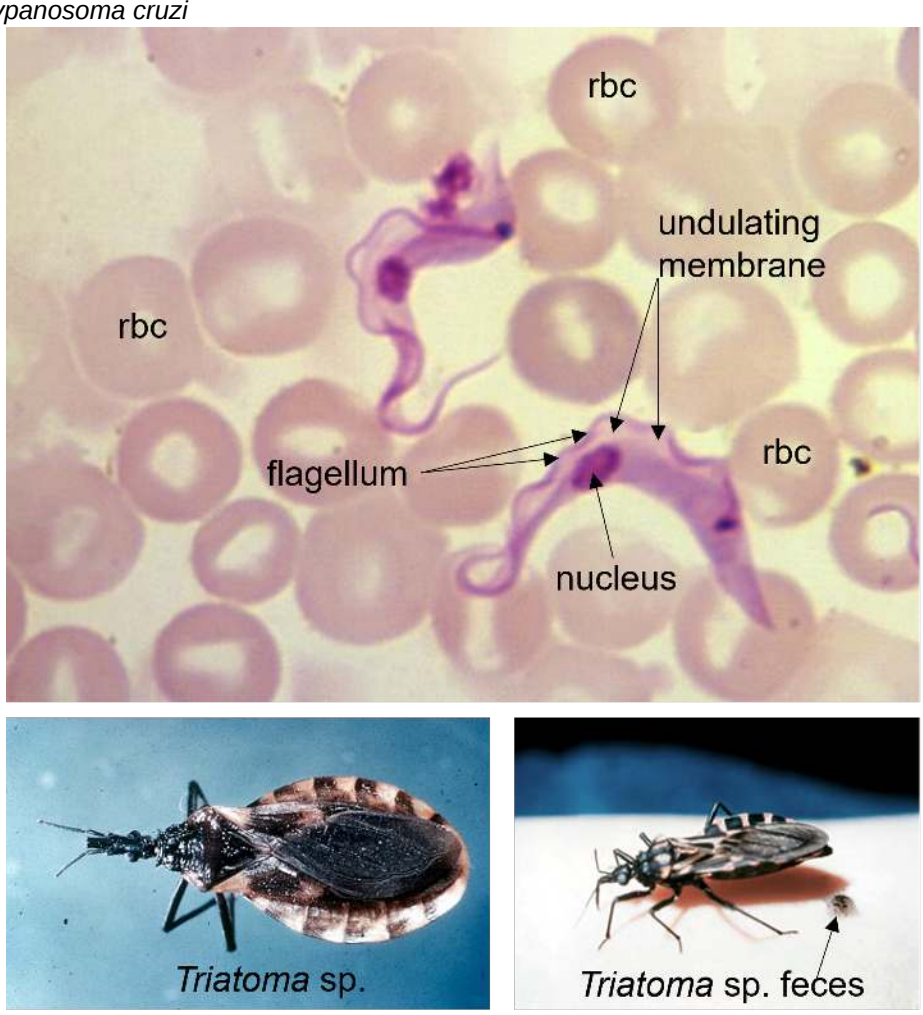


Figure 3: (Top) Human blood viewed under a light microscope. The normal human red blood cells (rbc) are round and pink. Trypanosoma cruzi inhabits the blood (purple). In this high magnification view of *T. cruzi*, visible are the flagellum wrapped around the cell and connected to the cell by its undulating membrane that waves to propel the parasite around. (Bottom left) Image of *Triatoma* sp., one of the types of reduviid bugs capable of spreading *T. cruzi* to humans. (Bottom right) Entry of *T. cruzi* from its vector to the human involves a break or opening in the skin (commonly from a bite from the bug) after which the bug defecates. *T. cruzi* passes from the feces into the skin wound to cause infection in the human.

Trypanosoma cruzi (trip-an'o-so'muh/kroo'zye) is the protozoan responsible for causing Chagas disease. *T. cruzi* is limited to the western hemisphere, including California, the southern United States, Central and South America.It is most often seen in rural areas in Latin America where poverty is widespread. The vector for *T. cruzi* is reduviid bug species known as kissing bugs. The most common genera responsible for transmission of the disease are *Triatoma*, *Rhodnius*, and *Panstrongylus*. Infection usually occurs after bugs defecate on the bite site and are rubbed into the wound by the host scratching. Humans contract Chagas disease (South American Trypanosomiasis) when an infected bug bites the human skin and subsequently defecates in the wound. Infection may be mild or asymptomatic. There may be fever or swelling around the bug bite. Parasites may also be found in the circulating blood. Chagas disease occurs immediately after infection and may last a few weeks or months. In 20-30% of infected people, acute infection may result in severe inflammation of the heart muscle or the brain and lining around the brain.

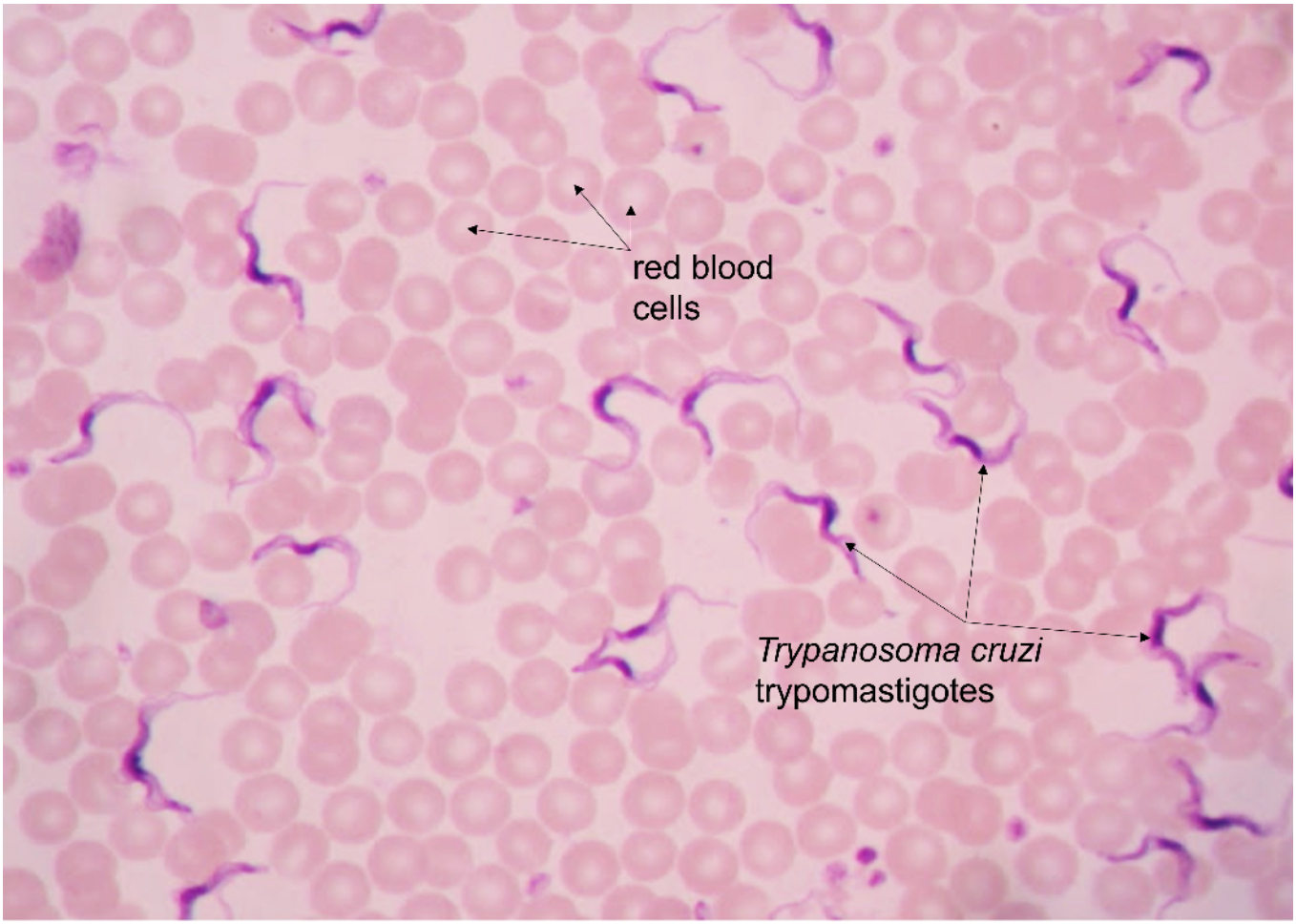


Figure 4: Human blood smear infected with *Trypanosoma cruzi* trypomastigotes. The round pink structures are normal human red blood cells. The purple structures between the red blood cells is the *T. cruzi* parasite.

Clinical Presentation of Chagas Disease

Chagas disease has an acute phase and chronic phase. The acute phase is usually asymptomatic, but can present with nonspecific somatic symptoms. Rarely, the acute phase may be more severe with potential cardiac or neurologic symptoms and signs. Nodular lesions or furuncles, usually called chagomas, may develop around the vector's feeding site. Chagomas occurring on the on the eyelids are commonly referred to as palpebral and periocular firm swelling. Most acute cases resolve over a period of a few weeks

or months into a subclinical chronic form of the disease (“indeterminate form”). Reactivation of Chagas disease from this asymptomatic form may occur in patients with HIV or those receiving immunosuppressive drugs.

The symptomatic chronic form (“determinate form”) may not occur for years or even decades after initial infection. This may include cardiac or gastrointestinal involvement, which occasionally occur together. The many complications of chronic Chagas disease can be fatal. Amastigote invasion of smooth muscle can lead to megaesophagus, megacolon, and dilated cardiomyopathy.



Life Cycle of *Trypanosoma cruzi*

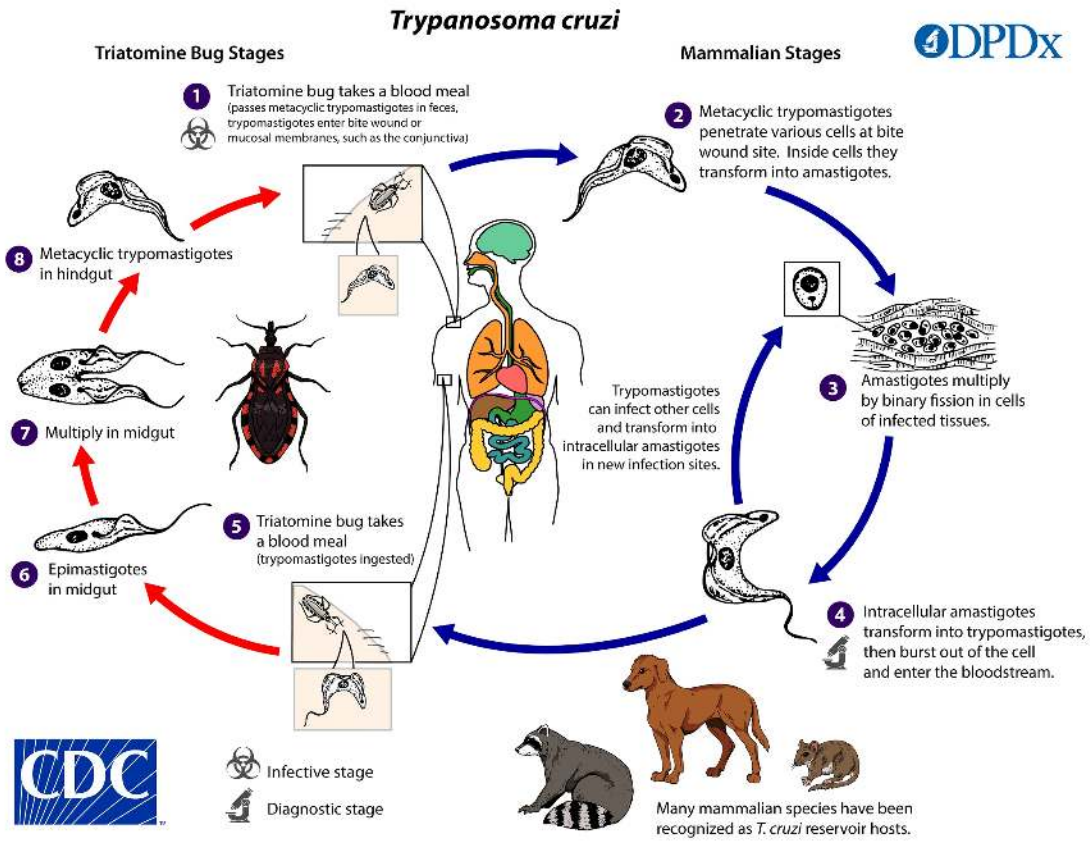


Figure 5: *Trypanosoma cruzi* life cycle. See table below for details about each stage in the diagram.

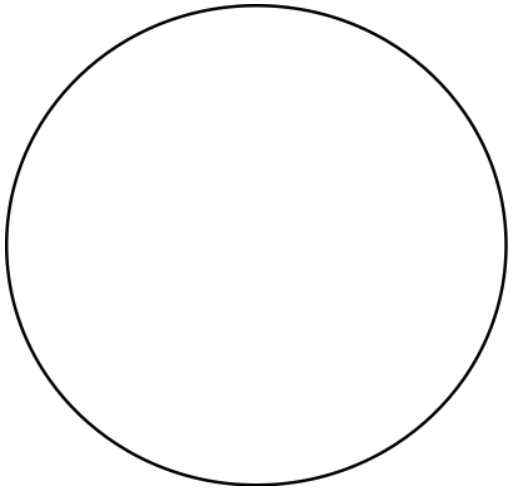
1	An infected triatomine insect vector (or “kissing” bug) takes a blood meal and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host through the bite wound or intact mucosal membranes, such as the conjunctiva.
2	Inside the host, the trypomastigotes invade cells near the site of inoculation, where they differentiate into intracellular amastigotes.
3	The amastigotes multiply by binary fission…
4	… and differentiate into trypomastigotes, and then are released into the circulation as bloodstream trypomastigotes.
	Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle. The bloodstream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector.
5	The “kissing” bug becomes infected by feeding on human or animal blood that contains circulating parasites.
6	The ingested trypomastigotes transform into epimastigotes in the vector’s midgut.
7	The parasites multiply and differentiate in the midgut…

8	… and differentiate into infective metacyclic trypomastigotes in the hindgut.
	Other less common routes of transmission include blood transfusions, organ transplantation, transplacental transmission, and foodborne transmission (via food/drink contaminated with the vector and/or its feces).

Laboratory Instructions: *Trypanosoma cruzi*

1. Examine a blood smear infected with *Trypanosoma cruzi* and identify the *T. cruzi* trophozoites (will require 400x or 1000x magnification).
2. Carefully illustrate the sample as you see it in the microscope. Label the following in your illustration:
   - "red blood cells"
   - "*Trypanosoma cruzi* trypomastigote"
   - "flagellum"
   - "undulating membrane"
   - "nucleus"

Results & Questions: *Trypanosoma cruzi*



specimen: _____

total magnification: _____

1. Carefully illustrate the sample as you see it in the microscope. Label the following in your illustration:
   - "red blood cells"
   - "*Trypanosoma cruzi* trypomastigote"
   - "flagellum"
   - "undulating membrane"
   - "nucleus"
2. Explain how humans become infected with *T. cruzi*.
3. What disease is caused by *T. cruzi* infection in humans?
4. What geographic regions of the world is *T. cruzi* infection possible?
5. Give at least three symptoms of Chagas disease.
6. Can Chagas disease be asymptomatic? Explain your answer.
7. Carefully examine the life cycle of *T. cruzi*. Inside of the human host, is *T. cruzi* an intracellular parasites (lives inside host cells) or an intercellular parasite (lives outside of host cells)? Explain your answer.

Trichomonas vaginalis (Cause of Trichomoniasis)

Introduction to *Trichomonas vaginalis*

Trichomoniasis a common, treatable, sexually transmitted disease (STD). Most people who have trichomoniasis do not have any symptoms.

Trichomonas vaginalis (trick'o-mo'nas/vadj-i-nay'lis) has no cyst stage so it cannot survive outside its host very long. It is transmitted by intimate contact between individuals. It is the cause of trichomoniasis, commonly called "ping-pong vaginitis" because it may be passed back and forth between sexual partners. In the United States, an estimated 2 million people have the infection, but only about 30% develop any symptoms of trichomoniasis. While most infections are asymptomatic, it can cause prostate and epididymis infections in men. Females report frequent urination, itching, burning, and a vaginal discharge. Diagnosis often occurs when people seek medical help for what they believe is a urinary tract infection. Flagyl is a medication used to treat infections. Diagnosis is usually made by microscopically identifying the tear-dropped shaped trophozoites in a wet preparation of a vaginal or urethral discharge. Three to five flagella may be visible as a tuft at the anterior end of the cell. *T. vaginalis* will exhibit a quick, jerky, darting motility as it zooms around the field of vision.



In the United States, CDC estimates that there were more than two million trichomoniasis infections in 2018. However, only about 30% develop any symptoms of trich. Infection is more common in women than in men. Older women are more likely than younger women to have the infection.

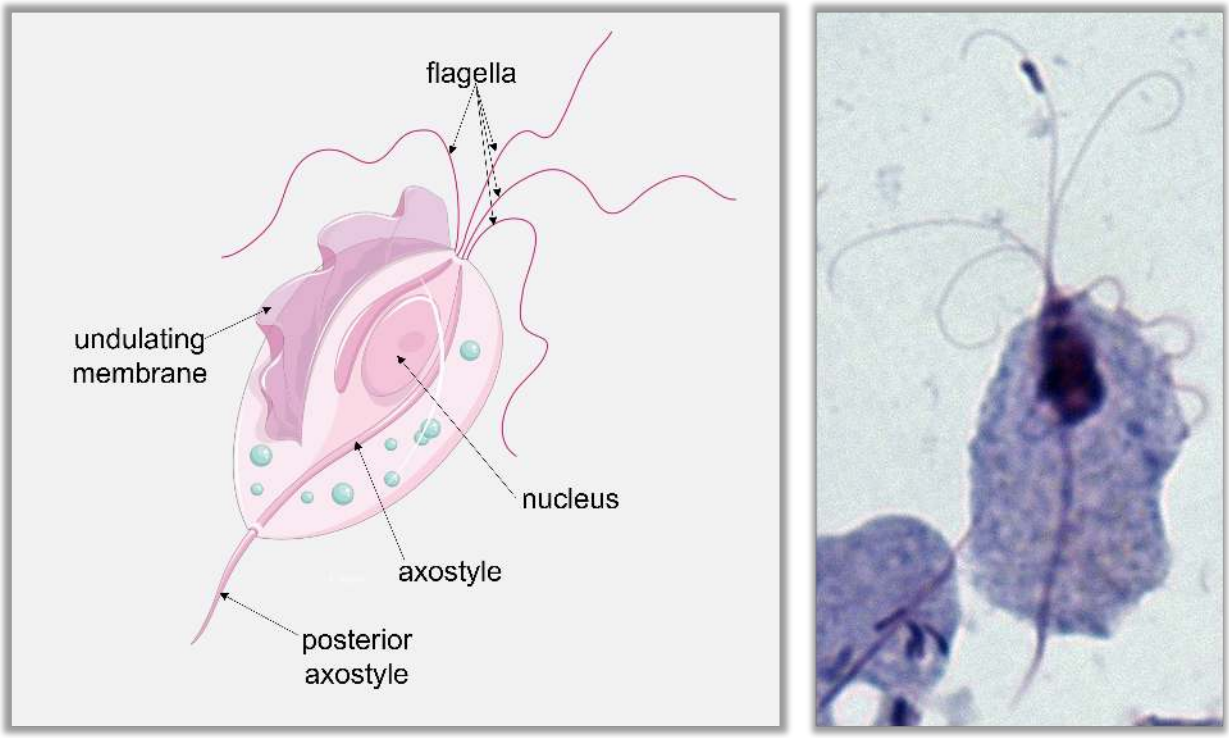


Figure 6: (Left) Illustration of a *Trichomonas vaginalis* trophozoite with its undulating membrane, axostyle, nucleus, and flagella labeled. (Right) Microscopic image of a *T. vaginalis* trophozoite.

Clinical Presentation of Trichomoniasis

About 70% of people with the infection do not have any signs or symptoms. When trich does cause symptoms, they can range from mild irritation to severe inflammation. Some people get symptoms within 5 to 28 days after getting the infection. Others do not develop symptoms until much later. Symptoms can come and go.

Men with trich may notice:

- Itching or irritation inside the penis;
- Burning after peeing or ejaculating; and
- Discharge from the penis.

Women with trich may notice:

- Itching, burning, redness or soreness of the genitals;
- Discomfort when peeing; and
- A clear, white, yellowish, or greenish vaginal discharge (i.e., thin discharge or increased volume) with a fishy smell.

Having trich can make sex feel unpleasant. Without treatment, the infection can last for months or even years.

Pregnant people with trich are more likely to have their babies early. Also, their babies are more likely to have a low birth weight (less than 5.5 pounds).

Life Cycle of *Trichomonas vaginalis*

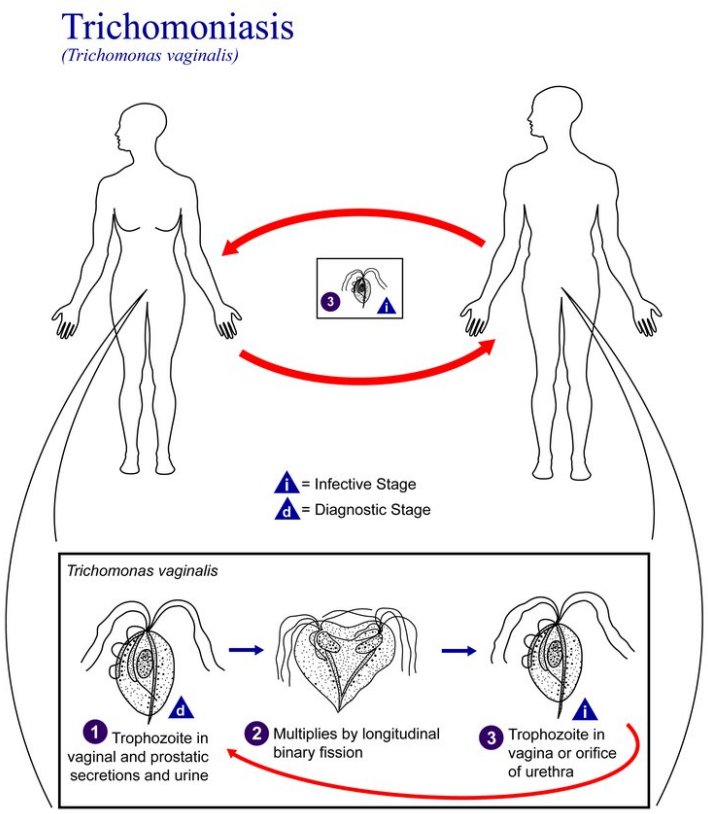
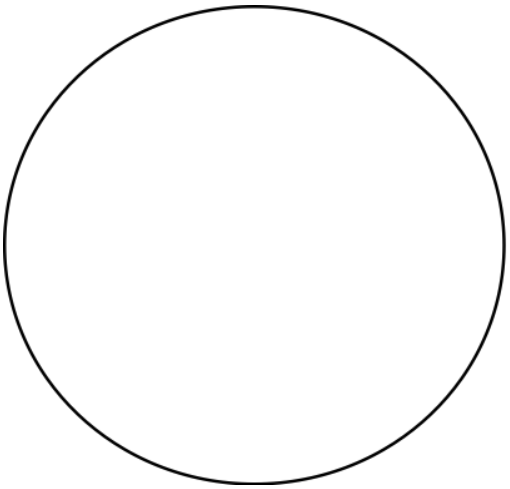


Figure 7: Life cycle of *T. vaginalis*.

Laboratory Instructions: *Trichomonas vaginalis*

1. Examine the sample of *T. vaginalis* and identify the *T. vaginalis* trophozoites (use 400x or 1000x magnification).
2. Carefully illustrate the sample as you see it in the microscope. Label the following in your illustration:
   - *"Trichomonas vaginalis*"
   - "flagella"
   - "undulating membrane"
   - "nucleus"
   - "axostyle"
   - "posterior axostyle"

Results & Questions: *Trichomonas vaginalis*



specimen: _____

total magnification: _____

1. Carefully illustrate the sample as you see it in the microscope. Label the following in your illustration:
   - *"Trichomonas vaginalis*"
   - "flagella"
   - "undulating membrane"
   - "nucleus"
   - "axostyle"
   - "posterior axostyle"
2. What disease is caused by *T. vaginalis*?
3. Give at least two common symptoms of trichomoniasis in men.
4. Give at least two common symptoms of trichomoniasis in women.
5. How frequently is trichomoniasis asymptomatic?
6. How is trichomoniasis spread?
7. How many host species does *T. vaginalis* have?
8. How is trichomoniasis diagnosed?

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1.35: Helminth Parasites

Learning Objectives

- Identify and name the following helminth parasites in microscopic samples: *Enterobius vermicularis*, *Dipylidium caninum*, and *Schistosoma* sp.
- Name the diseases caused by the following helminth parasites: *Enterobius vermicularis*, *Dipylidium caninum*, and *Schistosoma* sp.
- Explain how the following helminth parasites are transmitted to humans (or dogs/cats for *D. caninum*): *Enterobius vermicularis*, *Dipylidium caninum*, and *Schistosoma* sp.
- Interpret the life cycles of the following helminth parasites: *Enterobius vermicularis*, *Dipylidium caninum*, and *Schistosoma* sp.
- Examine the following helminth parasites using a microscope and illustrate and label the specimens: *Enterobius vermicularis*, *Dipylidium caninum*, and *Schistosoma* sp.

Enterobius vermicularis (Causes Enterobiasis)

Introduction to Enterobiasis

The pinworm, *Enterobius vermicularis* (en'tur-o'bee-us/vur-mick-yoo-lair'is), is thought to be the most common helminth parasite in temperate regions with a high level of sanitation. While children are more commonly infected than adults, pinworm infestations are found in all ages and socioeconomic groups. Humans are the only host of *Enterobius vermicularis*.

The life cycle of *Enterobius vermicularis* is 4-6 weeks. Adults worms inhabit the cecum (part of the large intestine/colon) and adjacent portions of the large and small intestine of the host. The male pinworm is inconspicuous, about 2 to 5 mm in length, and no more than 0.2 mm in width. The female may reach a length of 13 mm (1/2 inch) and a maximum width of 0.5 mm The female is distinguished by a long, thin, sharply pointed tail, which gives rise to the common name "pinworm". The gravid females, containing 11,000 to 15,000 eggs, migrate down the intestinal tract to the anus where they deposit their eggs. *Enterobius* ova, having a sticky albumin coating, adhere to the perianal region. The eggs mature quickly and are infective within a few hours after they are deposited.

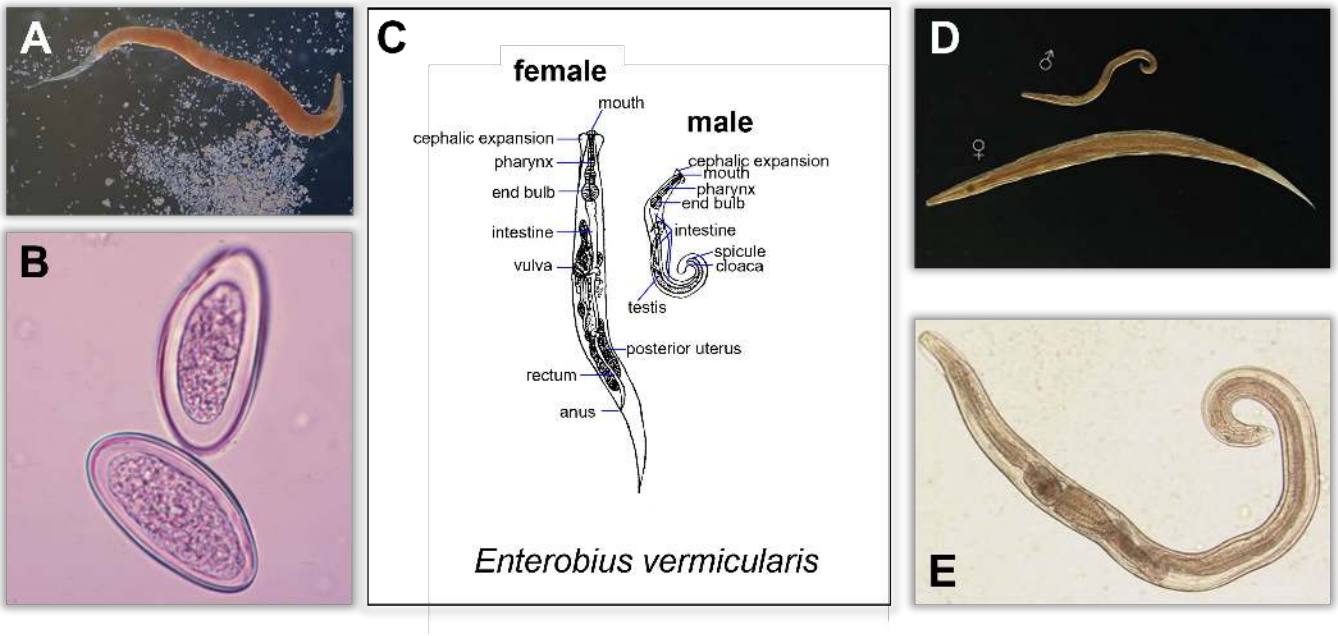


Figure 1: (A) Image of adult female *Enterobius vermicularis*. (B) Image of *E. vermicularis* eggs. (C) Diagram of the anatomy of male and female *E. vermicularis* worms. (D) Side-by-side image of male and female *E. vermicularis* worms. (E) Image of male *E. vermicularis* worm.

Recovery of eggs from the perianal region provides the most efficient method of diagnosis of pinworm infection. Repeated examinations on consecutive days are necessary because of the irregular migrations of the gravid female worms. Since pinworm eggs are generally deposited at night, the examination should be made in the early morning hours before the patient has washed or defecated. Typically, Graham's Scotch Tape Method is employed. The sticky side of cellophane tape is applied to the anal area of the patient. The tape is then pressed firmly on a microscope slide, sticky side down. The slide is examined under the low power of a microscope for the presence of pinworm eggs. The eggs are identified by their flattened oval (asymmetrical) shape and possibly the presence of a well-developed embryo.



Migration of the female worm may cause pruritus ani (anal itching). The local itching may cause insomnia and restlessness in children or adults who are infected, as the worms migrate from the anus during the resting hours. In about one-third of infected children, eggs may be obtained from beneath the fingernails. Reinfection of the patient by hand-to-mouth transmission (from scratching the perianal area or handling contaminated fomites) is common and makes control of the parasite difficult. *Enterobius* ova can survive for 2-3 weeks outside the body in high humidity and moderate temperatures. If towels or linens are shared, these eggs can be passed to others. It is recommended that all members of the infected household be treated simultaneously. Infection of others through contaminated bedding, clothing, bath tubs, toilet seats, dry dust, and air-borne eggs may serve to infect persons at some distance. The prescription drug, mebendazole, is used to treat pinworm. There are over-the-counter drugs available for treatment of pinworms also. Vermox, antiminth, and Povan (pyrvinium pamoate) are acceptable treatments. Patients using Povan should be forewarned that it colors the stool a bright red color!

E. vermicularis occurs worldwide, with infections occurring most frequently in school- or preschool-children and in crowded conditions.

Clinical Presentation of Enterobiasis

Enterobiasis is frequently asymptomatic. The most typical symptom is perianal pruritus, especially at night, which may lead to excoriations and bacterial superinfection. Occasionally, invasion of the female genital tract with vulvovaginitis and pelvic or peritoneal granulomas can occur. Other symptoms include, teeth grinding, enuresia, insomnia, anorexia, irritability, and abdominal pain, which can mimic appendicitis. *E. vermicularis* larvae are often found within the appendix on appendectomy, but the role of this nematode in appendicitis remains controversial. Very rare instances of eosinophilic colitis associated with *E. vermicularis* larvae have been reported.

Life Cycle of *Enterobius vermicularis*

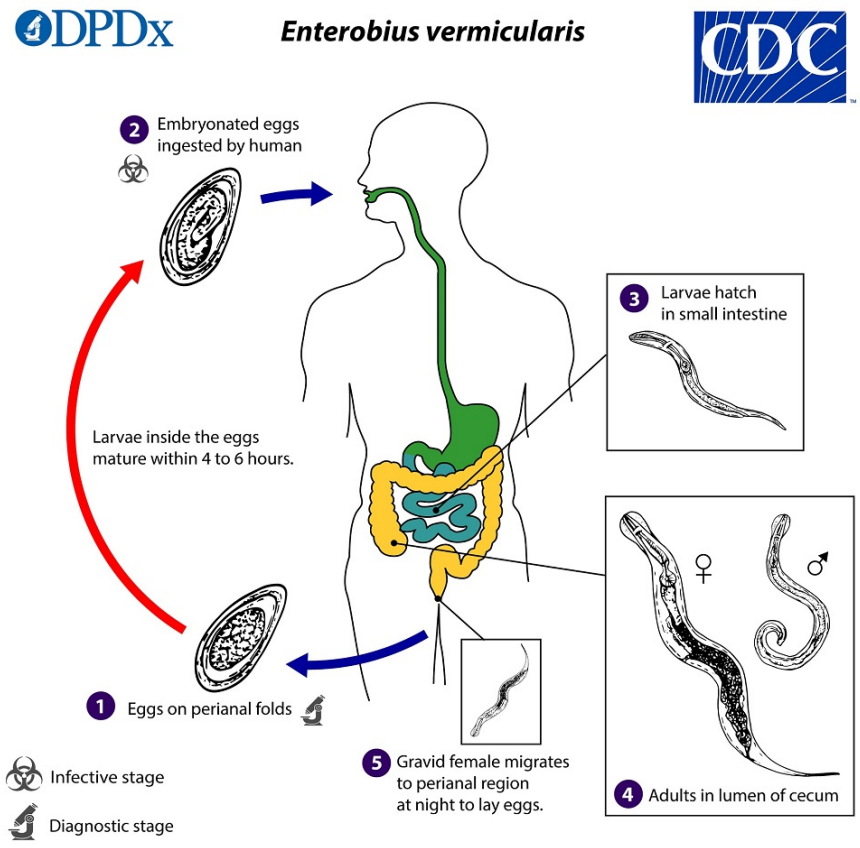


Figure 2: Life cycle of *Enterobius vermicularis*. See the table below for explanation of this life cycle.

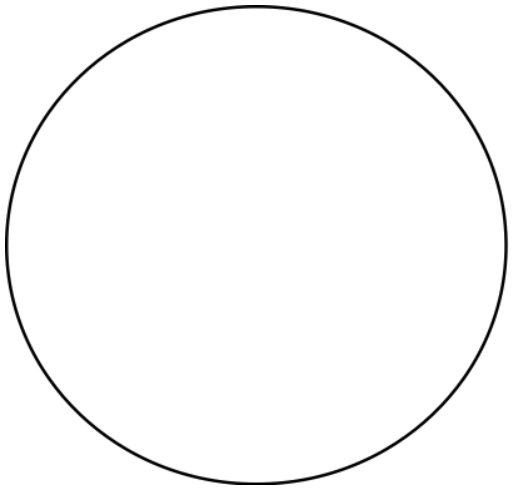
1	Gravid adult female *Enterobius vermicularis* deposit eggs on perianal folds.
2	Infection occurs via self-inoculation (transferring eggs to the mouth with hands that have scratched the perianal area) or through exposure to eggs in the environment (e.g. contaminated surfaces, clothes, bed linens, etc.).
3	Following ingestion of infective eggs, the larvae hatch in the small intestine...
4	...and the adults establish themselves in the colon, usually in the cecum.
	The time interval from ingestion of infective eggs to oviposition by the adult females is about one month. At full maturity adult females measure 8 to 13 mm, and adult males 2 to 5 mm; the adult life span is about two months.
5	Gravid females migrate nocturnally outside the anus and oviposit while crawling on the skin of the perianal area.
1	The larvae contained inside the eggs develop (the eggs become infective) in 4 to 6 hours under optimal conditions.
	Rarely, eggs may become airborne and be inhaled and swallowed. Retroinfection, or the migration of newly hatched larvae from the anal skin back into the rectum, may occur but the frequency with which this happens is unknown.

Laboratory Instructions: *Enterobius vermicularis*

1. Examine eggs of *E. vermicularis* (use 400x or 1000x magnification).
2. Carefully illustrate the egg sample as you see it in the microscope.
3. Examine an adult worm of *E. vermicularis*.
4. Carefully illustrate the adult worm sample as you see it in the microscope. Label the following in your illustration:
    - "male" or "female" (as appropriate)
    - "mouth"
    - "cephalic expansion"
    - "pharynx"
    - "end bulb"
    - "intestine"
    - if the worm is a male:
        - "testis"
        - "spicule"
        - "cloaca"
    - if the worm is a female:
        - "vulva"
        - "posterior uterus"

Results & Questions: *Enterobius vermicularis*

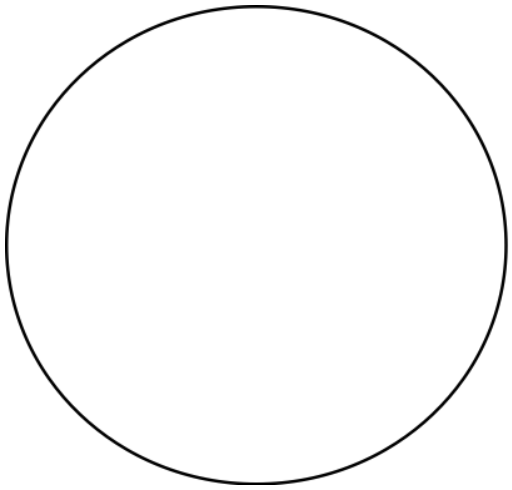
E. vermicularis eggs



specimen: _____

total magnification: _____

E. vermicularis adult helminth



specimen: _____

total magnification: _____

1. Carefully illustrate the egg sample as you see it in the microscope.
2. Carefully illustrate the adult worm sample as you see it in the microscope. Label the following in your illustration:
    - "male" or "female" (as appropriate)
    - "mouth"
    - "cephalic expansion"
    - "pharynx"
    - "end bulb"
    - "intestine"
    - if the worm is a male:
        - "testis"
        - "spicule"
        - "cloaca"
    - if the worm is a female:
        - "vulva"
        - "posterior uterus"
3. What disease is caused by *E. vermicularis*?
4. Where does *E. vermicularis* live inside its human host?
5. How can infection with *E. vermicularis* be diagnosed?
6. Give at least three symptoms of enterobiasis.
7. Can enterobiasis be asymptomatic?
8. Where geographically in the world does *E. vermicularis* infection occur?
9. What are three similarities of the male and female *E. vermicularis* worms?
10. What are four differences between the male and female *E. vermicularis* worms?

Dipylidium caninum **(Dog Tapeworm)**

Introduction to *Dipylidium caninum*

Dipylidium caninum is a common tapeworm of dogs and cats, but is occasionally found in humans. It has many common names including the “flea tapeworm”, “cucumber tapeworm”, and “double-pored tapeworm”. Geographic distribution of *D. caninum* is worldwide. This tapeworm is ubiquitous and common among pet dogs and cats. Human infection is rare, but has been reported from every inhabited continent.

Tapeworms are flatworms in the phylum Platyhelminthes. These helminths have a **scolex** (its "head") that is equipped with **suckers** and **hooks**, but no mouth. Tapeworms absorb nutrients through their **tegument** (their "skin"). The scolex and its suckers and hooks are used for attachment to the host intestine. Behind the scolex, a tapeworm is composed of segments called **proglottids**. The proglottids closest to the scolex are the smallest in size and are considered **immature proglottids**, that is, they lack functional reproductive organs and they lack eggs. New proglottids are produced just behind the scolex and neck of the tapeworm. As new proglottids are formed, the older proglottids will be pushed further from the scolex. As proglottids age, they will ultimately become larger and produce **mature proglottids** that contain mature **ovaries** and **testes**. Yes, that is correct, a tapeworm's mature proglottids contain both male and female genitalia, and are therefore capable of conducting sexual reproduction alone (a second worm is not involved in sexual reproduction in this case). The largest proglottids, and those most distant from the scolex, are called **gravid proglottids**. These gravid proglottids are distinct because sexual reproduction has already occurred and these proglottids contain a multitude of eggs. Gravid proglottids on the end of the tapeworm can break off and be released from its host in feces to release the eggs and continue the life cycle.

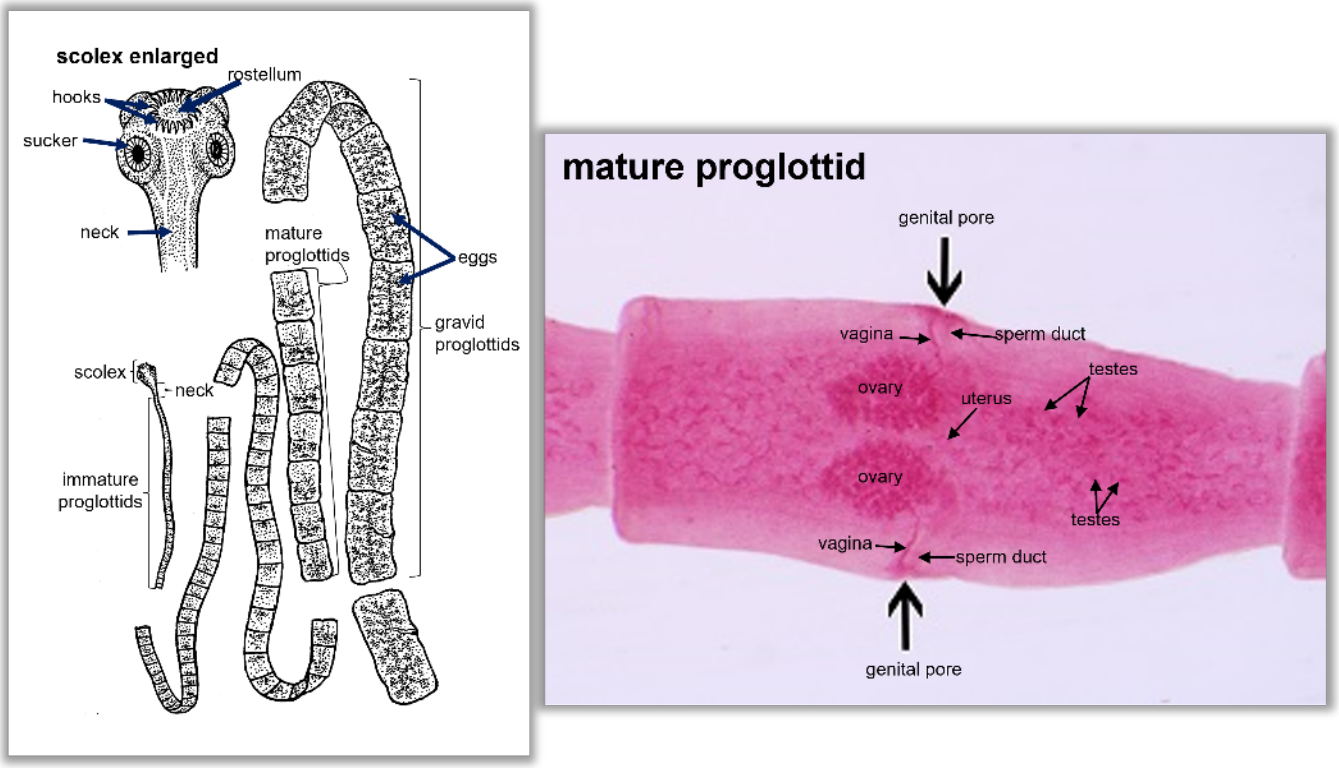


Figure 3: (Left) Overall anatomy of a tapeworm. (Right) Anatomy of *Dipyldidium caninum* mature proglottid with its genital pores, ovaries, testes, vaginas, sperm ducts, and uterus labeled.

Diagnosis is made by demonstrating the typical proglottids or egg packets in the stool or the environment. Although concentration methods are usually not necessary due to the large size of the proglottids, flotation methods may miss infections as egg packets are heavy and may fail to float.



Clinical Presentation of the Dog Tapeworm

Most infections with *Dipylidium caninum* are asymptomatic. Pets may exhibit behavior to relieve anal pruritis (such as scraping anal region across grass or carpeting). Mild gastrointestinal disturbances may occur. The most striking feature in animals and children consists of the passage of proglottids. These can be found in the perianal region, in the feces, on diapers, and occasionally on floor covering and furniture. The proglottids are motile when freshly passed and may be mistaken for maggots or fly larvae.



Figure 4: *Dipylidium caninum* egg packet containing 8 visible eggs.

Life Cycle of *Dipylidium caninum*

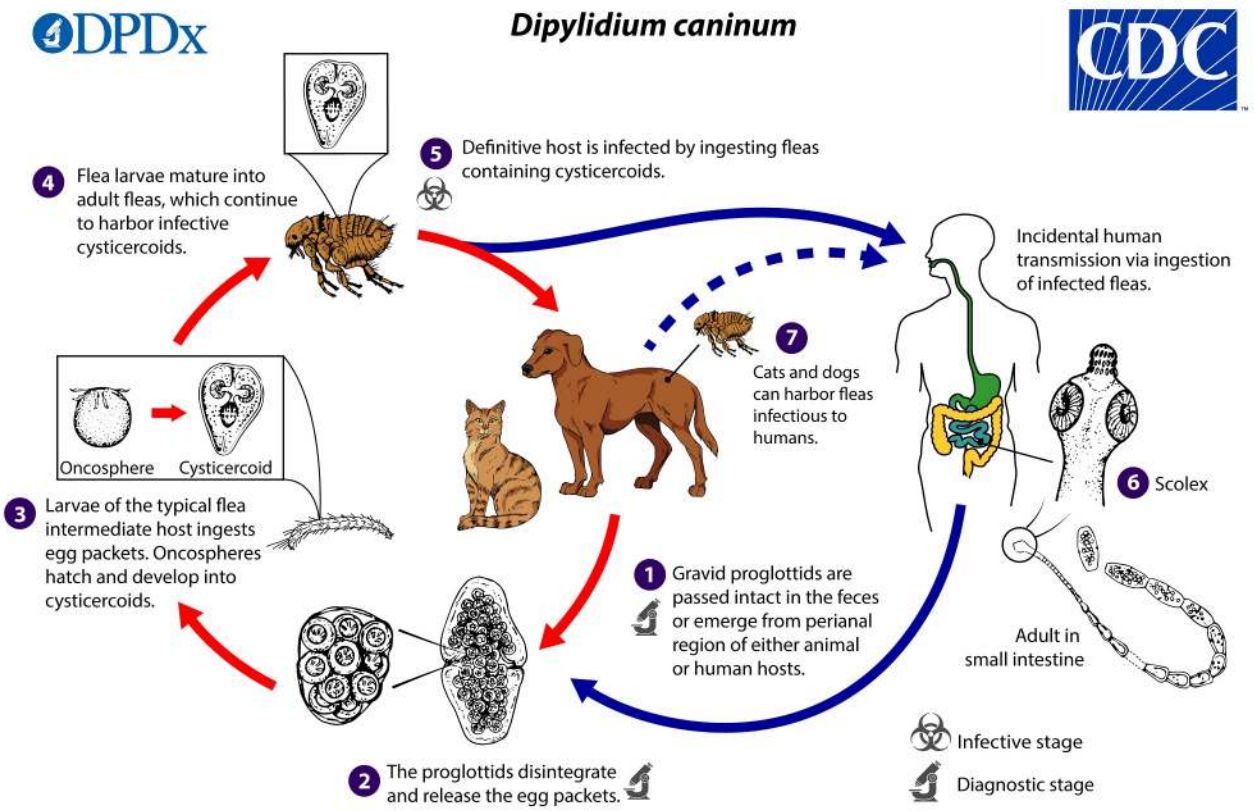


Figure 5: *Dipylidium caninum* life cycle. See the table below for details about this life cycle.

1	Gravid proglottids are passed intact in the feces or emerge from the perianal region of the host.
2	In the environment, the proglottids disintegrate and release egg packets, which are also occasionally found free in the feces.
3	The intermediate host (most often larval stages of the dog or cat flea *Ctenocephalides* spp.) ingests egg packets, and the oncosphere within is released into the larval flea's intestine. The oncosphere penetrates the intestinal wall, invades the insect's hemocoel (body cavity), and develops into a cysticercoid.
4	The cysticercoid remains in the flea as it matures from a larva into an adult.
5	The vertebrate host becomes infected by ingesting the adult flea containing the cysticercoid.
6	In the small intestine of the vertebrate host, the cysticercoid develops into the adult tapeworm after about one month. The adult tapeworms (measuring up to 60 cm in length and 3 mm in width) reside in the small intestine of the host, where they each attach by their scolex.
	Gravid, double-pored proglottids detach from the strobila (body) and are shed in the feces.

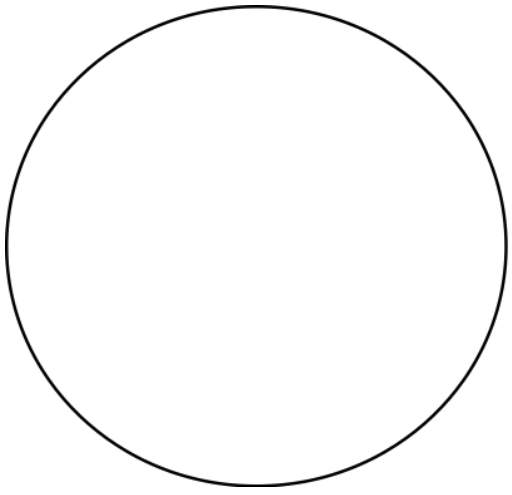
7	Humans also acquire infection by ingesting the cysticercoid contaminated flea. Children are most frequently infected, possibly due to close contact with flea-infested pets.

Laboratory Instructions: *Dipylidium caninum*

1. Examine eggs of *D. caninum* (use 400x or 1000x magnification).
2. Carefully illustrate the egg sample as you see it in the microscope.
3. Examine an adult worm of *D. caninum*. The specimen will be separated into the following segments: scolex with neck and immature proglottids, mature proglottids, and gravid proglottids.
4. Carefully illustrate the three segments of the adult worm sample as you see it in the microscope. Label the following in your illustration:
   - the smallest sample (scolex, neck, and immature proglottids):
     - "scolex"
     - "suckers"
     - "hooks"
     - "neck"
     - "immature proglottids"
   - intermediate-sized sample (mature proglottids)
     - "mature proglottid"
     - "genital pore"
     - "ovary"
     - "testes"
     - "uterus"
   - largest sample (gravid proglottids)
     - "gravid proglottid"
     - "eggs"

Results & Questions: *Dipylidium caninum*

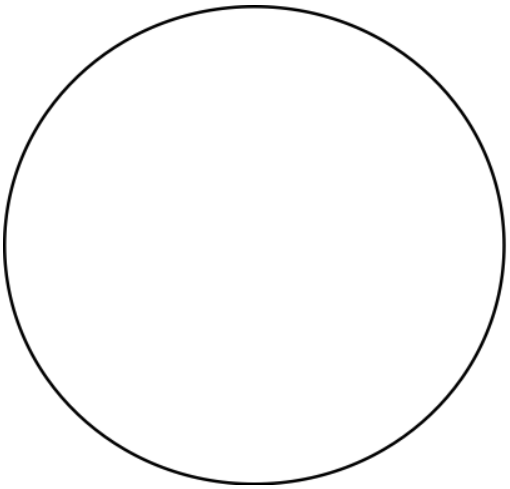
D. caninum eggs



specimen: _____

total magnification: _____

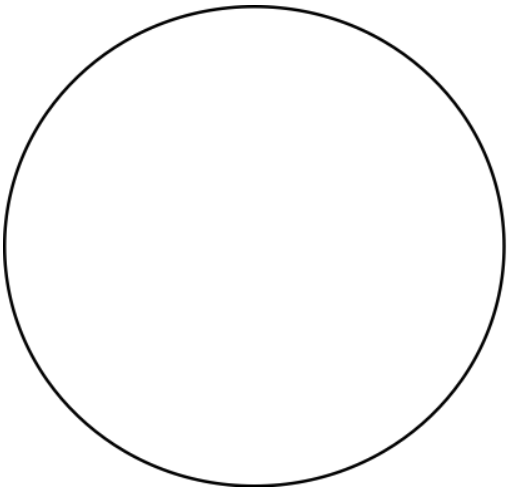
D. caninum adult helminth: scolex & immature proglottids



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total magnification: _____

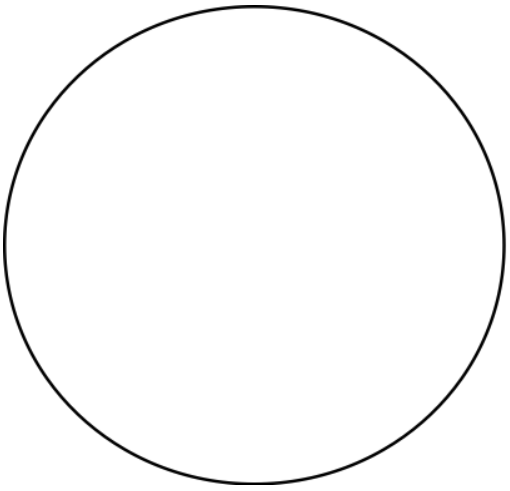
D. caninum adult helminth: mature proglottids



specimen: _____

total magnification: _____

D. caninum adult helminth: gravid proglottids



specimen: _____

total magnification: _____

1. Carefully illustrate the egg sample as you see it in the microscope.
2. Carefully illustrate the three segments of the adult worm sample as you see it in the microscope. Label the following in your illustration:
    - the smallest sample (scolex, neck, and immature proglottids):

- "scolex"
- "suckers"
- "hooks"
- "neck"
- "immature proglottids"

- intermediate-sized sample (mature proglottids)
  - "mature proglottid"
  - "genital pore"
  - "ovary"
  - "testes"
  - "uterus"
- largest sample (gravid proglottids)
  - "gravid proglottid"
  - "eggs"

3. Explain how immature proglottids become mature proglottids.
4. Explain how mature proglottids become gravid proglottids.
5. Explain how eggs are released from the host body to continue the life cycle.
6. What is the function of the scolex?
7. How does a tapeworm get its nutrients?
8. How does a tapeworm conduct sexual reproduction?
9. Give three signs/symptoms of *D. caninum* infection.
10. What species is/are the definitive host(s) of *D. caninum*?
11. What species is/are the intermediate host(s) of *D. caninum*?

Schistosoma sp. (Cause of Schistosomiasis)

Introduction to *Schistosoma* sp. (shis'to-so'muh)

There are several species of *Schistosoma* that cause disease in humans. Each species is unique to a particular area in the world. Since the United States does not have the specific host snails needed for its life cycle, infections are not contracted directly, yet many immigrants and travelers to endemic areas are infected. An estimated 236 million people require preventative drug treatment per year, out of which 105 million people were reported to have been treated. Preventative treatment, which should be repeated for a number of years, will reduce and prevent morbidity.

Infection occurs when your skin comes in contact with contaminated freshwater in which certain types of snails that carry schistosomes are living. Individuals infected with *Schistosoma* excrete the ova in the feces. Upon contact with water, the ova (which look like a cartoon talk bubble) develop into **miracidia** which enters the snail. Maturation in the snail results in the production of the free swimming **cercaria**. When the cercaria contacts the skin of a person who is in the water, it secretes enzymes that enable it to ***burrow through unbroken skin***. The *Schistosoma* are then carried in the bloodstream to the liver or urinary bladder where they mature into an adult. Adults can live up to seven years. *Schistosoma* is dioecious – the male and female are separate animals. If both male and female are present, fertilization occurs, and ova are produced. The cycle then continues in a new host. *Schistosoma* infection may result in destruction of the liver, lungs, and/or urinary system. This is second most devastating parasitic disease. (Malaria is the most common). Praziquantel is an effective treatment.



Birds in the United States suffer from a *Schistosoma* infection. The *Schistosoma* parasite that infects birds has an intermediate snail host that is predominantly found in water in the Great Lakes and on the east coast. Sometimes when the free swimming cercaria of this bird parasite encounters a human swimming in the water, it will attempt to burrow through the skin of the human host instead of the normal bird host. Since humans are not the correct host for this parasite, the cercaria are unable to penetrate the human skin and instead, become embedded in the skin. This causes a painful, itchy inflammatory dermatitis called “swimmer’s itch”.

Geographic regions where schistosomiasis occurs include:

- Africa: contact with any freshwater in southern and sub-Saharan Africa–including the great lakes and rivers as well as smaller bodies of water– should be considered a risk for schistosomiasis transmission. Transmission also occurs in the Mahgreb region of North Africa and the Nile River valley in Egypt and Sudan .
- South America: Brazil, Suriname, Venezuela
- Caribbean: Dominican Republic, Guadeloupe, Martinique, Saint Lucia (risk in Caribbean is very low)
- The Middle East: Iran, Iraq, Saudi Arabia, Yemen
- Southern China
- Parts of Southeast Asia and the Philippines, Laos
- A recent focus of ongoing transmission has been identified in Corsica.

Clinical Presentation of the Schistosomiasis

Within days after becoming infected, you may develop a rash or itchy skin. Fever, chills, cough, and muscle aches can begin within 1-2 months of infection. Most people have no symptoms at this early phase of infection.

When adult worms are present, the eggs that are produced usually travel to the intestine, liver or bladder, causing inflammation or scarring. Children who are repeatedly infected can develop anemia, malnutrition, and learning difficulties. After years of infection, the parasite can also damage the liver, intestine, lungs, and bladder. Rarely, eggs are found in the brain or spinal cord and can cause seizures, paralysis, or spinal cord inflammation.

Symptoms of schistosomiasis are caused by the body’s reaction to the eggs produced by worms, not by the worms themselves.

Life Cycle of *Schistosoma* sp.

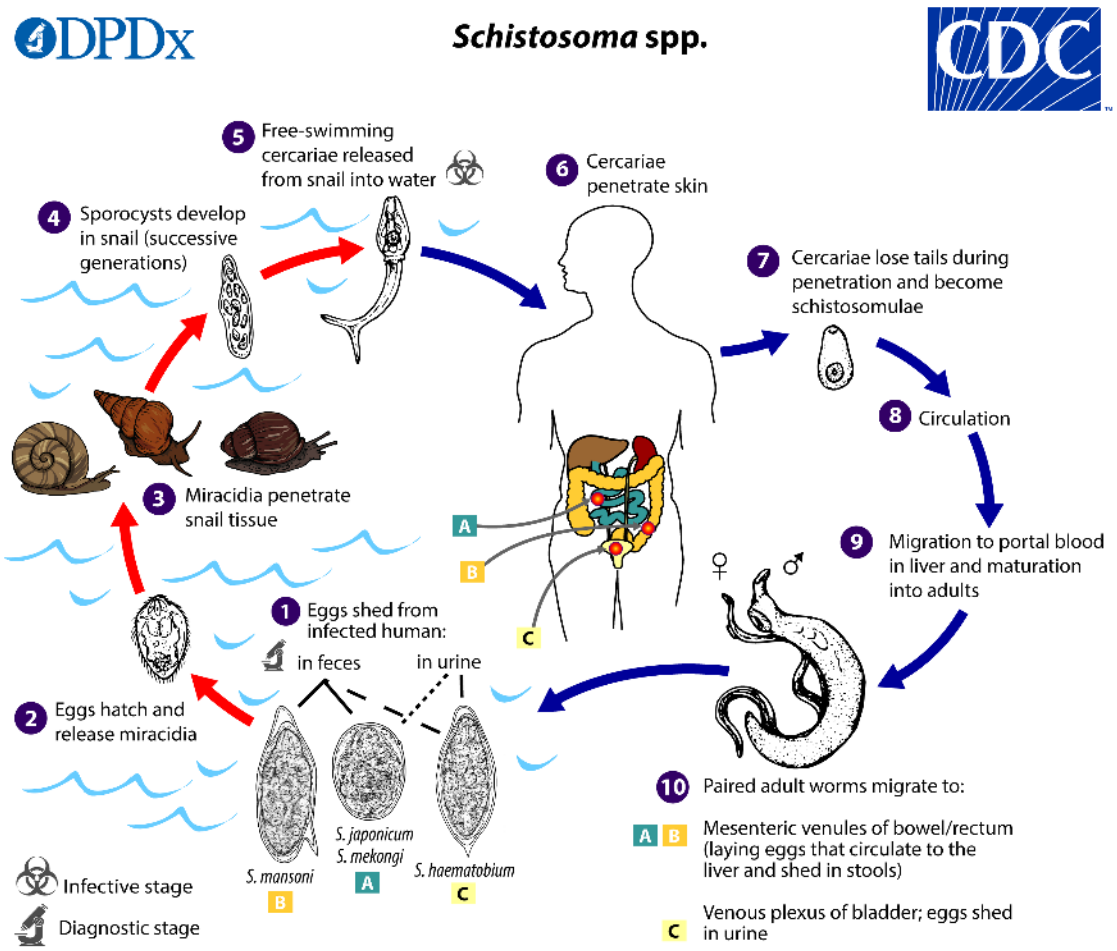


Figure 6: Life cycle of *Schistosoma* sp. See the table below for details about this life cycle.

1	*Schistosoma* eggs are eliminated with feces or urine, depending on species.
2	Under appropriate conditions the eggs hatch and release miracidia...
3	...which swim and penetrate specific snail intermediate hosts.
4	The stages in the snail include two generations of sporocysts...
5	...and the production of cercariae.
6	Upon release from the snail, the infective cercariae swim, penetrate the skin of the human host...
7	...and shed their forked tails, becoming schistosomulae.
8 9	The schistosomulae migrate via venous circulation to lungs, then to the heart, and then develop in the liver, exiting the liver via the portal vein system when mature,
10	Male and female adult worms copulate and reside in the mesenteric venules, the location of which varies by species (with

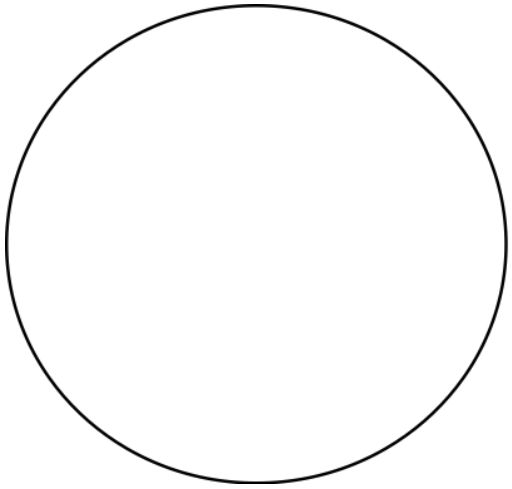
	some exceptions).
A	For instance, *S. japonicum* is more frequently found in the superior mesenteric veins draining the small intestine...
B	...and *S. mansoni* occurs more often in the inferior mesenteric veins draining the large intestine.
C	However, both species can occupy either location and are capable of moving between sites. *S. intercalatum* and *S. guineensis* also inhabit the inferior mesenteric plexus but lower in the bowel than *S. mansoni*. *S. haematobium* most often inhabits in the vesicular and pelvic venous plexus of the bladder...
1	...but it can also be found in the rectal venules. The females (size ranges from 7–28 mm, depending on species) deposit eggs in the small venules of the portal and perivesical systems. The eggs are moved progressively toward the lumen of the intestine (*S. mansoni*,*S. japonicum*, *S. mekongi*, *S. intercalatum/guineensis*) and of the bladder and ureters (*S. haematobium*), and are eliminated with feces or urine, respectively

Laboratory Instructions: *Schistosoma* sp.

1. Examine eggs of *Schistosoma* sp. (use 400x or 1000x magnification).
2. Carefully illustrate the egg sample as you see it in the microscope.
3. Examine an adult worm of *Schistosoma* sp.
4. Carefully illustrate the adult worm sample as you see it in the microscope.

Results & Questions: *Schistosoma* sp.

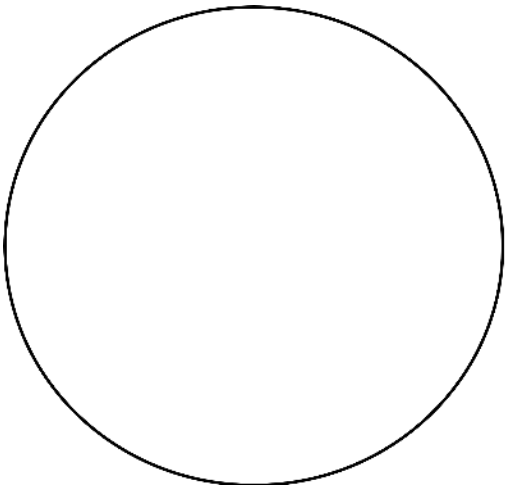
Schistosoma sp. eggs



specimen: _____

total magnification: _____

Schistosoma sp. adult helminth



specimen: _____

total magnification: _____

1. Carefully illustrate the egg sample as you see it in the microscope.
2. Carefully illustrate the adult worm sample as you see it in the microscope.
3. What disease does *Schistosoma* sp. cause?
4. How do people become infected with *Schistosoma* sp.?
5. What is the definitive host species of *Schistosoma* sp.?
6. What is the intermediate host species of *Schistosoma* sp.?
7. List four species of *Schistosoma* that causes schistosomiasis in humans.
8. Give at least five symptoms of schistosomiasis.
9. Where in the human body to adult worms live?
10. How does *Schistosoma* sp. leave a human to continue its life cycle?
11. What areas of the world can humans contract schistosomiasis?

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1.36: 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 **coencocytic 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**.

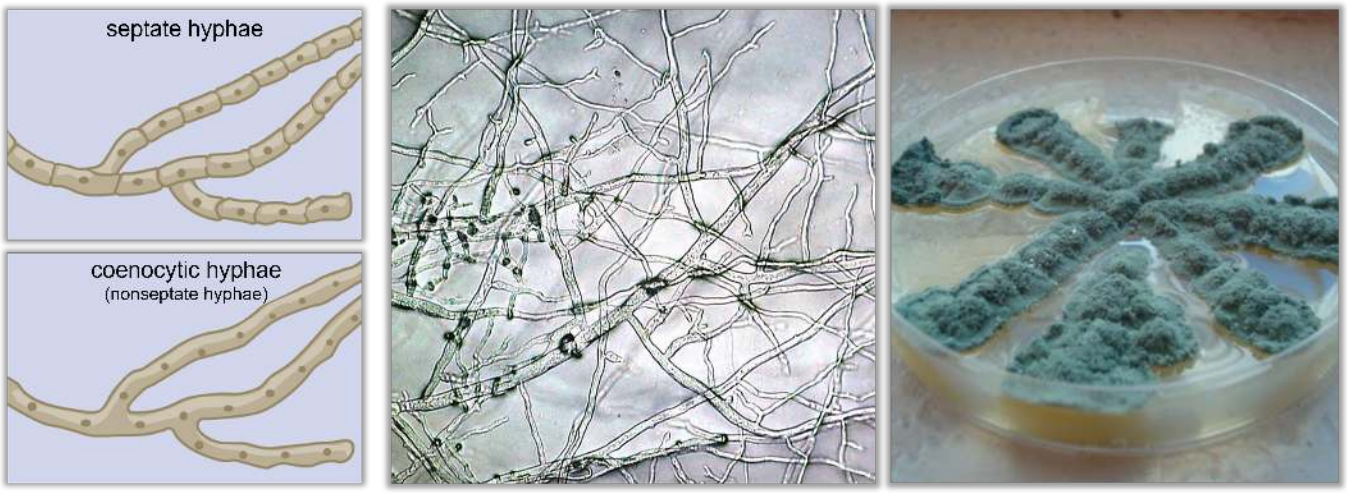


Figure 2: (Left top) Illustration of the structure of septate hyphae. These hyphae appear as long strands that are only one cell wide where cells occur end-to-end with septa that separate the cells. (Left bottom) Illustration of the structure of coenocytic hyphae or nonseptate hyphae. These hyphae appear 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*.

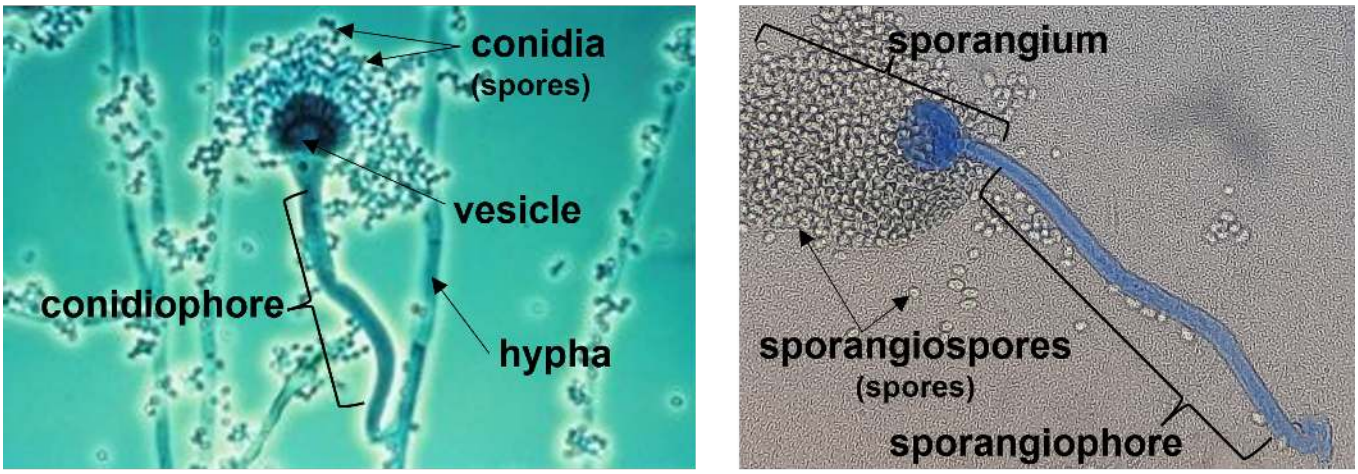


Figure 3: (Left) *Aspergillus* (a species of mold) with its asexual conidiophores leading to a vesicle supporting other cells that bear conidia (spores). (Right) Mucor (a species of mold) with its asexual sporangiophore leading to the sac of sporangiospores called the sporangium.

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*.

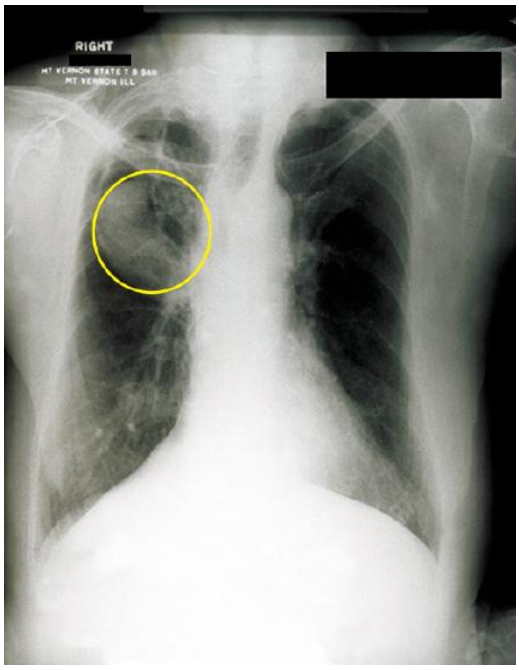


Figure 4: A fungal ball can be observed in the upper lobe of the right lung in this chest radiograph of a patient with aspergilloma. (credit: modification of work by Centers for Disease Control and Prevention)

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:

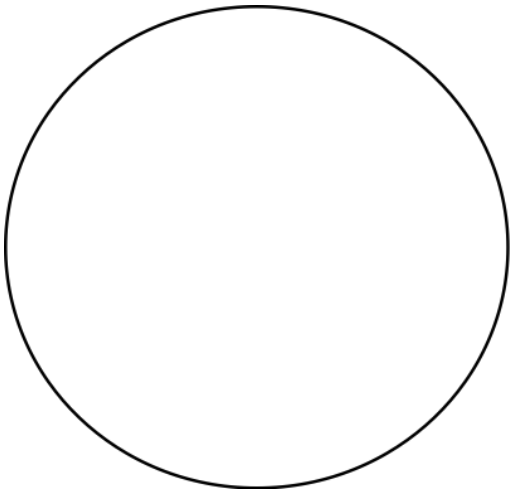
- Weight loss
- Cough
- Coughing up blood
- Fatigue
- Shortness of breath

Invasive aspergillosis[1] 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. View the sample at 400x or 1000x magnification.
2. Carefully illustrate the *Aspergillus* sample as you see it in the microscope. Label the following in your illustration:
    - "hyphae" or "hypha"
    - "conidiophore"
    - "vesicle"
    - "conidia (spores)" or "conidiospores"



specimen: _____

total magnification: _____

1. Carefully illustrate the *Aspergillus* sample as you see it in the microscope. Label the following in your illustration:
   - "hyphae" or "hypha"
   - "conidiophore"
   - "vesicle"
   - "conidia (spores)" or "conidiospores"
2. What areas of the human body can become infected with *Aspergillus*?
3. Where does *Aspergillus* live and how are humans exposed to *Aspergillus*?
4. Who populations are most at risk of developing aspergillosis?
5. What is the difference between a mold and yeast?
6. Is *Aspergillus* a mold or a yeast?
7. Name the *Aspergillus* structures that branch from the hyphae to produce asexual spores?
8. What are the *Aspergillus* asexual spores called?
9. 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.

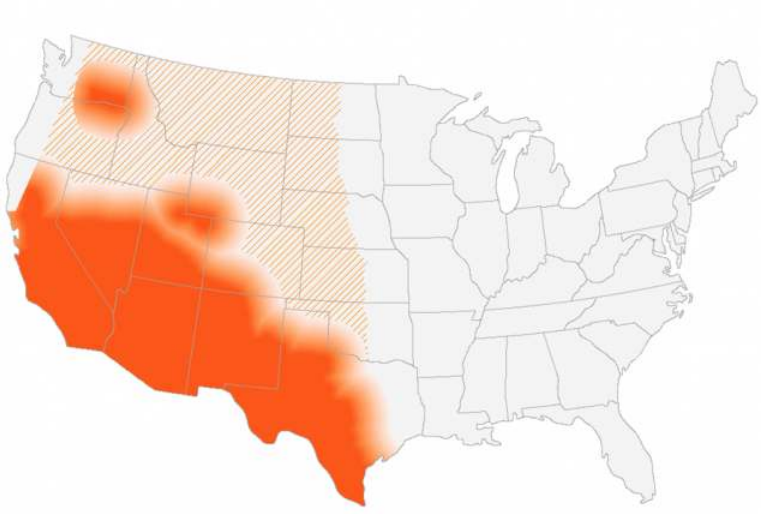


Figure 5: This map shows CDC's current estimate of where the fungus that causes Valley fever lives in the environment in the United States. The fungus is not distributed evenly in the shaded areas, might not be present everywhere in the shaded areas, and can also be outside the shaded areas. Darker shading 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 (*Coccidiodes*) 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.



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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1.37: Viruses and Viral Epidemic Simulation

Learning Objectives

- Name the following virus types and virus structures: helical capsid, icosahedral capsid, enveloped virus, complex virus, nucleic acid (DNA or RNA)/viral genome, capsid, protein, spike proteins, envelope, sheath, tail fibers
- Tell that viruses are not cells, but are particles that are almost always smaller than cells.
- Participate in viral epidemic simulation.
- Use data from viral epidemic simulation to determine the original carrier of the "virus."
- Calculate incidence rate and prevalence rate.
- Define, use, and recognize and name examples of the following: epidemiology, etiology, morbidity, morbidity rate, prevalence, incidence, mortality, sporadic diseases, endemic diseases, epidemic diseases, pandemic diseases, causative agent, reservoirs, passive carriers, active carriers, asymptomatic carriers, direct contact transmission, droplet transmission, indirect contact transmission, vehicle transmission, mechanical transmission, mechanical vector, quarantine, nosocomial infections, healthcare-associated infections

Introduction to Viruses

Viruses are noncellular parasitic entities that cannot be classified within any living kingdom. They can infect organisms as diverse as bacteria, plants, and animals. In fact, viruses exist in a sort of netherworld between a living organism and a nonliving entity. Living things grow, metabolize, and reproduce. In contrast, viruses are not cellular, do not have a metabolism or grow, and cannot divide by cell division. Viruses *can* copy, or replicate themselves; however, they are entirely dependent on resources derived from their host cells to produce progeny viruses—which are assembled in their mature form. No one knows exactly when or how viruses evolved or from what ancestral source because viruses have not left a fossil record. Some virologists contend that modern viruses are a mosaic of bits and pieces of nucleic acids picked up from various sources along their respective evolutionary paths.

Viruses are diverse entities: They vary in structure, methods of replication, and the hosts they infect. Nearly all forms of life—from prokaryotic bacteria and archaeans, to eukaryotes such as plants, animals, and fungi—have viruses that infect them. While most biological diversity can be understood through evolutionary history (such as how species have adapted to changing environmental conditions and how different species are related to one another through common descent), much about virus origins and evolution remains unknown.

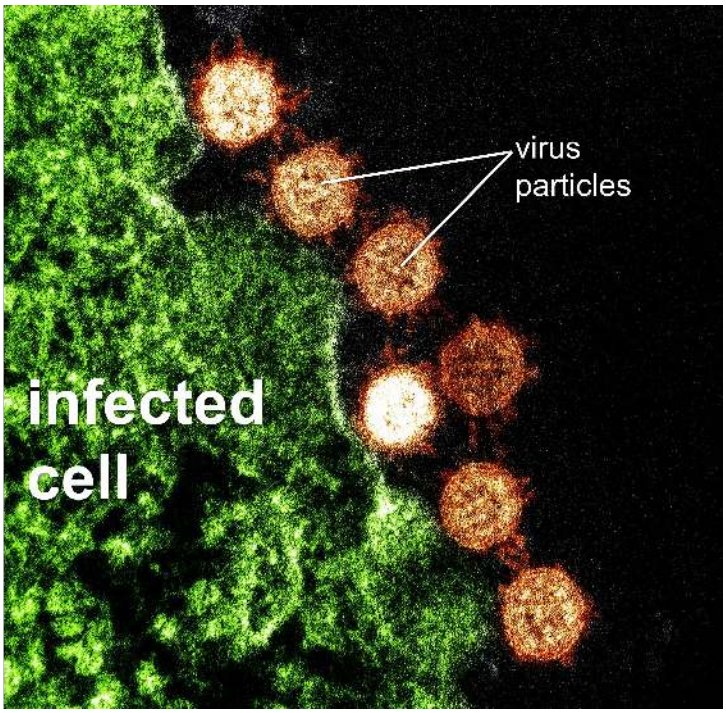


Figure 1: Electron micrograph of the SARS virus (red) attached to an infected cell (green). Note how very small virus particles are and they are not cellular.

Viruses were first discovered after the development of a porcelain filter—the Chamberland-Pasteur filter—that could remove all bacteria visible in the microscope from any liquid sample. In 1886, Adolph Meyer demonstrated that a disease of tobacco plants—**tobacco mosaic disease**—could be transferred from a diseased plant to a healthy one via liquid plant extracts. In 1892, Dmitri Ivanowski showed that this disease could be transmitted in this way even after the Chamberland-Pasteur filter had removed all viable bacteria from the extract. Still, it was many years before it was proved that these "filterable" infectious agents were not simply very small bacteria but were a new type of very small, disease-causing particle.

Virus Structures

Most **virions**, or single virus particles, are very small, about 20 to 250 nanometers in diameter. However, some recently discovered viruses from amoebae range up to 1000 nm in diameter. With the exception of large virions, like the poxvirus and other large DNA viruses, viruses cannot be seen with a light microscope. It was not until the development of the electron microscope in the late 1930s that scientists got their first good view of the structure of the tobacco mosaic virus, discussed above, and other viruses. The surface structure of virions can be observed by both scanning and transmission electron microscopy, whereas the internal structures of the virus can only be observed in images from a transmission electron microscope. The use of electron microscopy and other technologies has allowed for the discovery of many viruses of all types of living organisms.

Use this interactive tool to get a sense of how big viruses are in comparison to cells and familiar objects.*

***viruses in this interactive tool include:** measles virus, hiv, phage, influenza virus, hepatitis virus, and rhinovirus

Viruses are **noncellular**, meaning they are biological entities that do not have a cellular structure. They therefore lack most of the components of cells, such as organelles, ribosomes, and the plasma membrane. A virion consists of a nucleic acid core (DNA or RNA), an outer protein coating or **capsid**, and sometimes an outer **envelope** made of protein and phospholipid membranes derived from the host cell. Viruses may also contain additional proteins, such as enzymes, within the capsid or attached to the viral genome. The most obvious difference between members of different viral families is the variation in their morphology, which is quite

diverse. An interesting feature of viral complexity is that the complexity of the host does not necessarily correlate with the complexity of the virion. In fact, some of the most complex virion structures are found in the **bacteriophages**—viruses that infect the simplest living organisms, bacteria.

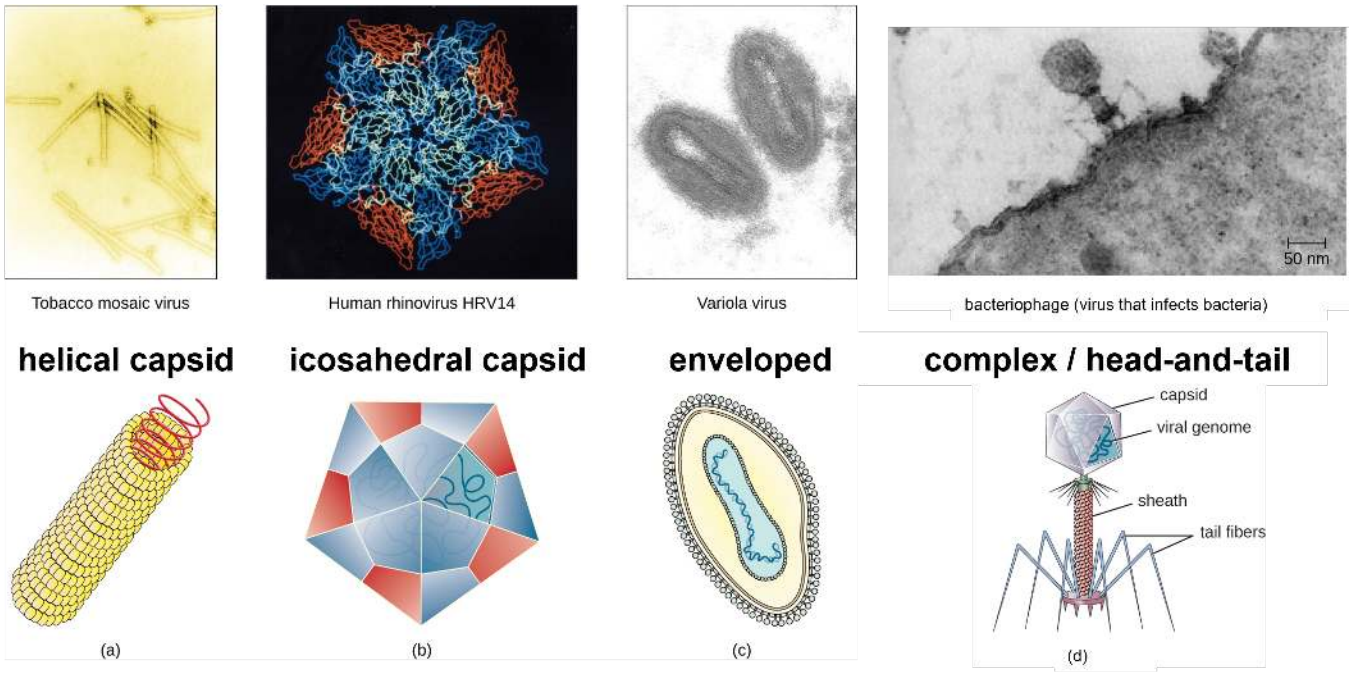


Figure 2: Viruses have diverse shapes. (a) Helical viruses have protein capsids form a hollow tube containing the viral genetic molecule(s). (b) Icosahedral viruses is a three-dimensional hollow structure formed by 20 faces (icosahedron) with the viral genetic material inside. (c) Enveloped viruses are surrounding by a lipid membrane that arose from the plasma membrane of a host cell. (d) Complex or head-and-tail viruses have a head-like structure formed by the capsid with genetic material inside, and a sheath or tail-like structure. (credit a "micrograph": modification of work by USDA ARS; credit b "micrograph": modification of work by U.S. Department of Energy) (credit d: modification of work by U.S. Department of Energy, Office of Science, LBL, PBD)

Viruses come in many shapes and sizes, but these features are consistent for each viral family. As we have seen, all virions have a nucleic acid genome covered by a protective capsid. The proteins of the capsid are encoded in the viral genome, and are called ***capsomeres***. Some viral capsids are simple helices or polyhedral "spheres," whereas others are quite complex in structure.

In general, viruses structures can be classified as: **helical**, **icosahedral**, **enveloped**, and **complex**, also known as **head-and-tail**. *Helical capsids* are long and cylindrical. Many plant viruses are helical, including TMV. *Icosahedral viruses* have shapes that are roughly spherical, such as those of poliovirus or herpesviruses. *Enveloped viruses* have membranes derived from the host cell that surrounds the capsids. Animal viruses, such as HIV, are frequently enveloped. *Head-and-tail viruses* infect bacteria and have a head that is similar to icosahedral viruses and a tail shaped like helical viruses.

Viral Reproduction

All viruses depend on cells for reproduction and metabolic processes. By themselves, viruses do not encode for all of the enzymes necessary for viral replication. But within a host cell, a virus can commandeer cellular machinery to produce more viral particles. Bacteriophages replicate only in the cytoplasm, since prokaryotic cells do not have a nucleus or organelles. In eukaryotic cells, most DNA viruses can replicate inside the nucleus, with an exception observed in the large DNA viruses, such as the poxviruses, that can replicate in the cytoplasm. With a few exceptions, RNA viruses that infect animal cells replicate in the cytoplasm. An important exception that will be highlighted later is Influenza virus.

The life cycle of bacteriophages has been a good model for understanding how viruses affect the cells they infect, since similar processes have been observed for eukaryotic viruses, which can cause immediate death of the cell or establish a latent or chronic infection.

The Lytic Cycle

During the **lytic cycle** of virulent phage, the bacteriophage takes over the cell, reproduces new phages, and destroys the cell. T-even phage is a good example of a well-characterized class of virulent phages. There are five stages in the bacteriophage lytic cycle. **Attachment** is the first stage in the infection process in which the phage interacts with specific bacterial surface receptors (e.g., lipopolysaccharides and OmpC protein on host surfaces). Most phages have a narrow host range and may infect one species of bacteria or one strain within a species. This unique recognition can be exploited for targeted treatment of bacterial infection by phage therapy or for phage typing to identify unique bacterial subspecies or strains. The second stage of infection is entry or **penetration**. This occurs through contraction of the tail sheath, which acts like a hypodermic needle to inject the viral genome through the cell wall and membrane. The phage head and remaining components remain outside the bacteria.

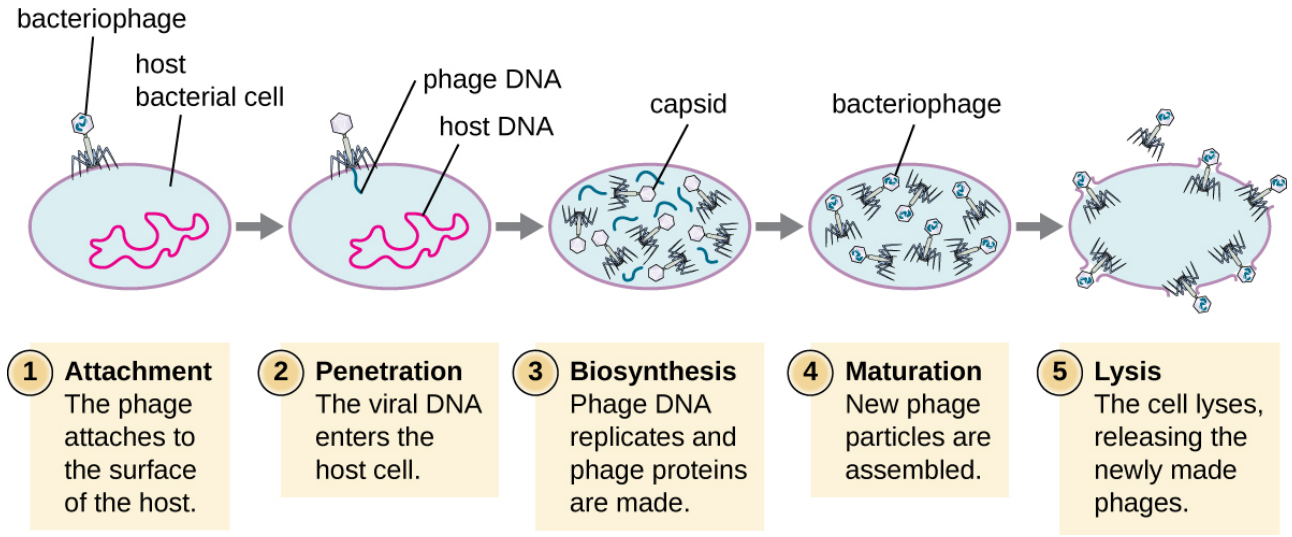


Figure 3: A virulent phage shows only the lytic cycle pictured here. In the lytic cycle, the phage replicates and lyses the host cell. 1. Attachment: the phage attaches to the surface of the host. 2. Penetration: the viral DNA enters the host cell. 3. Biosynthesis: phage DNA replicates and phage proteins are made. 4. Maturation: New phage particles are assembled. 5. Lysis: The cell lyses, releasing the newly made phages.

The third stage of infection is **biosynthesis** of new viral components. After entering the host cell, the virus synthesizes virus-encoded endonucleases to degrade the bacterial chromosome. It then hijacks the host cell to replicate, transcribe, and translate the necessary viral components (capsomeres, sheath, base plates, tail fibers, and viral enzymes) for the assembly of new viruses. Polymerase genes are usually expressed early in the cycle, while capsid and tail proteins are expressed later. During the **maturation** phase, new virions are created. To liberate free phages, the bacterial cell wall is disrupted by phage proteins such as holin or lysozyme. The final stage is release. Mature viruses burst out of the host cell in a process called **lysis** and the progeny viruses are liberated into the environment to infect new cells.

The Lysogenic Cycle

In a **lysogenic cycle**, the phage genome also enters the cell through attachment and penetration. A prime example of a phage with this type of life cycle is the lambda phage. During the lysogenic cycle, instead of killing the host, the phage genome integrates into the bacterial chromosome and becomes part of the host. The integrated phage genome is called a **prophage**. A bacterial host with a

prophage is called a **lysogen**. The process in which a bacterium is infected by a temperate phage is called **lysogeny**. It is typical of temperate phages to be latent or inactive within the cell. As the bacterium replicates its chromosome, it also replicates the phage's DNA and passes it on to new daughter cells during reproduction. The presence of the phage may alter the phenotype of the bacterium, since it can bring in extra genes (e.g., toxin genes that can increase bacterial virulence). This change in the host phenotype is called **lysogenic conversion** or **phage conversion**. Some bacteria, such as *Vibrio cholerae* and *Clostridium botulinum*, are less virulent in the absence of the prophage. The phages infecting these bacteria carry the toxin genes in their genome and enhance the virulence of the host when the toxin genes are expressed. In the case of *V. cholera*, phage encoded toxin can cause severe diarrhea; in *C. botulinum*, the toxin can cause paralysis. During lysogeny, the prophage will persist in the host chromosome until **induction**, which results in the excision of the viral genome from the host chromosome. After induction has occurred the temperate phage can proceed through a lytic cycle and then undergo lysogeny in a newly infected cell.

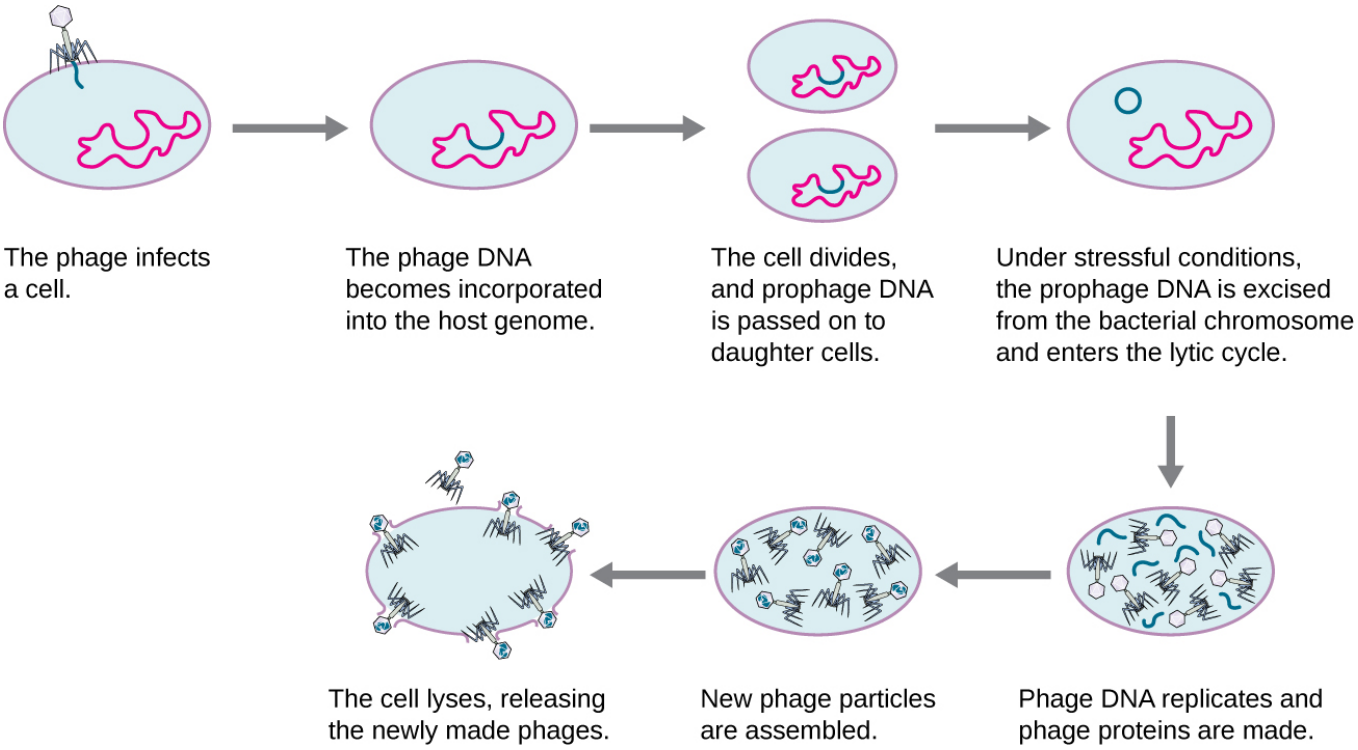


Figure 4: A temperate bacteriophage has both lytic and lysogenic cycles. In the lysogenic cycle, phage DNA is incorporated into the host genome, forming a prophage, which is passed on to subsequent generations of cells. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to be excised and enter the lytic cycle.

Epidemiology

In the United States and other developed nations, public health is a key function of government. A healthy citizenry is more productive, content, and prosperous; high rates of death and disease, on the other hand, can severely hamper economic productivity and foster social and political instability. The burden of disease makes it difficult for citizens to work consistently, maintain employment, and accumulate wealth to better their lives and support a growing economy.

Epidemiology is the science that underlies public health by examining the incidence, spread, transmission, and control of diseases in society. Epidemiology studies how disease originates and spreads throughout a population, with the goal of preventing outbreaks and containing them when they do occur. Over the past two centuries, discoveries in epidemiology have led to public health policies that have transformed life in developed nations, leading to the eradication (or near eradication) of many diseases (e.g. polio, smallpox, measles) that were once causes of great human suffering and premature death. However, the work of

epidemiologists is far from finished. Numerous diseases continue to plague humanity, and new diseases are always emerging. Moreover, in the developing world, lack of infrastructure continues to pose many challenges to efforts to contain disease.

The field of **epidemiology** concerns the geographical distribution and timing of infectious disease occurrences and how they are transmitted and maintained in nature, with the goal of recognizing and controlling outbreaks. The science of epidemiology includes **etiology** (the study of the causes of disease) and investigation of disease transmission (mechanisms by which a disease is spread).

Analyzing Disease in a Population

Epidemiological analyses are always carried out with reference to a population, which is the group of individuals that are at risk for the disease or condition. The population can be defined geographically, but if only a portion of the individuals in that area are susceptible, additional criteria may be required. Susceptible individuals may be defined by particular behaviors, such as intravenous drug use, owning particular pets, or membership in an institution, such as a college. Being able to define the population is important because most measures of interest in epidemiology are made with reference to the size of the population.

The state of being diseased is called **morbidity**. Morbidity in a population can be expressed in a few different ways. Morbidity or total morbidity is expressed in numbers of individuals without reference to the size of the population. The **morbidity rate** can be expressed as the number of diseased individuals out of a standard number of individuals in the population, such as 100,000, or as a percent of the population.

There are two aspects of morbidity that are relevant to an epidemiologist: a disease's **prevalence** and its **incidence**. Prevalence is the number, or proportion, of individuals with a particular illness in a given population at a point in time. For example, the Centers for Disease Control and Prevention (CDC) estimated that in 2012, there were about 1.2 million people 13 years and older with an active human immunodeficiency virus (HIV) infection. Expressed as a proportion, or rate, this is a prevalence of 467 infected persons per 100,000 in the population. On the other hand, incidence is the number or proportion of *new* cases in a period of time. For the same year and population, the CDC estimates that there were 43,165 newly diagnosed cases of HIV infection, which is an incidence of 13.7 new cases per 100,000 in the population. The relationship between incidence and prevalence can be seen in Figure 5. For a chronic disease like HIV infection, prevalence will generally be higher than incidence because it represents the cumulative number of new cases over many years minus the number of cases that are no longer active (e.g., because the patient died or was cured).

In addition to morbidity rates, the incidence and prevalence of **mortality** (death) may also be reported. A mortality rate can be expressed as the percentage of the population that has died from a disease or as the number of deaths per 100,000 persons (or other suitable standard number).

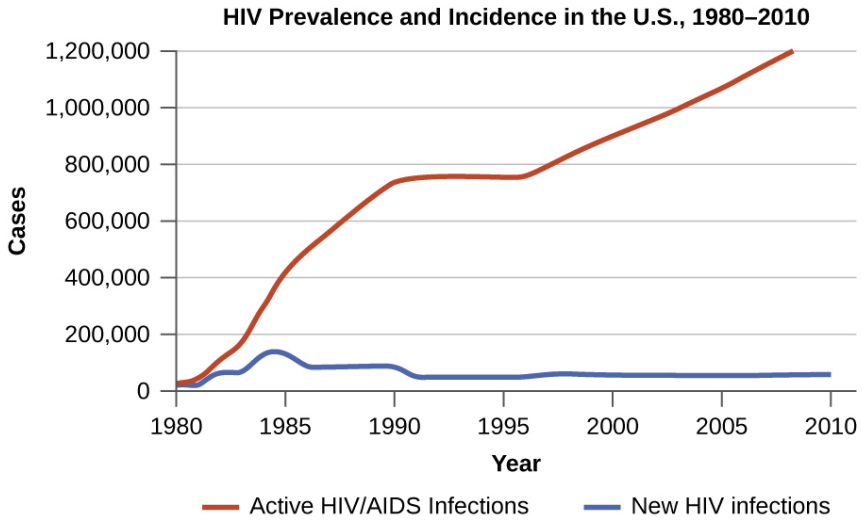


Figure 5: This graph compares the incidence of HIV (the number of new cases reported each year) with the prevalence (the total number of cases each year). Prevalence and incidence can also be expressed as a rate or proportion for a given population.

Patterns of Incidence

Diseases that are seen only occasionally, and usually without geographic concentration, are called **sporadic diseases**. Examples of sporadic diseases include tetanus, rabies, and plague. In the United States, *Clostridium tetani*, the bacterium that causes tetanus, is ubiquitous in the soil environment, but incidences of infection occur only rarely and in scattered locations because most individuals are vaccinated, clean wounds appropriately, or are only rarely in a situation that would cause infection. Likewise in the United States there are a few scattered cases of plague each year, usually contracted from rodents in rural areas in the western states.

Diseases that are constantly present (often at a low level) in a population within a particular geographic region are called **endemic diseases**. For example, malaria is endemic to some regions of Brazil, but is not endemic to the United States.

Diseases for which a larger than expected number of cases occurs in a short time within a geographic region are called **epidemic diseases**. Influenza is a good example of a commonly epidemic disease. Incidence patterns of influenza tend to rise each winter in the northern hemisphere. These seasonal increases are expected, so it would not be accurate to say that influenza is epidemic every winter; however, some winters have an usually large number of seasonal influenza cases in particular regions, and such situations would qualify as epidemics (Figure 6 and Figure 7).

An epidemic disease signals the breakdown of an equilibrium in disease frequency, often resulting from some change in environmental conditions or in the population. In the case of influenza, the disruption can be due to antigenic shift or drift (see Virulence Factors of Bacterial and Viral Pathogens), which allows influenza virus strains to circumvent the acquired immunity of their human hosts.

An epidemic that occurs on a worldwide scale is called a **pandemic disease**. For example, HIV/AIDS is a pandemic disease and novel influenza virus strains often become pandemic.

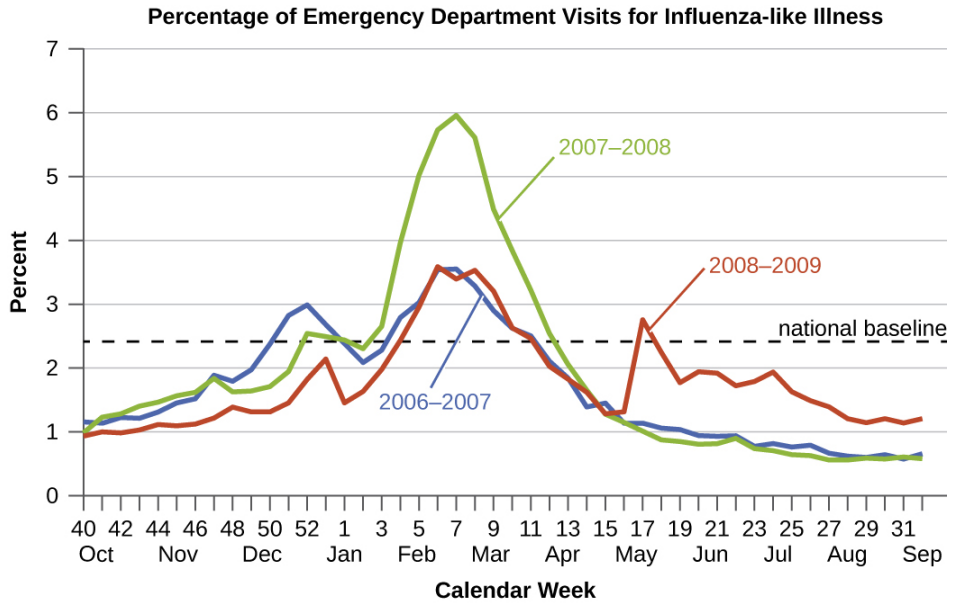


Figure 6: The 2007–2008 influenza season in the United States saw much higher than normal numbers of visits to emergency departments for influenza-like symptoms as compared to the previous and the following years. (credit: modification of work by Centers for Disease Control and Prevention)

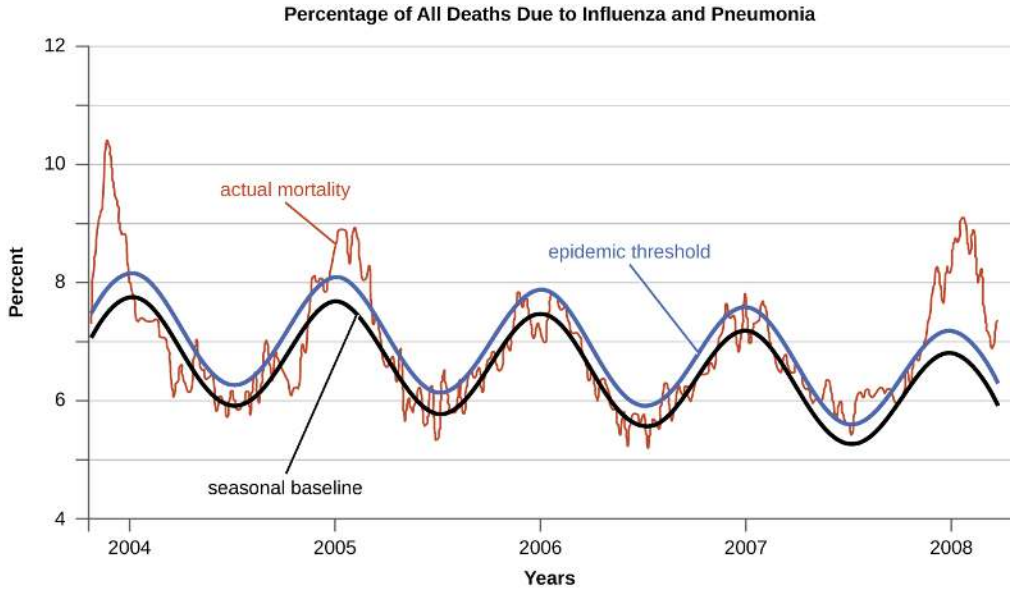


Figure 7: The seasonal epidemic threshold (blue curve) is set by the CDC-based data from the previous five years. When actual mortality rates exceed this threshold, a disease is considered to be epidemic. As this graph shows, pneumonia- and influenza-related mortality saw pronounced epidemics during the winters of 2003–2004, 2005, and 2008. (credit: modification of work by Centers for Disease Control and Prevention)

Etiology

When studying an epidemic, an epidemiologist's first task is to determinate the cause of the disease, called the **etiologic agent** or **causative agent**. Connecting a disease to a specific pathogen can be challenging because of the extra effort typically required to demonstrate direct causation as opposed to a simple association. It is not enough to observe an association between a disease and a suspected pathogen; controlled experiments are needed to eliminate other possible causes. In addition, pathogens are typically difficult to detect when there is no immediate clue as to what is causing the outbreak. Signs and symptoms of disease are also

commonly nonspecific, meaning that many different agents can give rise to the same set of signs and symptoms. This complicates diagnosis even when a causative agent is familiar to scientists.

Robert Koch was the first scientist to specifically demonstrate the causative agent of a disease (anthrax) in the late 1800s. Koch developed four criteria, now known as Koch's postulates, which had to be met in order to positively link a disease with a pathogenic microbe. Without Koch's postulates, the Golden Age of Microbiology would not have occurred. Between 1876 and 1905, many common diseases were linked with their etiologic agents, including cholera, diphtheria, gonorrhea, meningitis, plague, syphilis, tetanus, and tuberculosis. Today, we use the molecular Koch's postulates, a variation of Koch's original postulates that can be used to establish a link between the disease state and virulence traits unique to a pathogenic strain of a microbe.

Koch's Postulates

1. The suspected pathogen must be found in every case of disease and not be found in healthy individuals.
2. The suspected pathogen can be isolated and grown in pure culture.
3. A healthy test subject infected with the suspected pathogen must develop the same signs and symptoms of disease as seen in postulate 1.
4. The pathogen must be re-isolated from the new host and must be identical to the pathogen from postulate 2.

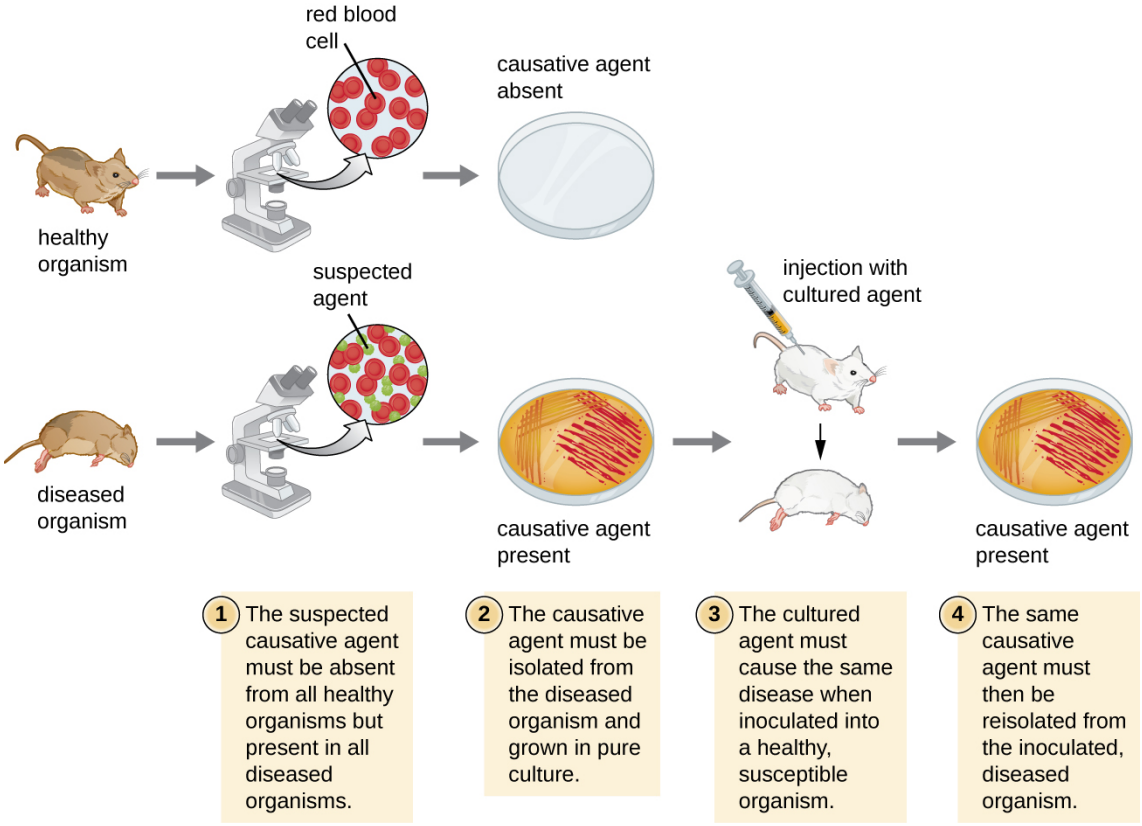


Figure 8: The steps for confirming that a pathogen is the cause of a particular disease using Koch's postulates. 1. The suspected causative agent must be absent from all healthy organisms but present in all diseased organisms. 2. The causative agent must be isolated from the diseased organism and grown in pure culture. 3. The cultured agent must cause the same disease when inoculated into a healthy, susceptible organisms. 4. The same causative agent must then be reisolated from the inoculated, diseased organism.

Spread of Pathogens

Understanding how infectious pathogens spread is critical to preventing infectious disease. Many pathogens require a living host to survive, while others may be able to persist in a dormant state outside of a living host. But having infected one host, all pathogens

must also have a mechanism of transfer from one host to another or they will die when their host dies. Pathogens often have elaborate adaptations to exploit host biology, behavior, and ecology to live in and move between hosts. Hosts have evolved defenses against pathogens, but because their rates of evolution are typically slower than their pathogens (because their generation times are longer), hosts are usually at an evolutionary disadvantage. This section will explore where pathogens survive—both inside and outside hosts—and some of the many ways they move from one host to another.

Reservoirs and Carriers

For pathogens to persist over long periods of time they require **reservoirs** where they normally reside. Reservoirs can be living organisms or nonliving locations. Nonliving reservoirs can include soil and water in the environment. These may naturally harbor the organism because it may grow in that environment. These environments may also become contaminated with pathogens in human feces, pathogens shed by intermediate hosts, or pathogens contained in the remains of intermediate hosts.

Pathogens may have mechanisms of dormancy or resilience that allow them to survive (but typically not to reproduce) for varying periods of time in nonliving environments. For example, *Clostridium tetani* survives in the soil and in the presence of oxygen as a resistant endospore. Although many viruses are soon destroyed once in contact with air, water, or other non-physiological conditions, certain types are capable of persisting outside of a living cell for varying amounts of time. For example, a study that looked at the ability of influenza viruses to infect a cell culture after varying amounts of time on a banknote showed survival times from 48 hours to 17 days, depending on how they were deposited on the banknote. On the other hand, cold-causing rhinoviruses are somewhat fragile, typically surviving less than a day outside of physiological fluids.

A human acting as a reservoir of a pathogen may or may not be capable of transmitting the pathogen, depending on the stage of infection and the pathogen. To help prevent the spread of disease among school children, the CDC has developed guidelines based on the risk of transmission during the course of the disease. For example, children with chickenpox are considered contagious for five days from the start of the rash, whereas children with most gastrointestinal illnesses should be kept home for 24 hours after the symptoms disappear.

An individual capable of transmitting a pathogen without displaying symptoms is referred to as a carrier. A **passive carrier** is contaminated with the pathogen and can mechanically transmit it to another host; however, a passive carrier is not infected. For example, a health-care professional who fails to wash their hands after seeing a patient harboring an infectious agent could become a passive carrier, transmitting the pathogen to another patient who becomes infected.

By contrast, an **active carrier** is an infected individual who can transmit the disease to others. An active carrier may or may not exhibit signs or symptoms of infection. For example, active carriers may transmit the disease during the incubation period (before they show signs and symptoms) or the period of convalescence (after symptoms have subsided). Active carriers who do not present signs or symptoms of disease despite infection are called **asymptomatic carriers**. Pathogens such as hepatitis B virus, herpes simplex virus, and HIV are frequently transmitted by asymptomatic carriers.

Mary Mallon, better known as Typhoid Mary, is a famous historical example of an asymptomatic carrier. An Irish immigrant, Mallon worked as a cook for households in and around New York City between 1900 and 1915. In each household, the residents developed typhoid fever (caused by *Salmonella typhi*) a few weeks after Mallon started working. Later investigations determined that Mallon was responsible for at least 122 cases of typhoid fever, five of which were fatal. See Eye on Ethics: Typhoid Mary for more about the Mallon case.

A pathogen may have more than one living reservoir. In zoonotic diseases, animals act as reservoirs of human disease and transmit the infectious agent to humans through direct or indirect contact. In some cases, the disease also affects the animal, but in other cases the animal is asymptomatic.

In parasitic infections, the parasite's preferred host is called the **definitive host**. In parasites with complex life cycles, the definitive host is the host in which the parasite reaches sexual maturity. Some parasites may also infect one or more **intermediate hosts** in which the parasite goes through several immature life cycle stages or reproduces asexually.

Transmission

Regardless of the reservoir, transmission must occur for an infection to spread. First, transmission from the reservoir to the individual must occur. Then, the individual must transmit the infectious agent to other susceptible individuals, either directly or indirectly. Pathogenic microorganisms employ diverse transmission mechanisms.

Contact Transmission

Contact transmission includes direct contact or indirect contact. Person-to-person transmission is a form of **direct contact transmission**. Here the agent is transmitted by physical contact between two individuals (Figure 9) through actions such as touching, kissing, sexual intercourse, or droplet sprays. Direct contact can be categorized as vertical, horizontal, or droplet transmission. **Vertical direct contact transmission** occurs when pathogens are transmitted from mother to child during pregnancy, birth, or breastfeeding. Other kinds of direct contact transmission are called **horizontal direct contact transmission**. Often, contact between mucous membranes is required for entry of the pathogen into the new host, although skin-to-skin contact can lead to mucous membrane contact if the new host subsequently touches a mucous membrane. Contact transmission may also be site-specific; for example, some diseases can be transmitted by sexual contact but not by other forms of contact.

When an individual coughs or sneezes, small droplets of mucus that may contain pathogens are ejected. This leads to direct **droplet transmission**, which refers to droplet transmission of a pathogen to a new host over distances of one meter or less. A wide variety of diseases are transmitted by droplets, including influenza and many forms of pneumonia. Transmission over distances greater than one meter is called airborne transmission.



Figure 9: Direct contact transmission of pathogens can occur through physical contact. Many pathogens require contact with a mucous membrane to enter the body, but the host may transfer the pathogen from another point of contact (e.g., hand) to a mucous membrane (e.g., mouth or eye). (credit left: modification of work by Lisa Doehnert)

Indirect Contact Transmission

Indirect contact transmission involves inanimate objects called fomites that become contaminated by pathogens from an infected individual or reservoir (Figure 10). For example, an individual with the common cold may sneeze, causing droplets to land on a fomite such as a tablecloth or carpet, or the individual may wipe her nose and then transfer mucus to a fomite such as a doorknob or towel. Transmission occurs indirectly when a new susceptible host later touches the fomite and transfers the contaminated material to a susceptible portal of entry. Fomites can also include objects used in clinical settings that are not properly sterilized, such as syringes, needles, catheters, and surgical equipment. Pathogens transmitted indirectly via such fomites are a major cause of healthcare-associated infections (see Controlling Microbial Growth).



Figure 10: Fomites are nonliving objects that facilitate the indirect transmission of pathogens. Contaminated doorknobs, towels, and syringes are all common examples of fomites. (credit left: modification of work by Kate Ter Haar; credit middle: modification of work by Vernon Swanepoel; credit right: modification of work by "Zaldylmg"/Flickr)

Vehicle Transmission

The term **vehicle transmission** refers to the transmission of pathogens through vehicles such as water, food, and air. Water contamination through poor sanitation methods leads to waterborne transmission of disease. Waterborne disease remains a serious problem in many regions throughout the world. The World Health Organization (WHO) estimates that contaminated drinking water is responsible for more than 500,000 deaths each year. Similarly, food contaminated through poor handling or storage can lead to foodborne transmission of disease (Figure 11).

Dust and fine particles known as aerosols, which can float in the air, can carry pathogens and facilitate the airborne transmission of disease. For example, dust particles are the dominant mode of transmission of hantavirus to humans. Hantavirus is found in mouse feces, urine, and saliva, but when these substances dry, they can disintegrate into fine particles that can become airborne when disturbed; inhalation of these particles can lead to a serious and sometimes fatal respiratory infection.

Although droplet transmission over short distances is considered contact transmission as discussed above, longer distance transmission of droplets through the air is considered vehicle transmission. Unlike larger particles that drop quickly out of the air column, fine mucus droplets produced by coughs or sneezes can remain suspended for long periods of time, traveling considerable distances. In certain conditions, droplets desiccate quickly to produce a droplet nucleus that is capable of transmitting pathogens; air temperature and humidity can have an impact on effectiveness of airborne transmission.

Tuberculosis is often transmitted via airborne transmission when the causative agent, *Mycobacterium tuberculosis*, is released in small particles with coughs. Because tuberculosis requires as few as 10 microbes to initiate a new infection, patients with tuberculosis must be treated in rooms equipped with special ventilation, and anyone entering the room should wear a mask.



Figure 11: Food is an important vehicle of transmission for pathogens, especially of the gastrointestinal and upper respiratory systems. Notice the glass shield above the food trays, designed to prevent pathogens ejected in coughs and sneezes from entering the food. (credit: Fort George G. Meade Public Affairs Office)

Vector Transmission

Diseases can also be transmitted by a mechanical or biological vector, an animal (typically an arthropod) that carries the disease from one host to another. **Mechanical transmission** is facilitated by a **mechanical vector**, an animal that carries a pathogen from one host to another without being infected itself. For example, a fly may land on fecal matter and later transmit bacteria from the feces to food that it lands on; a human eating the food may then become infected by the bacteria, resulting in a case of diarrhea or dysentery (Figure 12).

Biological transmission occurs when the pathogen reproduces within a **biological vector** that transmits the pathogen from one host to another (Figure 12). Arthropods are the main vectors responsible for biological transmission (Figure 13). Most arthropod vectors transmit the pathogen by biting the host, creating a wound that serves as a portal of entry. The pathogen may go through part of its reproductive cycle in the gut or salivary glands of the arthropod to facilitate its transmission through the bite. For example, hemipterans (called “kissing bugs” or “assassin bugs”) transmit Chagas disease to humans by defecating when they bite, after which the human scratches or rubs the infected feces into a mucous membrane or break in the skin.

Biological insect vectors include mosquitoes, which transmit malaria and other diseases, and lice, which transmit typhus. Other arthropod vectors can include arachnids, primarily ticks, which transmit Lyme disease and other diseases, and mites, which transmit scrub typhus and rickettsial pox. Biological transmission, because it involves survival and reproduction within a parasitized vector, complicates the biology of the pathogen and its transmission. There are also important non-arthropod vectors of disease, including mammals and birds. Various species of mammals can transmit rabies to humans, usually by means of a bite that transmits the rabies virus. Chickens and other domestic poultry can transmit avian influenza to humans through direct or indirect contact with avian influenza virus A shed in the birds’ saliva, mucous, and feces.

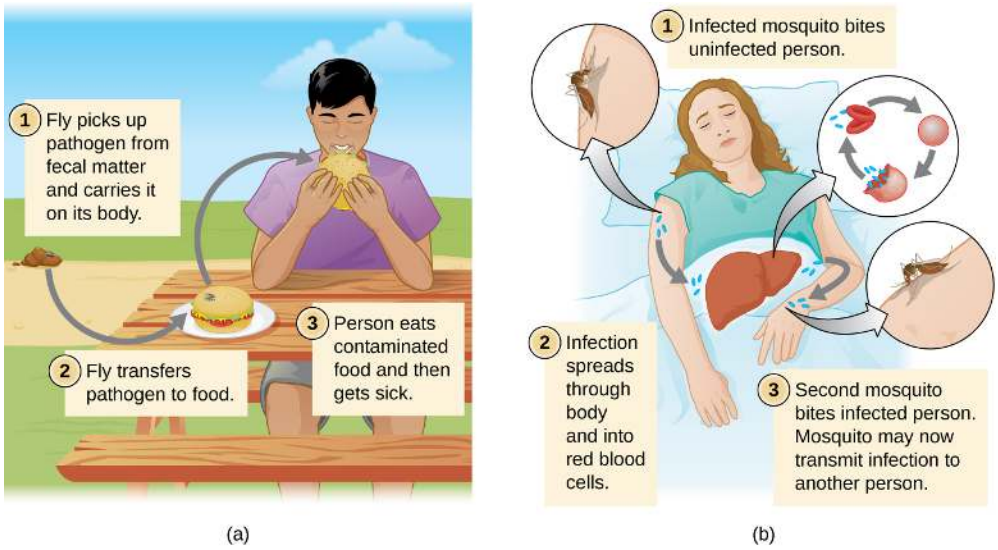


Figure 12: (a) A mechanical vector carries a pathogen on its body from one host to another, not as an infection. 1. Fly picks up pathogen from fecal matter and carries it on its body. 2. Fly transfers pathogen to food. 3. Person eats contaminated food and then gets sick. (b) A biological vector carries a pathogen from one host to another after becoming infected itself. 1. Infected mosquito bites uninfected person. 2. Infection spreads through body and into red blood cells. 3. Second mosquito bites infected person. Mosquito may now transmit infection to another person.

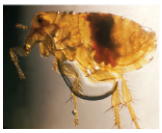
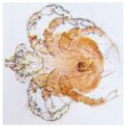
Common Arthropod Vectors and Select Pathogens			
Vector	**Species**	**Pathogen**	**Disease**
Black fly 	*Simulium* spp.	*Onchocerca volvulus*	Onchocerciasis (river blindness)
Flea 	*Xenopsylla cheopis*	*Rickettsia typhi*	Murine typhus
		Yersinia pestis	Plague
Kissing bug 	*Triatoma* spp.	*Trypanosoma cruzi*	Chagas disease
Louse 	*Pediculus humanus humanus*	*Bartonella quintana*	Trench fever
		Borrelia recurrentis	Relapsing fever
		Rickettsia prowazekii	Typhus
Mite (chigger) 	*Leptotrombidium* spp.	*Orientia tsutsugamushi*	Scrub typhus
	Liponyssoides sanguineus	*Rickettsia akari*	Rickettsialpox
Mosquito 	*Aedes* spp., *Haemagogus* spp.	*Yellow fever virus*	Yellow fever
	Anopheles spp.	*Plasmodium falciparum*	Malaria
	Culex pipiens	*West Nile virus*	West Nile disease
Sand fly 	*Phlebotomus* spp.	*Leishmania* spp.	Leishmaniasis
Tick 	*Ixodes* spp.	*Borrelia* spp.	Lyme disease
	Dermacentor spp. and others	*Rickettsia rickettsii*	Rocky Mountain spotted fever
Tsetse fly 	*Glossina* spp.	*Trypanosoma brucei*	African trypanosomiasis (sleeping sickness)

Figure 13: (credit “Black fly”, “Tick”, “Tsetse fly”: modification of work by USDA; credit: “Flea”: modification of work by Centers for Disease Control and Prevention; credit: “Louse”, “Mosquito”, “Sand fly”: modification of work by James Gathany, Centers for Disease Control and Prevention; credit “Kissing bug”: modification of work by Glenn Seplak; credit “Mite”: modification of work by Michael Wunderli)

Quarantining

Individuals suspected or known to have been exposed to certain contagious pathogens may be **quarantined**, or isolated to prevent transmission of the disease to others. Hospitals and other health-care facilities generally set up special wards to isolate patients with particularly hazardous diseases such as tuberculosis or Ebola (Figure 16.15). Depending on the setting, these wards may be equipped with special air-handling methods, and personnel may implement special protocols to limit the risk of transmission, such as personal protective equipment or the use of chemical disinfectant sprays upon entry and exit of medical personnel.

The duration of the quarantine depends on factors such as the incubation period of the disease and the evidence suggestive of an infection. The patient may be released if signs and symptoms fail to materialize when expected or if preventive treatment can be administered in order to limit the risk of transmission. If the infection is confirmed, the patient may be compelled to remain in isolation until the disease is no longer considered contagious.

In the United States, public health authorities may only quarantine patients for certain diseases, such as cholera, diphtheria, infectious tuberculosis, and strains of influenza capable of causing a pandemic. Individuals entering the United States or moving between states may be quarantined by the CDC if they are suspected of having been exposed to one of these diseases. Although the CDC routinely monitors entry points to the United States for crew or passengers displaying illness, quarantine is rarely implemented.

Healthcare-Associated (Nosocomial) Infections

Hospitals, retirement homes, and prisons attract the attention of epidemiologists because these settings are associated with increased incidence of certain diseases. Higher rates of transmission may be caused by characteristics of the environment itself, characteristics of the population, or both. Consequently, special efforts must be taken to limit the risks of infection in these settings.

Infections acquired in health-care facilities, including hospitals, are called **nosocomial infections** or **healthcare-associated infections (HAI)**. HAIs are often connected with surgery or other invasive procedures that provide the pathogen with access to the portal of infection. For an infection to be classified as an HAI, the patient must have been admitted to the health-care facility for a reason other than the infection. In these settings, patients suffering from primary disease are often afflicted with compromised immunity and are more susceptible to secondary infection and opportunistic pathogens.

In 2011, more than 720,000 HAIs occurred in hospitals in the United States, according to the CDC. About 22% of these HAIs occurred at a surgical site, and cases of pneumonia accounted for another 22%; urinary tract infections accounted for an additional 13%, and primary bloodstream infections 10%. Such HAIs often occur when pathogens are introduced to patients’ bodies through contaminated surgical or medical equipment, such as catheters and respiratory ventilators. Health-care facilities seek to limit nosocomial infections through training and hygiene protocols such as those described in Control of Microbial Growth.

Laboratory Instructions

Viral Epidemic Scenario

One of the most rapid ways for a pathogen to spread is when people are asymptomatic carriers or when people are in the incubation period before symptoms occur. Since these people are not experiencing symptoms, they are unaware that they are carrying a pathogen and able to spread that pathogen. This is particularly true when the incubation period is lengthy (length of the incubation period is different for different pathogens).

You are among a population who is at risk of catching a virus at the start of an epidemic. Everyone in the population begins either as an uninfected person, except one person who is an asymptomatic carrier of the virus. No one knows who the infected person is including that person.

This virus passes when two people are in close proximity and the carrier exhales respiratory droplets into the air. The uninfected person inhales respiratory droplets from the nearby carrier and infection then occurs.

As you go about normal day to day life in this scenario, you will come into contact with different individuals in the classroom each "day." Each "day" you will interact with one classmate and breath each others respiratory droplets. You will not know until the end whether you were the original infected person, or if you become infected and transmit the infection to others.

Epidemic Day 1

A well plate will be used to track fluid samples from each participant.

1. Choose/be assigned a vial number.
2. Collect the vial with your number and a transfer pipet.
3. Write your vial number down in the Results & Questions section below.
4. Put 5 drops from your vial into the well on the well plate corresponding with your vial number and epidemic day 1.

Epidemic Day 2 (Transfer #1)

1. Names will be randomly chosen using a group randomizer.
2. Find the person you are paired with and write their vial number in the table for epidemic day 2 in the Results & Questions section below.
3. Use your transfer pipet to collect fluid from your vial. Your partner will do the same.
4. Drop 5 drops from your transfer pipet into your partner's vial (this represents you transferring your respiratory droplets to your partner).
5. Your partner will drop 5 drops from their transfer pipet to your vial (this represents your partner transferring their respiratory droplets to you).
6. Put any liquid remaining in the transfer pipet back into your own vial.
7. Close your vial securely and turn the vial upside-down 10 times to mix the vial well.
8. Collect liquid from your vial with the transfer pipet.
9. Put 5 drops from your vial into the well on the well plate corresponding with your vial number and epidemic day 2.
10. Put any liquid remaining in the transfer pipet back into your own vial.

Epidemic Day 3 (Transfer #2)

1. Names will be randomly chosen using a group randomizer.
2. Find the person you are paired with and write their vial number in the table for epidemic day 3 in the Results & Questions section below.
3. Use your transfer pipet to collect fluid from your vial. Your partner will do the same.
4. Drop 5 drops from your transfer pipet into your partner's vial (this represents you transferring your respiratory droplets to your partner).
5. Your partner will drop 5 drops from their transfer pipet to your vial (this represents your partner transferring their respiratory droplets to you).
6. Put any liquid remaining in the transfer pipet back into your own vial.
7. Close your vial securely and turn the vial upside-down 10 times to mix the vial well.
8. Collect liquid from your vial with the transfer pipet.
9. Put 5 drops from your vial into the well on the well plate corresponding with your vial number and epidemic day 3.
10. Put any liquid remaining in the transfer pipet back into your own vial.

Epidemic Day 4 (Transfer #3)

1. Names will be randomly chosen using a group randomizer.
2. Find the person you are paired with and write their vial number in the table for epidemic day 4 in the Results & Questions section below.
3. Use your transfer pipet to collect fluid from your vial. Your partner will do the same.
4. Drop 5 drops from your transfer pipet into your partner's vial (this represents you transferring your respiratory droplets to your partner).
5. Your partner will drop 5 drops from their transfer pipet to your vial (this represents your partner transferring their respiratory droplets to you).
6. Put any liquid remaining in the transfer pipet back into your own vial.
7. Close your vial securely and turn the vial upside-down 10 times to mix the vial well.
8. Collect liquid from your vial with the transfer pipet.
9. Put 5 drops from your vial into the well on the well plate corresponding with your vial number and epidemic day 4.
10. Put any liquid remaining in the transfer pipet back into your own vial.

Revealing the Viral Infections & Spread of Virus in the Population

1. After epidemic day 4, your instructor will place a single drop of a solution into each person's vial. If the vial turns red, you are "infected" with the virus.
2. If you are infected, fill out the table on the board to indicate your vial number and the vial numbers you exchanged "respiratory drops" with.
3. Your instructor will also use the well plate to determine the number of "infected" people in the population each day if the epidemic. They will share this information with the class so you can calculate the prevalence rates and incidence rates.

Results & Questions

My vial number is: _____

Epidemic Day	Vial # Respiratory Drops Exchanged With
2	
3	
4	

Vial #	Mark with 'X' if infected on Epidemic Day 4	Vials that Exchanged Respiratory Droplets with this Vial
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		

1. Fill out the table above for each of the epidemic days.
2. Fill out the table above to indicate which of the vials were positive for the virus and which vials the virus-positive vials exchanged respiratory droplets with.
3. Put your epidemiologist hat on. Use the information above to determine which vial was the original one that was infected. Which vial contained virus on epidemic day 1? How can you tell?
4. How many chances did people in this scenario have to become "infected?"
5. At the beginning of this viral epidemic scenario, only one person was "infected" with the virus. How many people were "infected" with the virus by epidemic day 4?

6. Explain why there were more people who were "infected" at the end of the scenario than the number of fluid exchanges.
7. What type or types of transmission occurred in this scenario (direct contact transmission, droplet transmission, indirect contact transmission, vehicle transmission, mechanical transmission)
8. When a person is asymptomatic, are they aware that they are spreading a pathogen?
9. Calculate the prevalence rate and incidence rate of the virus in the population for each epidemic day.

Epidemic Day	Prevalence Rate	Incidence Rate
Day 1		
Day 2		
Day 3		
Day 4		

10. Define the following terms:
    - epidemiology:
    - etiology:
    - morbidity:
    - morbidity rate:
    - prevalence:
    - incidence:
    - mortality:
    - sporadic diseases:
    - endemic diseases:
    - epidemic diseases:
    - pandemic diseases:
    - causative agent:
    - reservoirs:
    - passive carriers:
    - active carriers:
    - asymptomatic carriers:
    - direct contact transmission:
    - droplet transmission:
    - indirect contact transmission:
    - vehicle transmission:
    - mechanical transmission:
    - mechanical vector:
    - quarantine:
    - nosocomial infections:
    - healthcare-associated infections:

Attributions

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1.38: Virus Bioassay

Learning Objectives

- Describe the structures of viruses, including their sizes.
- Identify examples of different virus morphologies (e.g. helical, icosohedral, etc.)
- Explain how tobacco mosaic virus infects cells, spreads, and replicates.
- Tell that specific viruses will only infect specific species.
- Describe how TMV has significant impacts on agriculture.
- Extract TMV from tobacco.
- Conduct a bioassay using TMV, analyze results of the experiment by t-test, and state conclusions of the experiment.

Introduction to Viruses

Viruses are noncellular parasitic entities that cannot be classified within any living kingdom. They can infect organisms as diverse as bacteria, plants, and animals. In fact, viruses exist in a sort of netherworld between a living organism and a nonliving entity. Living things grow, metabolize, and reproduce. In contrast, viruses are not cellular, do not have a metabolism or grow, and cannot divide by cell division. Viruses *can* copy, or replicate themselves; however, they are entirely dependent on resources derived from their host cells to produce progeny viruses—which are assembled in their mature form. No one knows exactly when or how viruses evolved or from what ancestral source because viruses have not left a fossil record. Some virologists contend that modern viruses are a mosaic of bits and pieces of nucleic acids picked up from various sources along their respective evolutionary paths.

Viruses are diverse entities: They vary in structure, methods of replication, and the hosts they infect. Nearly all forms of life—from prokaryotic bacteria and archaeans, to eukaryotes such as plants, animals, and fungi—have viruses that infect them. While most biological diversity can be understood through evolutionary history (such as how species have adapted to changing environmental conditions and how different species are related to one another through common descent), much about virus origins and evolution remains unknown.

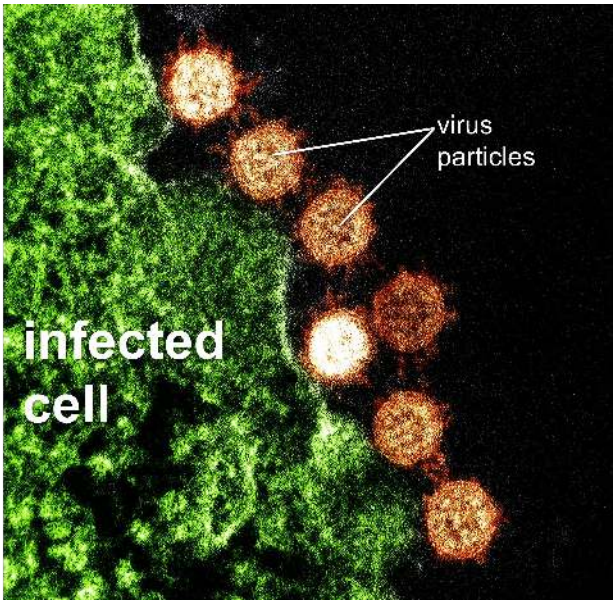


Figure 1: Electron micrograph of the SARS virus (red) attached to an infected cell (green). Note how very small virus particles are and they are not cellular.

Viruses were first discovered after the development of a porcelain filter—the Chamberland-Pasteur filter—that could remove all bacteria visible in the microscope from any liquid sample. In 1886, Adolph Meyer demonstrated that a disease of tobacco plants—**tobacco mosaic disease**—could be transferred from a diseased plant to a healthy one via liquid plant extracts. In 1892, Dmitri Ivanowski showed that this disease could be transmitted in this way even after the Chamberland-Pasteur filter had removed all viable bacteria from the extract. Still, it was many years before it was proved that these "filterable" infectious agents were not simply very small bacteria but were a new type of very small, disease-causing particle.

Virus Structures

Most **virions**, or single virus particles, are very small, about 20 to 250 nanometers in diameter. However, some recently discovered viruses from amoebae range up to 1000 nm in diameter. With the exception of large virions, like the poxvirus and other large DNA viruses, viruses cannot be seen with a light microscope. It was not until the development of the electron microscope in the late 1930s that scientists got their first good view of the structure of the tobacco mosaic virus, discussed above, and other viruses. The surface structure of virions can be observed by both scanning and transmission electron microscopy, whereas the internal structures of the virus can only be observed in images from a transmission electron microscope. The use of electron microscopy and other technologies has allowed for the discovery of many viruses of all types of living organisms.

Use this interactive tool to get a sense of how big viruses are in comparison to cells and familiar objects.*

***viruses in this interactive tool include:** measles virus, hiv, phage, influenza virus, hepatitis virus, and rhinovirus

V

iruses are **noncellular**, meaning they are biological entities that do not have a cellular structure. They therefore lack most of the components of cells, such as organelles, ribosomes, and the plasma membrane. A virion consists of a nucleic acid core (DNA or RNA), an outer protein coating or **capsid**, and sometimes an outer **envelope** made of protein and phospholipid membranes derived from the host cell. Viruses may also contain additional proteins, such as enzymes, within the capsid or attached to the viral genome. The most obvious difference between members of different viral families is the variation in their morphology, which is quite diverse. An interesting feature of viral complexity is that the complexity of the host does not necessarily correlate with the complexity of the virion. In fact, some of the most complex virion structures are found in the **bacteriophages**—viruses that infect the simplest living organisms, bacteria.

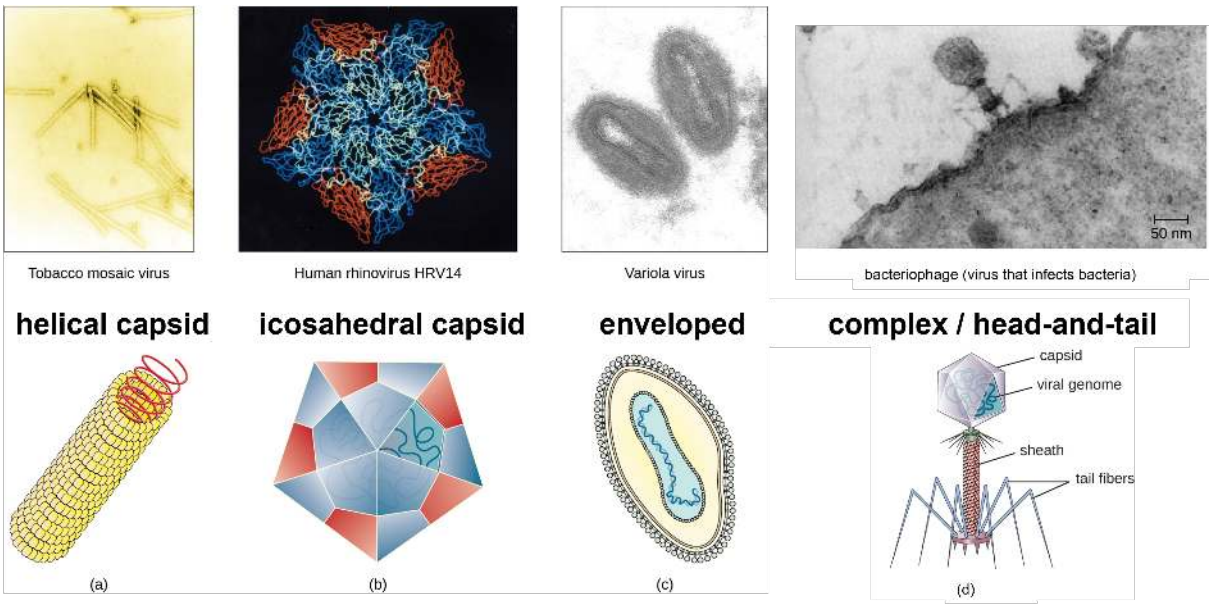


Figure 2: Viruses have diverse shapes. (a) Helical viruses have protein capsids form a hollow tube containing the viral genetic molecule(s). (b) Icosahedral viruses is a three-dimensional hollow structure formed by 20 faces (icosahedron) with the viral genetic material inside. (c) Enveloped viruses are surrounding by a lipid membrane that arose from the plasma membrane of a host cell. (d) Complex or head-and-tail viruses have a head-like structure formed by the capsid with genetic material inside, and a sheath or tail-like structure. (credit a "micrograph": modification of work by USDA ARS; credit b "micrograph": modification of work by U.S. Department of Energy) (credit d: modification of work by U.S. Department of Energy, Office of Science, LBL, PBD)

Viruses come in many shapes and sizes, but these features are consistent for each viral family. As we have seen, all virions have a nucleic acid genome covered by a protective capsid. The proteins of the capsid are encoded in the viral genome, and are called ***capsomeres***. Some viral capsids are simple helices or polyhedral "spheres," whereas others are quite complex in structure.

In general, viruses structures can be classified as: **helical**, **icosahedral**, **enveloped**, and **complex**, also known as **head-and-tail**. *Helical capsids* are long and cylindrical. Many plant viruses are helical, including TMV. *Icosahedral viruses* have shapes that are roughly spherical, such as those of poliovirus or herpesviruses. *Enveloped viruses* have membranes derived from the host cell that surrounds the capsids. Animal viruses, such as HIV, are frequently enveloped. *Head-and-tail viruses* infect bacteria and have a head that is similar to icosahedral viruses and a tail shaped like helical viruses.

Viral Reproduction

All viruses depend on cells for reproduction and metabolic processes. By themselves, viruses do not encode for all of the enzymes necessary for viral replication. But within a host cell, a virus can commandeer cellular machinery to produce more viral particles. Bacteriophages replicate only in the cytoplasm, since prokaryotic cells do not have a nucleus or organelles. In eukaryotic cells, most DNA viruses can replicate inside the nucleus, with an exception observed in the large DNA viruses, such as the poxviruses, that can replicate in the cytoplasm. With a few exceptions, RNA viruses that infect animal cells replicate in the cytoplasm. An important exception that will be highlighted later is Influenza virus.

The life cycle of bacteriophages has been a good model for understanding how viruses affect the cells they infect, since similar processes have been observed for eukaryotic viruses, which can cause immediate death of the cell or establish a latent or chronic infection.

The Lytic Cycle

During the **lytic cycle** of virulent phage, the bacteriophage takes over the cell, reproduces new phages, and destroys the cell. T-even phage is a good example of a well-characterized class of virulent phages. There are five stages in the bacteriophage lytic cycle. **Attachment** is the first stage in the infection process in which the phage interacts with specific bacterial surface receptors (e.g., lipopolysaccharides and OmpC protein on host surfaces). Most phages have a narrow host range and may infect one species of bacteria or one strain within a species. This unique recognition can be exploited for targeted treatment of bacterial infection by phage therapy or for phage typing to identify unique bacterial subspecies or strains. The second stage of infection is entry or **penetration**. This occurs through contraction of the tail sheath, which acts like a hypodermic needle to inject the viral genome through the cell wall and membrane. The phage head and remaining components remain outside the bacteria.

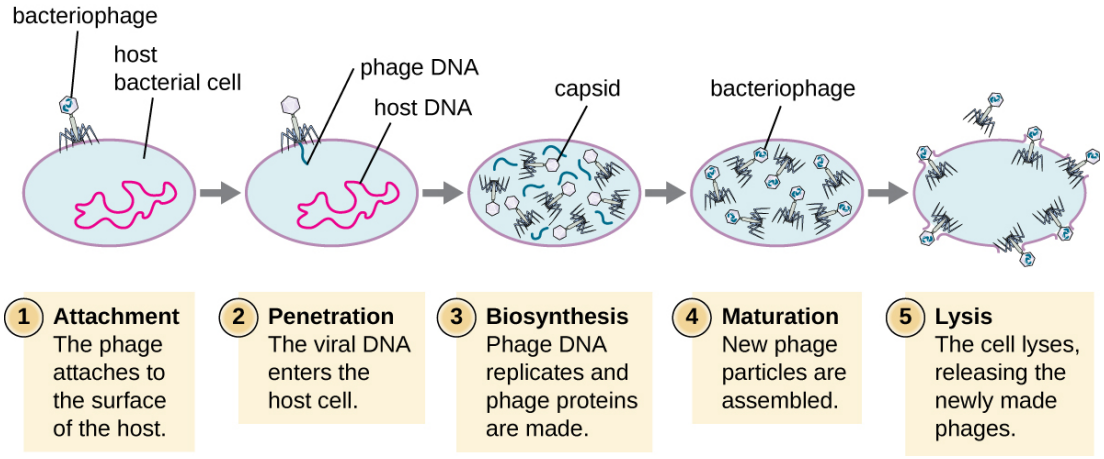


Figure 3: A virulent phage shows only the lytic cycle pictured here. In the lytic cycle, the phage replicates and lyses the host cell. 1. Attachment: the phage attaches to the surface of the host. 2. Penetration: the viral DNA enters the host cell. 3. Biosynthesis: phage DNA replicates and phage proteins are made. 4. Maturation: New phage particles are assembled. 5. Lysis: The cell lyses, releasing the newly made phages.

The third stage of infection is **biosynthesis** of new viral components. After entering the host cell, the virus synthesizes virus-encoded endonucleases to degrade the bacterial chromosome. It then hijacks the host cell to replicate, transcribe, and translate the necessary viral components (capsomeres, sheath, base plates, tail fibers, and viral enzymes) for the assembly of new viruses. Polymerase genes are usually expressed early in the cycle, while capsid and tail proteins are expressed later. During the **maturation** phase, new virions are created. To liberate free phages, the bacterial cell wall is disrupted by phage proteins such as holin or lysozyme. The final stage is release. Mature viruses burst out of the host cell in a process called **lysis** and the progeny viruses are liberated into the environment to infect new cells.

The Lysogenic Cycle

In a **lysogenic cycle**, the phage genome also enters the cell through attachment and penetration. A prime example of a phage with this type of life cycle is the lambda phage. During the lysogenic cycle, instead of killing the host, the phage genome integrates into the bacterial chromosome and becomes part of the host. The integrated phage genome is called a **prophage**. A bacterial host with a prophage is called a **lysogen**. The process in which a bacterium is infected by a temperate phage is called **lysogeny**. It is typical of temperate phages to be latent or inactive within the cell. As the bacterium replicates its chromosome, it also replicates the phage's DNA and passes it on to new daughter cells during reproduction. The presence of the phage may alter the phenotype of the bacterium, since it can bring in extra genes (e.g., toxin genes that can increase bacterial virulence). This change in the host phenotype is called **lysogenic conversion** or **phage conversion**. Some bacteria, such as *Vibrio cholerae* and *Clostridium botulinum*, are less virulent in the absence of the prophage. The phages infecting these bacteria carry the toxin genes in their genome and enhance the virulence of the host when the toxin genes are expressed. In the case of *V. cholera*, phage encoded toxin

can cause severe diarrhea; in *C. botulinum*, the toxin can cause paralysis. During lysogeny, the prophage will persist in the host chromosome until **induction**, which results in the excision of the viral genome from the host chromosome. After induction has occurred the temperate phage can proceed through a lytic cycle and then undergo lysogeny in a newly infected cell.

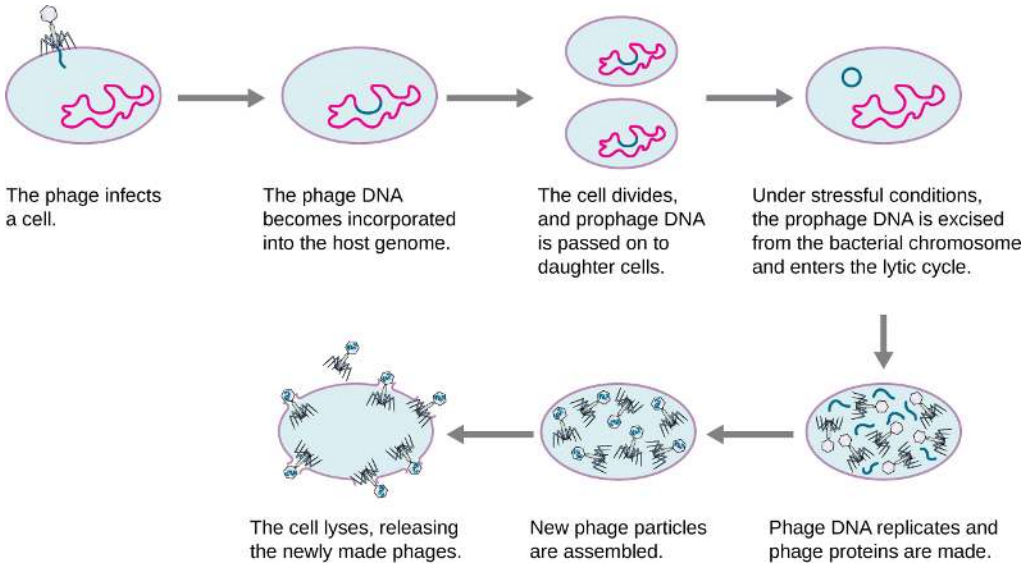


Figure 4: A temperate bacteriophage has both lytic and lysogenic cycles. In the lysogenic cycle, phage DNA is incorporated into the host genome, forming a prophage, which is passed on to subsequent generations of cells. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to be excised and enter the lytic cycle.

Introduction to Plant Viruses

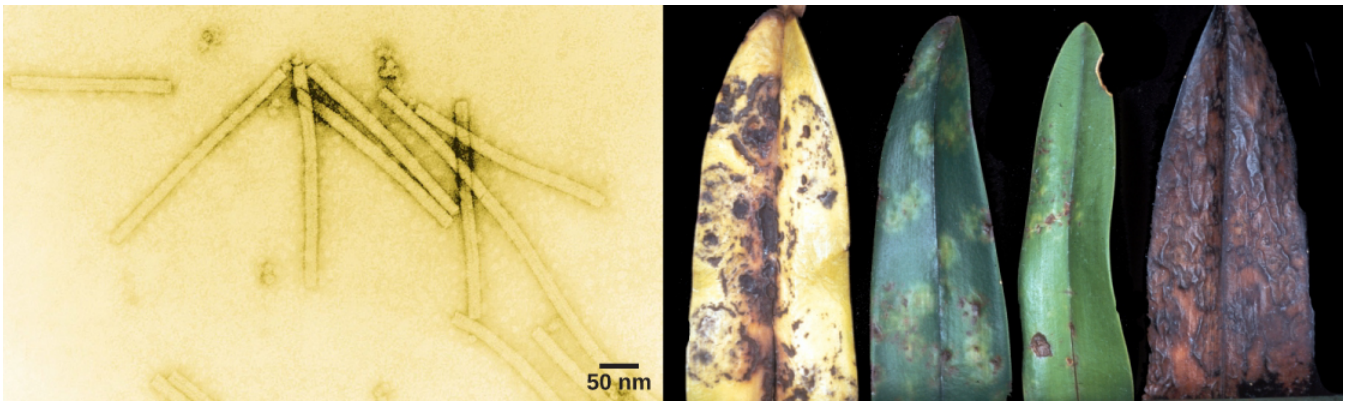


Figure 5: The tobacco mosaic virus (TMV), seen here by transmission electron microscopy (left), was the first virus to be discovered. The virus causes disease in tobacco and other plants, such as the orchid (right). (credit a: USDA ARS; credit b: modification of work by USDA Forest Service, Department of Plant Pathology Archive North Carolina State University; scale-bar data from Matt Russell)

Most plant viruses, like the tobacco mosaic virus, have single-stranded (+) RNA genomes. However, there are also plant viruses in most other virus categories. Unlike bacteriophages, plant viruses do not have active mechanisms for delivering the viral genome across the protective cell wall. For a plant virus to enter a new host plant, some type of mechanical damage must occur. This damage is often caused by weather, insects, animals, fire, or human activities like farming or landscaping. Movement from cell to cell within a plant can be facilitated by viral modification of plasmodesmata (cytoplasmic threads that pass from one plant cell to

the next). Additionally, plant offspring may inherit viral diseases from parent plants. Plant viruses can be transmitted by a variety of vectors, through contact with an infected plant's sap, by living organisms such as insects and nematodes, and through pollen. The transfer of a virus from one plant to another is known as **horizontal transmission**, whereas the inheritance of a virus from a parent is called **vertical transmission**.

Symptoms of viral diseases vary according to the virus and its host. One common symptom is ***hyperplasia***, the abnormal proliferation of cells that causes the appearance of plant tumors known as **galls**. Other viruses induce ***hypoplasia***, or decreased cell growth, in the leaves of plants, causing thin, yellow areas to appear. Still other viruses affect the plant by directly killing plant cells, a process known as ***cell necrosis***. Other symptoms of plant viruses include malformed leaves; black streaks on the stems of the plants; altered growth of stems, leaves, or fruits; and ring spots, which are circular or linear areas of discoloration found in a leaf.

Table 1: Some Common Symptoms of Plant Viral Diseases

Symptom	Appears as
Hyperplasia	Galls (tumors)
Hypoplasia	Thinned, yellow splotches on leaves
Cell necrosis	Dead, blackened stems, leaves, or fruit
Abnormal growth patterns	Malformed stems, leaves, or fruit
Discoloration	Yellow, red, or black lines, or rings in stems, leaves, or fruit

Plant viruses can seriously disrupt crop growth and development, significantly affecting our food supply. They are responsible for poor crop quality and quantity globally, and can bring about huge economic losses annually. Others viruses may damage plants used in landscaping. Some viruses that infect agricultural food plants include the name of the plant they infect, such as tomato spotted wilt virus, bean common mosaic virus, and cucumber mosaic virus. In plants used for landscaping, two of the most common viruses are peony ring spot and rose mosaic virus. There are far too many plant viruses to discuss each in detail, but symptoms of bean common mosaic virus result in lowered bean production and stunted, unproductive plants. In the ornamental rose, the rose mosaic disease causes wavy yellow lines and colored splotches on the leaves of the plant.

Tobacco Mosaic Virus

The tobacco mosaic virus (TMV) is a plant pathogen that causes tobacco mosaic disease. TMV infects tobacco plants, tomato plants, bean plants, and other plant species within the plant family Solanaceae (sometimes called "nightshades") (Scholthof, 2000). Tobacco mosaic disease has been reported as early as the early 1800s in Colombia where tobacco was an important agricultural crop (Scholthof, 2008). This virus impacted the quality of the tobacco by causing it to taste incredibly bitter, and therefore could devastate the value of tobacco crops (Scholthof, 2008).

TMV is an RNA virus with a helical capsid. When TMV infects a tobacco plant, it can cause abnormal growth patterns (e.g. curling leaves), cell necrosis, and discoloration - see **Figure 5** above (Scholthof, 2000). The impacts of TMV on tomato plant today are more profound (~20% crop loss) than on tobacco crops, since TMV-resistant tobacco crops are typically selected and grown agriculturally (~1% crop loss by TMV) (Scholthof, 2000).

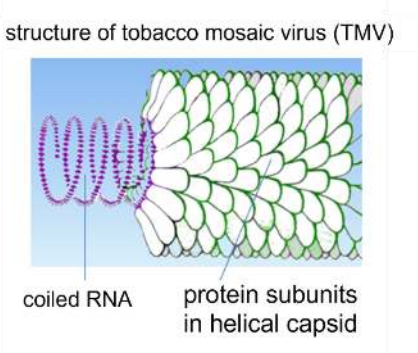


Figure 6: Structure of tobacco mosaic virus (TMV).

As mentioned above in the **Introduction to Plant Viruses** section, plant cells have a thick cell wall that viruses cannot penetrate, and TMV is no different. A plant cell must be injured in order for TMV to cause infection (Scholthof, 2000). Transfer of the TMV particles involves mechanical transmission from a farm tool carrying the virus, a farm worker's hand that is carrying the virus from another plant or from tobacco they have used, or from an infected leaf to non-infected leaf when they make contact with each other (Scholthof, 2000). After TMV enters a plant cell, the protein capsid disassembles to release the RNA genome of the virus (Scholthof, 2000). Within minutes of a plant cell being infected with TMV, the released viral RNA starts to be used by the host cell's ribosomes to begin making viral proteins (Scholthof, 2000). In summary, the TMV viral proteins replicate the viral RNA genome (to make more virus particles), form subunits for the capsid (to make more virus particles), and facilitate transfer of the virus between adjacent plant cells through cell-to-cell plant communication junctions called plasmodesmata (Scholthof, 2000). Newly synthesized virus particles can not only travel through plasmodesmata, they can also enter the plant's phloem (passages that connect the entire plant), and therefore infect the entire plant (Scholthof, 2000). If an infected cell has its cell wall damaged, virus particles can be transmitted from that cell to infect other plants (Scholthof, 2000).

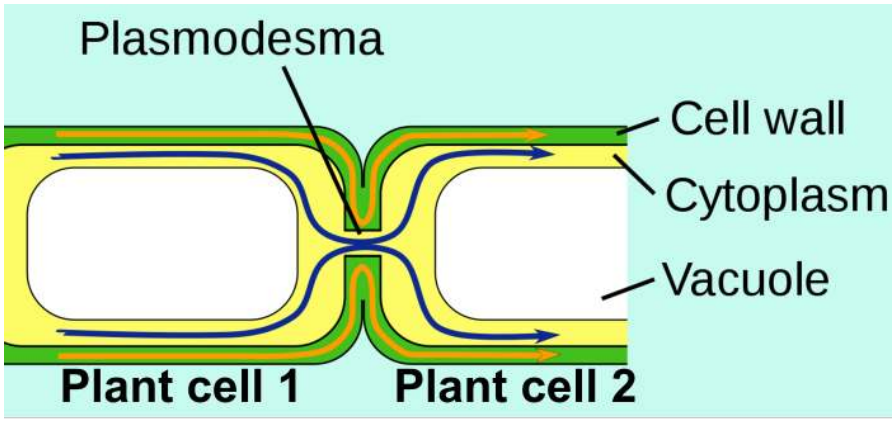


Figure 7: Two adjacent plant cells are shown and their connection with plasmodesmata. A "movement protein" coded in the TMV RNA genome facilitates movement of the virus particles and infection of adjacent cells using these plasmodesmata (Scholthof, 2000).

Laboratory Instructions

Grow Plants to Infect

1. Prepare a plant pot with soil.
2. Plant 5-6 tomato or bean seeds as instructed on the seed package.
3. Water and provide light for the plants for 4-6 weeks as instructed on the seed package.

What is a "Bioassay?"

An **assay** is a type of scientific test used to determine the composition of a substance. In this case, we will assay tobacco to determine:

1. Does this tobacco contain TMV?
2. How much infective TMV is in the tobacco?
3. Does milk have an impact on the ability of TMV to infect plant cells?

"Bio-" relates to life. In this case, "bio-" refers to the use of a plant used in this assay. Viruses cannot be grown on their own - they require cells. In this case, a living organism (a plant) is used to enable us to experiment with this virus.

Bioassay Instructions

Important

Wash your hands with soap anytime they may have touched anything that might contain tobacco mosaic virus. You may need to wash your hands multiple times throughout this laboratory protocol and be sure to wash your hands at the end of the laboratory so you do not carry and spread the virus into the environment.

This will prevent spread of the virus and prevent cross-contamination in the experiment.

Important

Thoroughly clean any laboratory materials (test tubes, stir rods, etc.) that could have come into contact with the tobacco mosaic virus ***with disinfectant***.

This will prevent spread of the virus and prevent cross-contamination in the experiment.

Create a Virus Extract from Tobacco

1. Mix tobacco from 2-3 different sources (different brands of tobacco).
2. Use a mortar and pestle to produce finely ground tobacco. This will damage the cell walls of the tobacco to release the tobacco mosaic virus from infected cells.
3. Weigh 1 g of tobacco.
4. Measure 15 mL of diatomaceous earth dissolved in phosphate buffer and pour into a small beaker. The diatomaceous earth will be important for creating friction and causing damage to plant leaves to enable infection.
5. Add the 1 g of tobacco to the 15 mL of phosphate buffer with diatomaceous earth.
6. Use a stir rod to further mash the tobacco and stir it into the liquid. This will release tobacco mosaic virus into the phosphate buffer.
7. Put a funnel into a test tube.
8. Line the inside of the funnel with 3-4 layers of cheesecloth.
9. Pour the tobacco-phosphate buffer-diatomaceous earth mixture through the cheesecloth. This will remove tobacco particles, but allow the diatomaceous earth and buffer carrying the tobacco mosaic virus into the test tube.
10. The liquid in this test tube will hereafter be referred to as the "virus extract."

11. Wash your hands thoroughly.

> **Note**
>
> The "virus extract" contains tobacco mosaic virus. Any solution that this virus extract is added to should also contain the virus. Any solution that does not contain this virus extract should not contain the virus.

Prepare Three Test Samples & Apply to Plant Leaves

Prepare Plant for Sample Application

1. Choose three different branches of the plant that have healthy-looking leaves.
2. Use masking tape to create a "flag" on each branch, each labeled as:
    1. Control (No Virus)
    2. Virus in Water
    3. Virus in Milk

Control (No Virus)

1. Label a clean test tube as "Control (No Virus)."
2. Measure 1.5 mL of the phosphate buffer containing diatomaceous earth and pour into the test tube.
3. Measure 1.5 mL of deionized water and pour into the test tube.
4. Gently mix the test tube by lightly swirling of lightly flicking the bottom of the test tube with your finger.
5. Dip a sterile cotton swab into the **Control (No Virus)** solution.
6. Gently swab the top surface of one leaf on the plant branch you labeled "Control (No Virus)"
7. Repeat steps 5-6 until all leaves on the "Control (No Virus)" branch have been swabbed. If any of the leaves on this branch already have discoloration, pull the leaf off the plant instead of swabbing it.

Virus in Water

1. Label a clean test tube as "Virus in Water."
2. Measure 1.5 mL of the **virus extract** solution and pour into the test tube.
3. Measure 1.5 mL of deionized water and pour into the test tube.
4. Gently mix the test tube by lightly swirling of lightly flicking the bottom of the test tube with your finger.
5. Dip a sterile cotton swab into the **Virus in Water** solution.
6. Gently swab the top surface of one leaf on the plant branch you labeled "Virus in Water" being careful not to touch any other leaves on the plant.
7. Repeat steps 5-6 until all leaves on the "Virus in Water" branch have been swabbed. If any of the leaves on this branch already have discoloration, pull the leaf off the plant instead of swabbing it.
8. Wash your hands well after this step to prevent spreading the virus to tools, bench tops, etc.

Virus with Milk

1. Label a clean test tube as "Virus with Milk."
2. Measure 1.5 mL of the **virus extract** solution and pour into the test tube.
3. Measure 1.5 mL of milk and pour into the test tube.
4. Gently mix the test tube by lightly swirling of lightly flicking the bottom of the test tube with your finger.
5. Dip a sterile cotton swab into the **Virus with Milk** solution.
6. Gently swab the top surface of one leaf on the plant branch you labeled "Virus with Milk" being careful not to touch any other leaves on the plant.
7. Repeat steps 5-6 until all leaves on the "Virus with Milk" branch have been swabbed. If any of the leaves on this branch already have discoloration, pull the leaf off the plant instead of swabbing it.

8. Wash your hands well after this step to prevent spreading the virus to tools, bench tops, etc.

Post-Inoculation Plant Care

1. Continue caring for plants as you did before Inoculation (light and water as indicated on the seed package). Take care that the leaves of the plants do not touch each other.
2. Wait 2-3 weeks before examining plant leaves and collecting data.

Results & Questions

	# of Lesions on Leaf 1	# of Lesions on Leaf 2	# of Lesions on Leaf 3	# of Lesions on Leaf 4	# of Lesions on Leaf 5	# of Lesions on Leaf 6	Average # of Lesions
Control (No Virus)							
Virus in Water							
Virus with Milk							

	Class Average # of Lesions	t-test Range
Control (No Virus)		
Virus in Water		
Virus with Milk		

1. Fill in the table above with data from the tobacco mosaic bioassay. Depending on the number of leaves on the plant, you may or may not need every box in the table above.
2. Share the average number of lesions per leaf for Control (No Virus), Virus in Water, and Virus with Milk with your instructor. They will calculate the class average for each and t-test ranges. Write these data in the table above.
3. Examine the class average of lesions and the t-test ranges and interpret the t-test statistic. Write conclusions about whether or not these data are statistically significantly different from each other (see the t-test Statistical Analysis section in the Control of Microbial Growth lab for guidance):
   - Control vs. Virus in Water:
   - Control vs. Virus with Milk:
   - Virus in Water vs. Virus with Milk:
4. Explain how TMV infects plant cells.
5. Why was diatomaceous earth used in this experiment (think about how TMV infects plant cells)?
6. How can TMV spread in a plant?
7. What plant species does TMV infect?
8. Can TMV infect humans? Explain your answer by discussing the specificity of viruses.
9. Define bioassay.

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1.39: 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 study[1] 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 item**s include surgical instruments, catheters, and intravenous fluids. Gastrointestinal endoscopes and various types of equipment for respiratory therapies are examples of **semicritical item**s; 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 item**s; 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, **disinfectant**s 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, **antiseptic**s 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
Hyberbaric 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 Ozone gas		Acne medication 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

t-test Statistical Analysis

The t-test is a statistical analysis that enables scientists to objectively determine, based on their data and not their opinions, whether the results from one experimental treatment are statistically different from another experimental treatment. For example, in this experiment, we will be able to determine if the soap-cleaned surfaces produced a statistically significantly different number of microbial colonies on petri plates than the uncleaned surfaces. If the t-test indicates that there is ***not*** a statistical difference, regardless of what the means (i.e. averages) are an how different those means may appear, they are statistically considered ***not different from each other***.

To determine if a pair of experimental treatments are statistically different from each other, consider the means (i.e. averages) and the t-test ranges:

- If the mean of one treatment falls into the t-test range of the other treatment, and the mean of the other treatment falls into the t-test range of the first treatment, these results indicate that these treatments are ***not statistically significantly different from each other***.
- If the mean of one treatment falls outside of the t-test range of the other treatment, and the mean of the other treatment falls outside of the t-test range of the first treatment, these results indicate these treatments ***are statistically significantly different from each other***.

Example 38.1

	uncleaned surface	soap cleaned surface
mean number of colonies on the petri plate	98	12
t-test range	54 - 142	0 - 24

Solution

To determine if the microbial load on the uncleaned surface is statistically significantly different than the soap cleaned surface as the following questions:

1. Does the mean of the uncleaned surface fall into the t-test range of the soap cleaned surface? **Answer** No. 98 does not fall between 0 and 24.
2. Does the mean of the soap cleaned surface fall into the t-test range of the uncleaned surface? **Answer:** No. 12 does not fall between 54 and 142.
3. Check with the bullet points above this example. According to those, since the means do not fit into the t-test ranges of the other experimental groups, they are statistically significantly different.

Conclusion Statement (Option 1)

The soap cleaned surface had statistically significantly lower microbial load than the uncleaned surface.

Conclusion Statement (Option 2)

The uncleaned surface had a statistically significantly higher microbial load than the soap cleaned surface.

Example 38.2

	disinfectant cleaned surface	soap cleaned surface
mean number of colonies on the petri plate	9	12
t-test range	2 - 16	0 - 24

Solution

To determine if the microbial load on the disinfectant cleaned surface is statistically significantly different than the soap cleaned surface as the following questions:

1. Does the mean of the disinfectant cleaned surface fall into the t-test range of the soap cleaned surface? **Answer** Yes. 9 falls between 0 and 24.
2. Does the mean of the soap cleaned surface fall into the t-test range of the disinfectant cleaned surface? **Answer:** Yes. 12 falls between 2 and 16.
3. Check with the bullet points above this example. According to those, since the means fall into the t-test ranges of the other experimental groups, they are ***not*** statistically significantly different.

Conclusion Statement (Option 1)

The soap cleaned surface did not have a statistically significantly different microbial load than the disinfectant cleaned surface.

Conclusion Statement (Option 2)

The disinfectant cleaned surface did not have a statistically significantly different microbial load than the soap cleaned surface.

Laboratory Instructions

Purpose

The goal of this experiment is to swab various surfaces for microbes and to compare the number of microorganisms that grow on a Petri plate from the same surface in three different conditions:

1. The surface as it is, untreated
2. The surface that has been cleaned with soap
3. The surface that has been cleaned with disinfectant

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 dH_2O 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 dH_2O 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 dH_2O 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).

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. Record your individual results (colony number) in the table below:

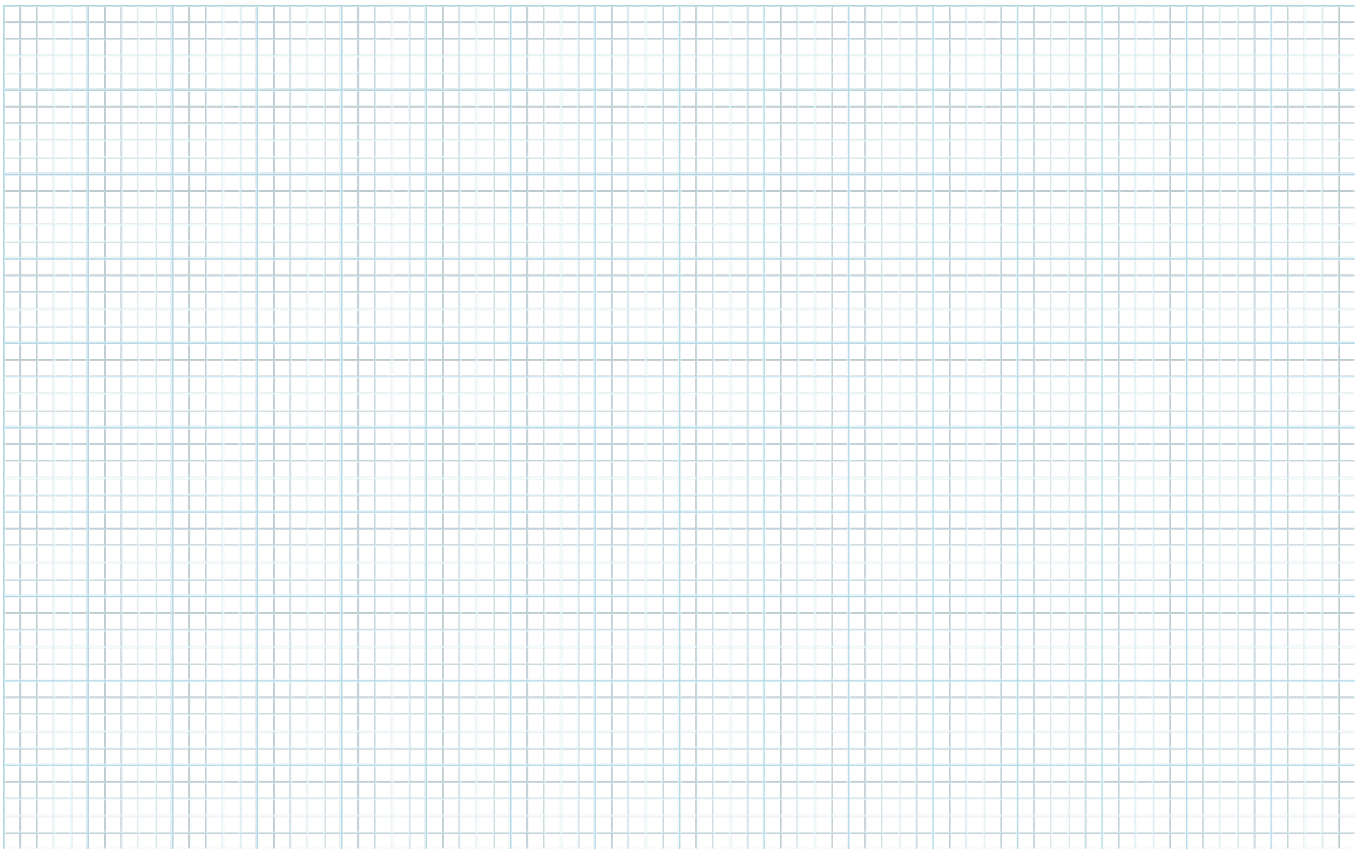
uncleaned	soap cleaned	disinfectant cleaned

5. Record class results (average colony number) in the table below:

	uncleaned	soap cleaned	disinfectant cleaned
mean (i.e. average) colony number			
t-test range			

6. Discuss your results.
   - Which treatment produced the most microbial growth?
   - Which treatment produced the least microbial growth?
   - See questions 2. and 3. above. Were your predictions for the control of bacterial growth correct? Explain your answer.
7. What type of control of microbes did we employ in this laboratory – physical or chemical?
8. Use your **individual data** to create a bar graph (you may use a graphing software or graph paper – ***handwritten graphs should be done neatly with a ruler***). Your bar graph should have 3 bars, one for each Petri plate. The y-axis should be the number of colonies. Remember to label all axes and give your graph a title.
9. Use your **class data** to create a bar graph (you may use a graphing software or graph paper – ***handwritten graphs should be done neatly with a ruler***). Your bar graph should have 3 bars, one for each Petri plate. The y-axis should be the average number of colonies. Remember to label all axes and give your graph a title. Standard error should be shown as error bars above and below the average colony numbers on each bar of the bar graph.
   - How to analyze data using t-test:
10. Interpret results of the t-test analysis. Give a proper conclusion statement for these results indicating that you conducted a statistical analysis (i.e. use the phrase "statistically signigicant" or statistically significantly").

Graph Paper



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1.40: 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.

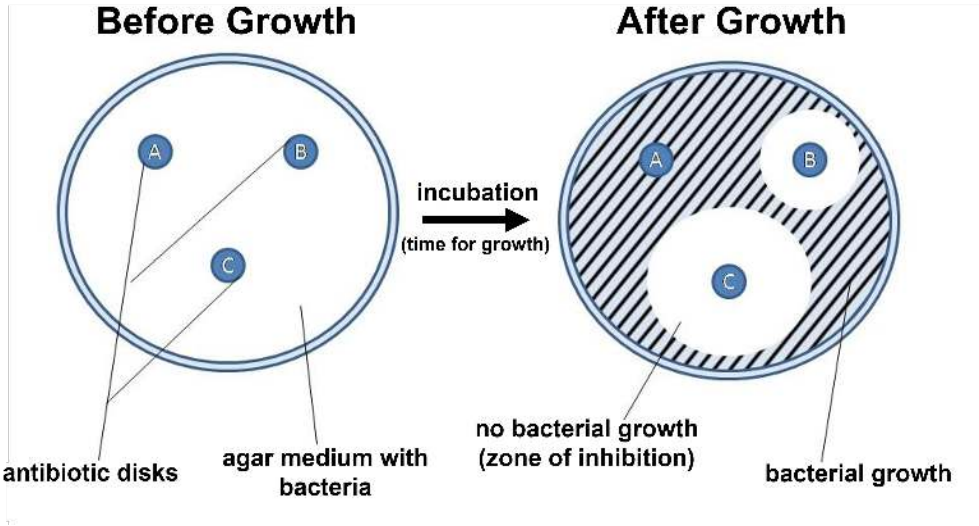


Figure 1: A summary diagram showing how the Kirby-Bauer approach works. A petri plate with nutrient agar is spread with the bacteria being tested. Antibiotic disks are placed on the agar. After incubation, zones on inhibition will occur surrounding the antibiotic disks based on the bacteria's sensitivity 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.

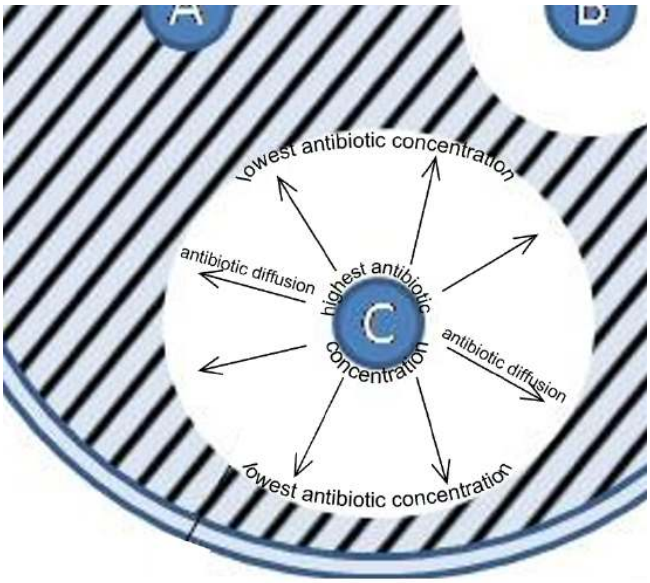


Figure 2: A closer look at antibiotic diffusion and how this produces zones of inhibition. Closest to the antibiotic disk is the highest antibiotic concentration and the antibiotic diffuses outward to produce a concentration gradient of antibiotic. The further from the antibiotic disk, the lower the 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.

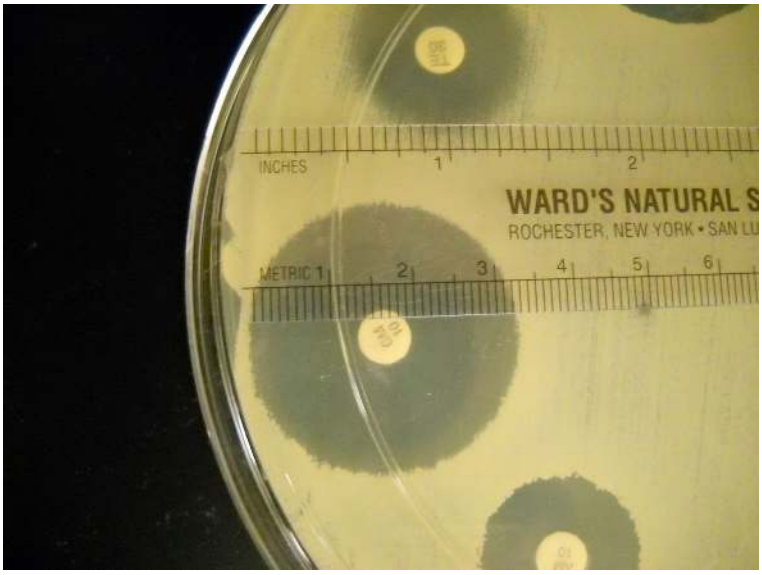


Figure 3: Example of how to measure the diameter of a zone of inhibition in mm. The example shown here has a zone of inhibition with a diameter of 32 mm.

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 *S. aureus* 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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1.41: Human Microbiome

Learning Objectives

- Define the terms microbial flora, microbiota, and microbiome.
- List locations in the human body where it is normal and healthy to have microbes growing.
- Explain how microorganisms keep humans healthy and give at least three ways they are beneficial to our health.
- Give at least three ways humans are not cultivating as healthy a microbiome as we could.
- Tell that the microbial communities are distinct in different areas of the human body.
- Tell that the microbial communities are distinct in different individuals.
- Grow microbes from your own skin microbiome.
- Characterize microbes grown from your own skin microbiome and tell the likely species they are.

Introduction to the Human Microbiome

The importance of the normal **microbial flora** (a.k.a. **microbiota** or **microbiome**) of the human body has been an area of increasing interest in both research and the popular media. One frequently cited statistic is that there are 10-100 times more bacterial than human cells in the body. More recent calculations, however, result in a ratio closer to 1:1, with an estimated 10^{13} human cells and 10^{13} – 10^{15} bacterial cells. The cellular contribution of microbes to the human body, however, is small compared to the genetic contribution. The human genome contains approximately 20,000 genes, but there are 3.3 million unique bacterial genes in the gut microbiota alone. That is a 150-fold difference between the human and bacterial genetic contribution. No matter the exact proportion of bacteria in the human body, the impact of the microbiota on our physiology is substantial.

It has been known for decades that animals raised without normal flora display a variety of health effects across many body systems. Not surprisingly, neither the digestive system nor the immune system develops properly. The cecum (part of the large intestine/colon) tends to be enlarged and other gastrointestinal (GI) abnormalities appear. The immune system is underdeveloped. More recently it has been shown that the central nervous system, including the brain, does not develop properly in these animals. Because bacteria produce vitamins necessary for animal nutrition (most notably vitamin K), animals without normal flora suffer from vitamin deficiencies. Lack of normal flora also makes animals more susceptible to infection with a variety of pathogens, particularly those that infect the GI tract. Although lack of normal flora generally has negative effects, it does also result in an absence of dental caries (cavities) and lower body fat.

Normal flora is found in all areas of the human body exposed to the environment (one exception is the lungs), but internal organs and body fluids are considered sterile in a healthy individual. This is generally true, although bacteria are sometimes found in these "sterile" tissues even in healthy people. For instance, a person's blood will become bacteremic (contain bacteria) for up to three hours after brushing their teeth.

Each area of the human body contains a characteristic population of microbes, although the exact composition of each person's flora is unique. The diversity of the bacteria populating the human gut alone is enormous, with an estimated 40,000 species. An increasing number of studies associates such shifts in the gut microbiota with outcomes such as susceptibility to infection, immune disorders, metabolic changes, and altered mood and behavior. Each of these physiological effects can be linked directly to chemical communication within the microbiota and between the microbiota and human.

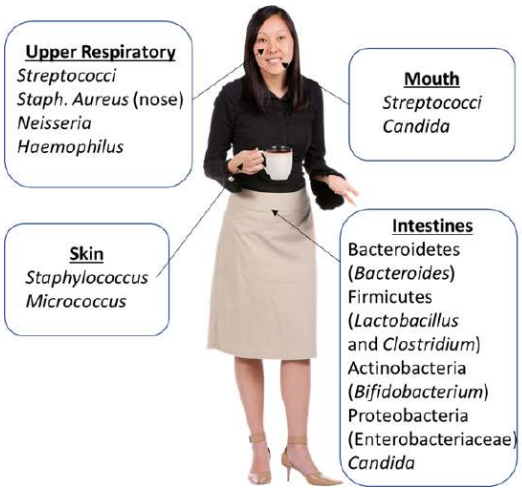


Figure 1: Locations of normal flora and the types of species commonly found in the different human habitats for microorganisms. (2021; Jeanne Kagle)

Flora of the Skin

The exact microbial population on the skin depends on the specific body area. Moist areas, such as axilla (armpits) and groin, tend to have more (and different) bacterial growth compared to drier areas. The most common bacteria of the skin flora are the Gram-positive, catalase positive cocci of the genera *Staphylococcus* and *Micrococcus*. Although *Staphylococcus aureus* can occasionally be found on the skin, it is more commonly found in the nose in those people that carry it in their normal flora.

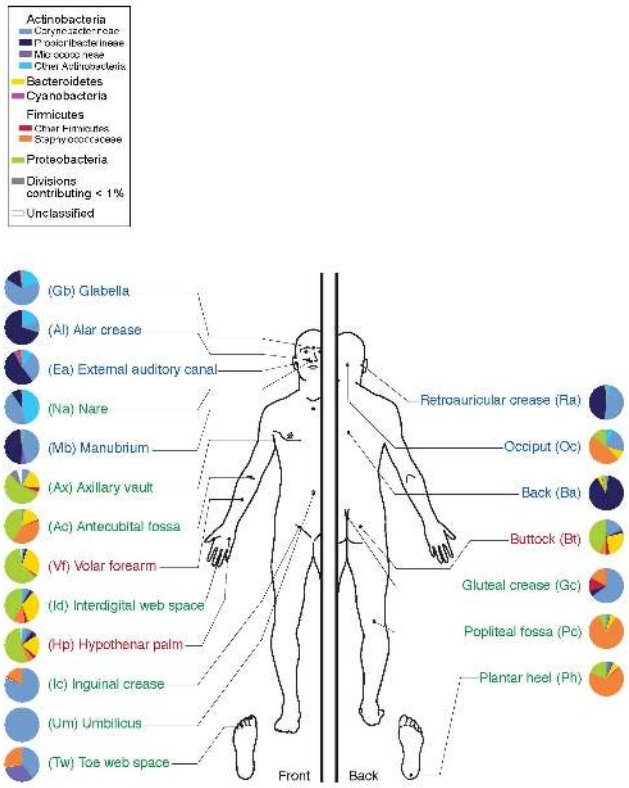


Figure 2: The microbial community compositions differ based on the human skin locations. The microbial community compositions are shown for each skin location with pie charts representing the types of species present and the quantities of these species.

Flora of the Mouth and Upper Respiratory Tract

The flora of the mouth and upper respiratory tract is typically associated with a more diverse set of microbes. *Streptococci*, specifically, alpha-hemolytic *Streptococci* often referred to collectively as the "viridans *Streptococci*"are very prominent in the mouth. These include *S. mutans*, *S. sanguis*, and *S. mitis*. *S. mutans* in particular plays a critical role in the formation of plaque and dental caries (cavities). Although both *Staphylococci* and *Streptococci* are Gram-positive cocci, unlike the *Staphylococci* the *Streptococci* are catalase-negative, consistent with the low-oxygen environment of the mouth.

Other inhabitants of the mouth and upper respiratory tract include bacteria in the genera *Neisseria* and *Haemophilus*. As mentioned above, *Staphylococcus aureus* is most often found in the nose of those individuals who carry it in their normal flora. The fungal genus *Candida* is also common in the mouth and upper respiratory tract.

Flora of the Intestines

The most studied population of normal flora in the microbes living in the intestines. This population of microbes is commonly referred to as the **gut microbiota** or **gut microbiome**. Although the bacterial species most commonly associated with the intestines is *Escherichia coli*, it is actually not the most numerous in the intestine. Bacteria in the phylum Bacteroidetes form a large proportion of bacteria in the gut. The Gram-positive Firmicutes (such as *Lactobacillus* and *Clostridium*) and Actinobacteria (including *Bifidobacterium*) can be equally numerous. In healthy individuals, Proteobacteria (including *E. coli* and other Enterobacteriaceae) are the least abundant of the major bacterial groups in the intestines. There are many other groups of microbes found in the intestines, including fungi such as *Candida*. It is shifts in the proportions of these groups of microbes that are typically studied when investigating the role of normal flora on human health.

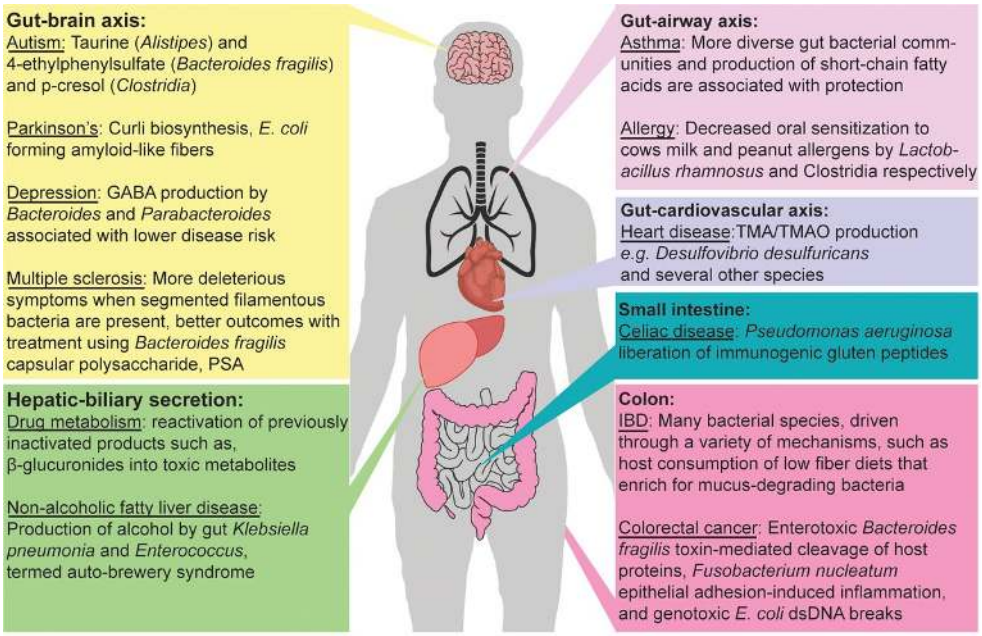


Figure 3: A summary of some of the know effects of the human gut microbiome relating to conditions affective multiple human organ systems.

Laboratory Instructions

In this experiment, a few locations of the skin will be swabbed in order to examine microbes present in different locations.

Prepare Petri Plates and Swab Skin

1. Prepare 2 TSA plates per person.
2. Label the 2 TSA plates with your name.
3. Choose the 2 skin locations you would like to swab and label one petri plate with each location you chose.
4. Moisten a sterile cotton swab with DI or distilled water.
5. Swab the first skin location you chose and then do a zig-zag streak on the TSA plate you labeled with that skin location.
6. Repeat steps 4 and 5 for the other skin location you chose.
7. Invert the petri plates and incubate for 24 – 48 hours.

Examine Results

1. Examine the TSA plates for colony morphologies. Choose one isolated colony from each petri plate. Choose colonies that have different morphologies. Describe the colony morphologies in the results & questions section below.
2. Create two bacterial smears from the two isolated colonies you chose from each plate. You can use a single slide with one smear on each side. Label the slide so you do not get the two smears mixed up.
3. Heat fix the two bacterial smears. Fix each individually (heat-fix one smear, let the slide cool off, then heat-fix the other smear).
4. Conduct a Gram stain on the two bacterial smears (can be done at the same time.
5. Examine the Gram-stained cells with a microscope.
6. Record the cell shape, cell arrangement, and Gram for each of the colonies you chose.
7. Use the table below to "identify" each species found on your skin. Write the species you found for each skin location.

Skin Organism	Gram	Microscopic Morphology	Microscopic Arrangement	Colony Appearance on TSA
Staphylococcus epidermidis*	Positive	Cocci	Clusters	Large, round, white, glistening
Staphylococcus aureus	Positive	Cocci	Clusters	Large, round glistening, typically yellow
Corynebacterium* spp.	Positive	Pleomorphic rods	Club-shaped, bipolar	Dry and wrinkled
Yeasts	Positive	Large budding cells	Single	Large, round, moist
Bacillus* spp.	Positive	Bacilli	Single, chains	Round or irregular, surface dull becoming thick and opaque
Streptococcus viridans*	Positive	Cocci	Chains	Small, dome-shaped colony surrounded by an area of discoloration.
Micrococcus luteus	Positive	Cocci	Single, tetrads	Large, round, yellow, glistening colony

* These are the most commonly isolated microorganisms from healthy skin.

Results & Questions

	Skin location #1: _____ Results from colony on skin location #1	Skin location #2: _____ Results from colony on skin location #2
colony color		
colony appearance (shiny, dull, dry, moist, glistening, etc.)		
colony form		
Gram		
cell shape		
cell arrangement		
likely species (based on the table above)		

1. Complete the table above to summarize results of the two isolated colonies you chose.
2. Examining the results from the skin swabs, answer the following:
   - How many distinct types of microbes can you identify on the petri plates (each distinct type would have a different colony morphology)?
   - Which of the skin locations you swabbed had the most microbes present?
3. **True or false.** The microbes growing on your skin, under normal circumstances, are dangerous to your health.
4. Explain why microbes growing on your skin may actually be beneficial to your health.
5. List at least 3 ways microbes in your "gut" are important for keeping you healthy.
6. What happens to your microbiome when you take antibiotics?
7. What circumstances prevent humans from getting enough exposure to microbes to build up a healthy microbiome? Give at least three.
8. **True or false.** All microbial communities living on the skin have the same composition.
9. **True or false.** All humans have the same species composition in their gut microbiome.

Attributions

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1.42: 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. Empolyers want you to be able to communicate effectively with others. You are building your job and career skills here!

Activity Log

For this project you will work in a pair. You are BOTH responsible for contributing equally to identifying the unknown bacteria.

Keep the following activity log to show your contributions to identifying your unknown bacteria. This insures that this group project is fair and all members of the group are contributing equally to the project. You will submit your activity log in your report. If your contributions to the project don't demonstrate equal contributions, deductions will be made to the report.

Date	Task	Student(s) In the Group Working On This Task	Instructor Signature

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

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. Record results in the table below. Refer back to this table in Step 2 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

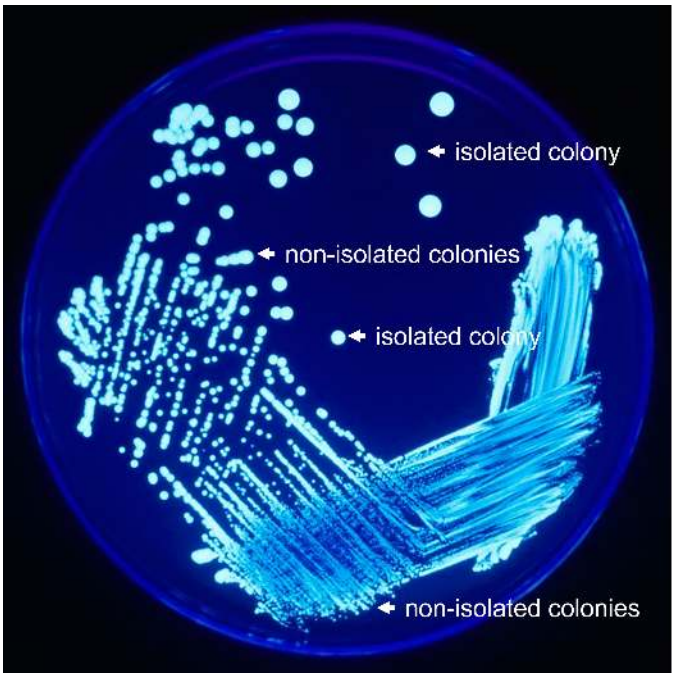


Figure 1: A quadrant streak plate after growth. Use this as a guide to help identify what are and are not isolated colonies. Isolated colonies originate from a single cell, and therefore should contain only one bacterial species. Therefore, growing a culture from an isolated colony is an effective way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

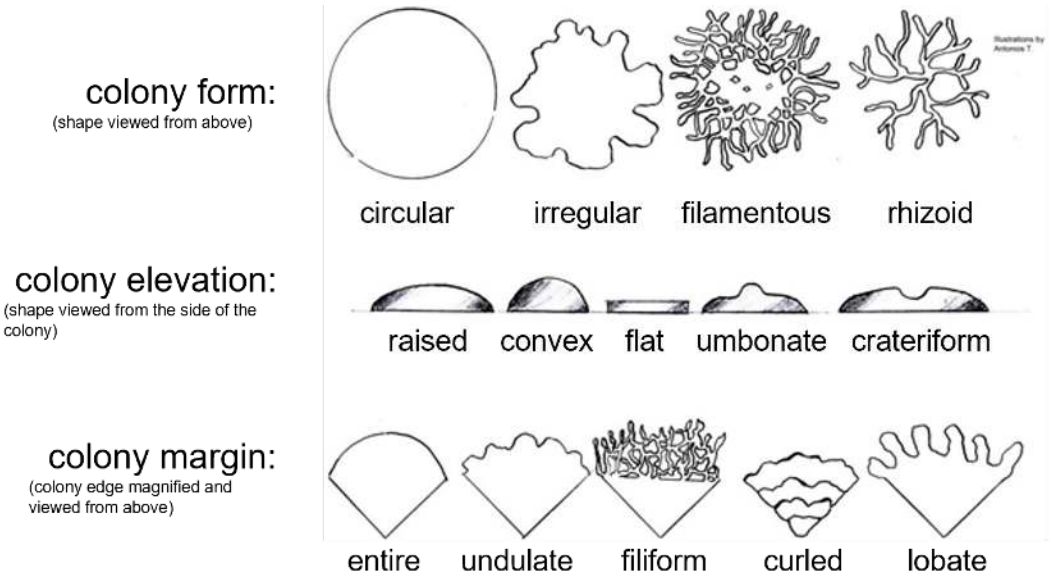


Figure 2: A guide for classifying colony characteristics. Colony form is the overall shape of the colony (circular, irregular, filamentous, or rhizoid) when viewed from above. Colony elevation examines the height and shape of the growth above the surface of the petri plate agar (raised, convex, flat, 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 half (1/2)*** of one of the isolated colonies to one of the TSA slants.
5. Transfer ***one half (1/2)*** of the other isolated colony to one of the TSA slants.
6. Use ***one half (1/2)*** of each of the colonies to make separate bacterial smears on a slide and conduct a Gram stain.
7. 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.
8. 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	

1. 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 to inoculate a SIM deep culture to test for H_2S 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
     - H_2S test

- oxidase test
- coagulase test and/or mannitol salts agar
- fermentation test

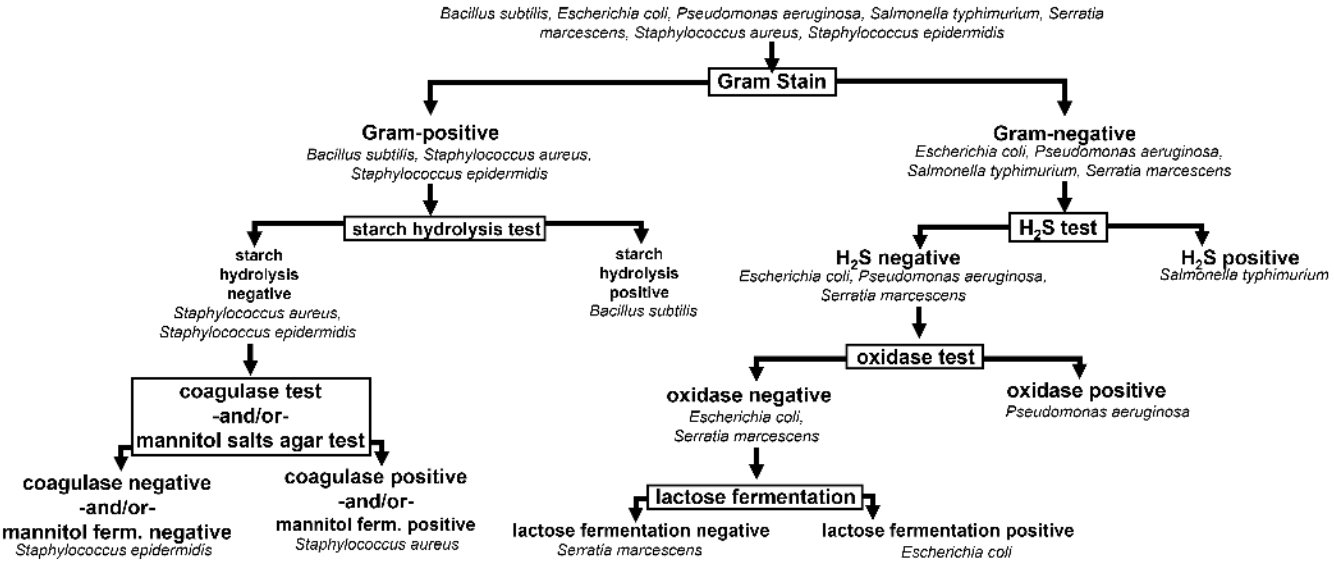


Figure 3: Unknown bacterial species identification flow chart. Once your bacterial species are isolated and you have good Gram stain results, begin to follow the flow chart for both of your unknown bacterial species. The tests that are a part of this project are in boxes. Use the results from each test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

Attributions

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1.43: 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 ALL the sources of information you used at the end of each sentence***. 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
- use at least three (3) different reputable sources (try adding "NCBI," "ASM," or "CDC" to a Google search to obtain some quality sources).

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_Microbiology_Lab_ Manual_(Pakpour_and_Horgan)/Lab_03%3A_Simple_Negative_and_Gram_Stain

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 that 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 sub-sections in the Introduction on the following topics:

- Streak plate
- Gram stain
- Starch hydrolysis
- H_2S 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 protocals). 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.

H_2S Production Test

Text here about what you did to do the H_2S 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 Methds 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

H_2S Production

To test whether Unknown Bacteria A produces H_2S, 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

Isolation of Unknown Bacterial Species

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

H_2S Production Test

Text & figures here about H_2S 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:

"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."

"Unknown Bacteria A, since they were Gram positive, have cell walls composed of a thick layer of peptidoglycan that adhered to the crystal violet during the Gram stain making it appear purple. These bacterial also would have teichoic acid in their cell walls."

"Unknown Bacteria B, since they were Gram negative, have cell walls composed of a thin layer of peptidoglycan that decolorized when ethanol was added resulting in crystal violet being washed out of the peptidoglycan layer. When these bacteria were subsequently stained with safranin, this results in their pink coloration upon observation. These bacteria also have cell walls with an outer membrane which contains lipopolysaccharide, also known as endotoxin."

In the example text above, the description would be further supported with images of 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.

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

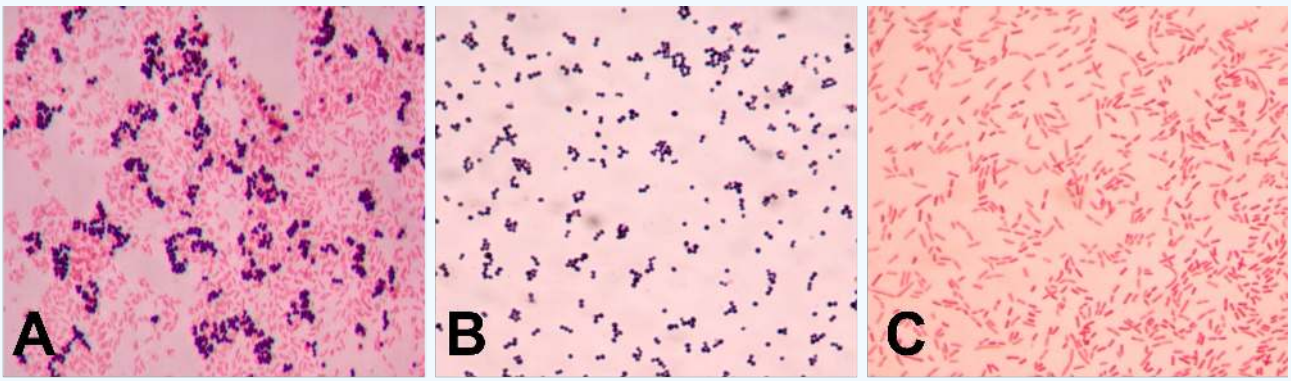


Figure 1: (A) Gram stain of mixed Unknown Bacterial Culture showing both species mixed together in this culture. (B) Gram stain of isolated unknown bacteria "A." (C) Gram stain of isolated unknown bacteria "B."

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.

Grading

Low Participation Activity Log Deductions

- Reports with activity logs that show 20% less work than your partner will receive a 10% deduction.
- Reports with activity logs that show 40% less work than your partner will receive a 20% deduction.
- Reports with activity logs that show 50%-70% less work than your partner will receive a 50% deduction.
- Reports with activity logs that show 75%-100% less work than your partner will receive an 80% deduction.

Grading Rubric

[ChatGPT-generated content]

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	Excellent (100%)	Well Done (82%)	Good (70%)	Developing (55%)	Needs Improvement (40%)	Missing/ Unacceptable (0%)
Introduction (40 pts total)						
Importance of Identifying Bacterial Species (10 pts)	Clearly explains the importance of bacterial species identification, linking it to real-world applications and the impact on public health, medical treatments, or the environment.	Effectively explains the importance of identification with good detail, and partly addresses some broader applications.	Provides a general explanation of the importance, but lacks detail or real-world context.	Mentions the importance but provides limited or unclear reasoning.	Vague or minimal explanation of the importance.	No explanation or completely off-topic.
Theory of Microbiological Techniques (25 pts)	Thorough explanation of the theory behind each microbiological technique used, emphasizing connections with bacterial biology (enzymes, genes, etc.).	Good explanation of most techniques, with adequate connections with bacterial biology, but lacks some depth.	Adequate explanation of the techniques, but with some gaps in scientific theory or biological features/ implications.	Limited explanation of the theory and biology of techniques.	Poor or incomplete explanation of techniques; lacks connection to biology of bacteria.	No explanation of microbiological techniques or completely incorrect.
Experimental Overview Paragraph (5 pts)	Provides a clear, comprehensive summary of the experimental design, including the procedures and tests used, and how they contribute to the identification of the bacterial species.	Good summary of the experimental design, with a clear outline of procedures and their purpose in the project.	Adequate summary of the experimental design, but lacks some clarity or key procedures.	The experimental overview is unclear, missing important steps or lacking detail on the tests performed.	The overview is vague, incomplete, or poorly structured, making it difficult to understand the experimental process.	No overview provided or completely irrelevant.
Materials and Methods (10 pts total)						
Protocol Descriptions (10 pts)	The methods are described in clear, logical, and detailed	The methods are described in a clear and organized	The methods are adequately described, but some details are	The methods are partially described, with several missing	The methods are poorly described, lacking clarity, important details,	No description of the methods, or the description is

	Excellent (100%)	Well Done (82%)	Good (70%)	Developing (55%)	Needs Improvement (40%)	Missing/ Unacceptable (0%)
	steps. All laboratory techniques, reagents, supplies are mentioned, and is easy to follow.	manner, with only minor omissions in details about techniques, reagents, or supplies.	missing or unclear, making it harder to fully understand the procedure.	steps or unclear explanations of key procedures.	or the correct sequence of steps.	entirely incorrect or unrelated to the procedure.
Results and Discussion (40 pts total)						
Observations and Results (10 pts)	Results are clearly and accurately described with all observations included. Information is organized and easy to interpret.	Results are described clearly, with most observations included and well-organized, though some details may be missing or unclear.	Results are described, but some important observations are missing or not presented clearly.	Results are incomplete or poorly organized, with key observations missing or difficult to understand.	Results are poorly presented, with major observations missing or unclear.	No results described, or the information is completely incorrect or irrelevant.
Discussion of Implications of the Results (15 pts)	Each result is thoroughly discussed with emphasis on what it indicates about the bacterial species (e.g., enzymes, genetic factors). The discussion connects experimental results to biological principles.	Most results are discussed in detail, with some focus on their biological implications, but the analysis may lack depth or connection to certain biological aspects.	Results are discussed but with minimal detail or clarity regarding their biological implications. Some connections to the biology of the bacteria may be missing.	Results are partially discussed, but biological implications are vague or largely absent. Limited explanation of how the results relate to bacterial biology.	Results are not discussed in relation to bacterial biology, with minimal or no mention of the biological significance.	No discussion of results or biological implications provided.
Progression of Identification Process (5 pt)	The progression of identification is clearly communicated, with each step logically leading to the next.	The progression of identification is clear, but may have minor gaps or lack some detail in explaining how each test contributed to the identification.	The progression of identification is generally clear, but some steps are missing or unclear, lacks detail and/or coherence.	The identification process is confusing or incomplete, with unclear progression between steps or missing tests.	The identification process is poorly explained or disorganized, making it difficult to follow or understand.	No explanation of the identification process is provided, or the process is entirely incorrect.

	Excellent (100%)	Well Done (82%)	Good (70%)	Developing (55%)	Needs Improvement (40%)	Missing/ Unacceptable (0%)
Figures and Captions (10 pts)	Figures are well-organized and clearly show each test result. Captions are detailed and explain what is shown.	Figures are provided and show most test results clearly. Captions are included but may lack some detail or explanation.	Figures are included but may not be clearly labeled or explained. Captions are brief or unclear.	Figures are poorly organized, incomplete, or not clearly labeled. Captions are minimal or missing.	Figures are poorly presented or absent; captions are vague or entirely missing.	No figures or captions provided.
Conclusion (10 pts total)						
Bacterial Species and Evaluation of Correctness (5 pts)	Clearly and confidently identifies the two bacterial species with correct identification based on the results. A clear evaluation of the accuracy of the identifications is provided.	Names the two bacterial species with minor uncertainty. The correctness of the identification is stated.	Names the two bacterial species, but could be stated more clearly and confidently. The correctness of the identification is addressed, but may lack clarity.	Species are named, but unclear about what the species identified were versus the actual species.	Identifies only one or no species, and evaluation if species identified were the same as the actual species addresses one or neither.	No identification of species or completely incoherent. No evaluation whether species are correct is provided.
Factors Contributing to Successful Identification and Misidentification (5 pt)	Clearly identifies multiple factors (test results, methodology, environmental conditions) that contributed to successful identification and any potential misidentification. Demonstrates a comprehensive understanding of the process.	Identifies several factors contributing to correct identification and/or possible misidentification, but may lack some depth or detail.	Identifies a few factors that contributed to the identification or misidentification but misses some key details/ understanding of the process.	Mentions a factor that could have contributed to the identification or misidentification but lacks depth or clarity.	Identifies one or no factors contributing to the identification or misidentification.	No discussion of contributing factors, or completely incorrect.
Works Cited (10 pts total)						
Citation Consistency,	At least 3 professional sources are used	At least 3 professional sources are used	At least 3 professional sources are	Fewer than 3 professional sources are	Sources are incomplete or improperly	No citations or completely incorrect

	Excellent (100%)	Well Done (82%)	Good (70%)	Developing (55%)	Needs Improvement (40%)	Missing/ Unacceptable (0%)
Formatting, and Source Quality (10 pts)	and cited consistently throughout the paper. Citation and reference formats are professional, consistent, and complete with no errors.	and cited consistently throughout the paper. Citation and reference formats are mostly professional, with minor inconsistencies or errors.	used, but citation and reference formatting has noticeable inconsistencies or errors.	used, or citation format is inconsistent or incomplete.	formatted, or fewer than 3 professional sources are used.	citation formatting.
Report Quality (50 pts total)						
Organization and Writing Quality (15 pts)	Writing is clear, concise, and highly organized; no grammatical or spelling errors.	Well-organized and clear writing with minimal grammatical or spelling issues.	Generally clear writing, but with several grammatical or spelling errors that detract from readability.	Writing lacks clarity or has frequent grammatical or spelling errors.	Writing is difficult to follow due to numerous grammatical or spelling errors.	Writing is incoherent or extremely difficult to understand.
Adherence to Guidelines (10 pts)	Fully follows all guidelines; report is well-structured and meets all requirements.	Follows most guidelines; minor deviations or omissions.	Adequately follows guidelines but with some notable omissions or deviations.	Does not fully follow guidelines; significant omissions or structural issues.	Lacks adherence to guidelines; many structural or content issues.	Completely ignores guidelines.
Overall Quality (15 pts)	The report is exceptionally well-done, clear, and professional in presentation.	The report is well-written and professional with minimal errors.	The report is adequate, but there are several issues with clarity or organization.	The report is incomplete or lacks clarity in several areas.	The report is poorly written or lacks essential content.	The report is unacceptable or not submitted.

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2.1: Exercise 2.1

Answer

6 g ("six grams")

How this Answer is Calculated

1. **Calculation setup:** 400 mL x [15 g /1000 mL]
2. **Calculate parentheses first:** 400 mL x 0.015 g/mL
3. **Multiply (mL units cancel out):** 6 g

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2.2: Exercise 2.2

13 g ("thirteen grams")

1. **Calculation setup:** 650 mL x [20 g /1000 mL]
2. **Calculate parentheses first:** 650 mL x 0.02 g/mL
3. **Multiply (mL units cancel out):** 13 g

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2.3: Exercise 2.3

6 g ("six grams")

1. **Calculation setup:** 200 mL x [30 g /1000 mL]
2. **Calculate parentheses first:** 200 mL x 0.03 g/mL
3. **Multiply (mL units cancel out):** 6 g

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2.4: Exercise 2.4

13.5 g ("thirteen point five grams")

1. **Calculation setup:** 300 mL x [45 g /1000 mL]
2. **Calculate parentheses first:** 300 mL x 0.045 g/mL
3. **Multiply (mL units cancel out):** 13.5 g

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2.5: Exercise 2.5

5.25 g ("five point two five grams")

1. **Calculation setup:** 150 mL x [35 g /1000 mL]
2. **Calculate parentheses first:** 150 mL x 0.035 g/mL
3. **Multiply (mL units cancel out):** 5.25 g

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2.6: Exercise 4.1

Answer

0.0025 m ("zero point zero zero two five meters")

How this Answer is Calculated

1. **Calculation setup:** 2,500 µm x 10^{-6}
2. **Calculate the exponent first:** 2,500 µm x 0.000001
3. **Multiply:** 0.0025 m

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2.7: Exercise 4.2

Answer

0.000012 m ("zero point zero zero zero zero one two meters")

How this Answer is Calculated

1. **Calculation setup:** 12,000 nm x 10^{-9}
2. **Calculate the exponent first:** 12,000 nm x 0.000000001
3. **Multiply:** 0.000012 m

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2.8: Exercise 4.3

Answer

0.035 m ("zero point zero three five meters")

How this Answer is Calculated

1. **Calculation setup:** 35 mm x 10^{-3}
2. **Calculate the exponent first:** 35 mm x 0.001
3. **Multiply:** 0.035 m

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2.9: Exercise 4.4

Answer

4,000 µm ("four thousand micrometers")

How this Answer is Calculated

1. **Calculation setup:** 0.004 m ÷ 10^{-6}
2. **Calculate the exponent first:** 0.004 m ÷ 0.000001
3. **Divide:** 4,000 µm

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2.10: Exercise 4.5

Answer

300 mm ("three hundred millimeters")

How this Answer is Calculated

1. **Calculation setup:** 0.3 m ÷ 10^{-3}
2. **Calculate the exponent first:** 0.3 m ÷ 0.001
3. **Divide:** 300 mm

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2.11: Exercise 4.6

Answer

850 nm ("eight hundred and fifty nanometers")

How this Answer is Calculated

1. **Calculation setup:** 0.00000085 m ÷ 10^{-9}
2. **Calculate the exponent first:** 0.00000085 m ÷ 0.000000001
3. **Divide:** 850 nm

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2.12: Exercise 5.1

Answer

40X

Calculation

1. **Formula:** total magnification = (objective lens magnification) x (ocular lens magnification)
2. **Plug numbers into the formula:** total magnification = (4X) x (10X)
3. **Multiply:** 40X

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2.13: Exercise 5.2

Answer

100X

Calculation

1. **Formula:** total magnification = (objective lens magnification) x (ocular lens magnification)
2. **Plug numbers into the formula:** total magnification = (10X) x (10X)
3. **Multiply:** 100X

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2.14: Exercise 5.3

Answer

400X

Calculation

1. **Formula:** total magnification = (objective lens magnification) x (ocular lens magnification)
2. **Plug numbers into the formula:** total magnification = (40X) x (10X)
3. **Multiply:** 400X

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2.15: Exercise 5.4

Answer

1000X

Calculation

1. **Formula:** total magnification = (objective lens magnification) x (ocular lens magnification)
2. **Plug numbers into the formula:** total magnification = (100X) x (10X)
3. **Multiply:** 1000X

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2.16: Exercise 5.5-A

Answer

eyepiece

> **Note**
>
> the eyepiece contains the ocular lens (usually 10X magnification)

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2.17: Exercise 5.5-B

Answer

revolving nosepiece

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2.18: Exercise 5.5-C

Answer

objective lens

> **Note**
>
> There are multiple objective lenses. Revolving the nosepiece will change the objective lens in use to increase or decrease magnification of the sample.

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2.19: Exercise 5.5-D

Answer

stage

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2.20: Exercise 5.5-E

Answer

stage clip

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2.21: Exercise 5.5-F

Answer

light source / illuminator

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2.22: Exercise 5.5-G

Answer

base

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2.23: Exercise 5.5-H

Answer

diaphragm

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2.24: Exercise 5.5-I

Answer

stage controls

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2.25: Exercise 5.5-J

Answer

fine focus

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2.26: Exercise 5.5-K

Answer

course focus

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2.27: Exercise 5.5-L

Answer

arm

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2.28: Exercise 5.6-ocular lens

Answer

E. A magnifying lens located inside the microscope part where a person looks into the microscope.

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2.29: Exercise 5.6-revolving nosepiece

Answer

D. A structure capable of rotating to change the objective lens being used to magnify the sample.

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2.30: Exercise 5.6-arm

Answer

L. A structural component that serves to support the eyepiece, revolving nosepiece, and stage.

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2.31: Exercise 5.6-stage control

Answer

A. Adjusts the position of a slide on the stage.

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2.32: Exercise 5.6-base

Answer

H. A structural component that serves to support the weight of the microscope from underneath.

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2.33: Exercise 5.6-course focus

Answer

B. Adjusts the focus of the microscope by moving the stage up and down in large increments.

> **Note**
>
> The course focus is used with the 4X objective lens and the 10X objective lens only and should never be used with the 40X or 100X objective lenses (it could crack a slide or damage the lenses).

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2.34: Exercise 5.6-fine focus

Answer

K. Adjusts the focus of the microscope by moving the stage up and down in smaller increments.

> **Note**
>
> The fine focus is mostly used with the 40X objective and 100X objective.

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2.35: Exercise 5.6-light source / illuminator

Answer

F. Provides light that shines on the sample and carries the image of the specimen through the magnifying lenses.

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2.36: Exercise 5.6-objective lens

Answer

M. A magnifying lens which increases magnification of the specimen that can easily be changed by rotating between lenses of different magnifications.

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2.37: Exercise 5.6-stage

Answer

C. A surface where a slide is positioned under the objective lens and over the light source.

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2.38: Exercise 5.6-stage clip

Answer

I. Secures a slide in place on the microscope.

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2.39: Exercise 5.6-diaphragm

Answer

G. A structure capable of changing the amount of light passing through the specimen.

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2.40: Exercise 5.6-eyepiece

Answer

J. Where the microscope user looks into the microscope to view a magnified image of the specimen.

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2.41: Exercise 6.1-1

Answer

organic molecule

Explanation

This molecule contains a carbon atom (C) ***and*** hydrogen atoms (H). Since it has both C and H, it is an organic molecule.

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2.42: Exercise 6.1-2

Answer

organic molecule

Explanation

This molecule contains carbon atoms (C) ***and*** hydrogen atoms (H). Since it has both C and H, it is an organic molecule.

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2.43: Exercise 6.1-3

Answer

inorganic molecule

Explanation

This molecule does not contain carbon atoms (C) or hydrogen atoms (H). Since it does not have ***both*** C and H, it is an inorganic molecule.

> **Note**
>
> Don't be thrown off by the "C" in NaCl. This is not a carbon atom. "Cl" is the element chlorine.

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2.44: Exercise 6.1-4

Answer

inorganic molecule

Explanation

This molecule does not contain carbon atoms (C) or hydrogen atoms (H). Since it does not have ***both*** C and H, it is an inorganic molecule.

> **Note**
>
> Don't be thrown off by the "C's" in $CaCl_2$. These are not a carbon atoms. "Ca" is the element calcium. "Cl" is the element chlorine.

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2.45: Exercise 6.1-5

Answer

inorganic molecule

Explanation

Even though this molecule does contain hydrogen atoms (H), it does not contain any carbon atoms (C). Since it does not have ***both*** C and H, it is an inorganic molecule.

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2.46: Exercise 6.1-6

Answer

organic molecule

Explanation

This molecule contains carbon atoms (C) ***and*** hydrogen atoms (H). Since it has both C and H, it is an organic molecule.

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2.47: Exercise 6.1-7

Answer

organic molecule

Explanation

This molecule contains carbon atoms (C) ***and*** hydrogen atoms (H). Since it has both C and H, it is an organic molecule.

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2.48: Exercise 6.1-8

Answer

inorganic molecule

Explanation

This molecule does not contain any hydrogen atoms (H). Since it does not have ***both*** C and H, it is an inorganic molecule.

> **Note**
>
> Most inorganic molecules do not contain carbon atoms, but they can. Note that this molecule does not contain any hydrogen atoms (H) with the carbon atom (C), thereby making it inorganic.

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2.49: Exercise 10.1

Pink cells after the Gram stain (assuming the Gram stain was completed successfully) means that this is a species that is considered **Gram-negative**.

A species that is Gram-negative has a thin layer of peptidoglycan in its cell wall and an outer membrane containing lipopolysaccharide (LPS).

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2.50: Exercise 10.2

Purple cells after the Gram stain (assuming the Gram stain was completed successfully) means that this is a species that is considered **Gram-positive**.

A species that is Gram-positive has a thick layer of peptidoglycan in its cell wall and does not have an outer membrane.

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2.51: Exercise 10.3

Purple colored cells and pink colored cells after the Gram stain (assuming the Gram stain was completed successfully) means that **there are at least two species of bacteria** in the sample and **one species is Gram-positive and one species is Gram-negative**.

The species that is Gram-positive has a thick layer of peptidoglycan in its cell wall and does not have an outer membrane.

The species that is Gram-negative has a thin layer of peptidoglycan in its cell wall and an outer membrane containing lipopolysaccharide (LPS).

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2.52: Exercise 10.4

1. Two different species of bacteria (Look for different cell shapes and different Gram results).
2. One species is Gram-positive (purple) and one species is Gram-negative (pink).
3. The Gram-positive cells are cocci and the Gram-negative cells are bacilli.

> **Note**
>
> While only two species of bacteria can be distinguished, it is possible that there could be more than two. There are many species of Gram-positive cocci and many species of Gram-negative bacilli.

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2.53: Exercise 10.5

1. One species of bacteria (look for different cell shapes and different Gram results - all cells appear as pink rods).
2. Gram-negative (pink)
3. bacilli

> **Note**
>
> Just because all cells appear as Gram-negative bacilli, that doesn't mean that it is for sure only one species of bacteria. There are many different species of Gram-negative bacilli.

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2.54: Exercise 10.6

1. Two different species of bacteria (look for different cell shapes and different Gram results).
2. One species is Gram-positive (purple) and one species is Gram-negative (pink).
3. The Gram-positive cells are bacilli and the Gram-negative cells are cocci.

> **Note**
>
> While only two species of bacteria can be distinguished, it is possible that there could be more than two. There are many species of Gram-positive bacilli and many species of Gram-negative cocci.

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2.55: Exercise 15.1

Answer

211,000,000,000 cells/mL in original sample **or** 2.11 x 10^{11} cells/mL in original sample

Solution

- **# of colonies counted** = 211
- **amount of diluted sample added to the petri plate in mL** = 100 µL = 0.1 mL
- **dilution of the petri plate counted** = 10^{-8}

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

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = CFU in diluted sample (cells/mL)

(211 colonies) ÷ (0.1 mL diluted sample added to petri plate) = 2,110 cells/mL in the diluted sample

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

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = CFU in original sample (cells/mL)

(2,110 cells/mL in diluted sample) ÷ (10^{-8} dilution of the petri plate counted) = 211,000,000,000 cells/mL in original sample = 2.11 x 10^{11} cells/mL in original sample

CFU = 211,000,000,000 cells/mL in original sample **or** 2.11 x 10^{11} cells/mL in original sample

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2.56: Exercise 15.2

Answer

32,400,000 cells/mL in original sample **or** 3.24 x 10^7 cells/mL in original sample

Solution

- **# of colonies counted** = 162
- **amount of diluted sample added to the petri plate in mL** = 50 µL = 0.05 mL
- **dilution of the petri plate counted** = 10^{-4}

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

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = CFU in diluted sample (cells/mL)

(162 colonies) ÷ (0.05 mL diluted sample added to petri plate) = 3,240 cells/mL in the diluted sample

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

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = CFU in original sample (cells/mL)

(3,240 cells/mL in diluted sample) ÷ (10^{-4} dilution of the petri plate counted) = 32,400,000 cells/mL in original sample **or** 3.24 x 10^7 cells/mL in original sample

CFU = 32,400,000 cells/mL in original sample **or** 3.24 x 10^7 cells/mL in original sample

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2.57: Exercise 15.3

Answer

356,000,000 cells/mL in original sample **or** 3.56 x 10^8 cells/mL in original sample

Solution

- **# of colonies counted** = 89
- **amount of diluted sample added to the petri plate in mL** = 250 µL = 0.25 mL
- **dilution of the petri plate counted** = 10^{-6}

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

(# of colonies counted on the petri plate) ÷ (amount of diluted sample added to the petri plate in mL) = CFU in diluted sample (cells/mL)

(89 colonies) ÷ (0.25 mL diluted sample added to petri plate) = 356 cells/mL in the diluted sample

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

(CFU in diluted sample) ÷ (dilution of the petri plate counted) = CFU in original sample (cells/mL)

(356 cells/mL in diluted sample) ÷ (10^{-6} dilution of the petri plate counted) = 356,000,000 cells/mL in original sample **or** 3.56 x 10^8 cells/mL in original sample

CFU = 356,000,000 cells/mL in original sample **or** 3.56 x 10^8 cells/mL in original sample

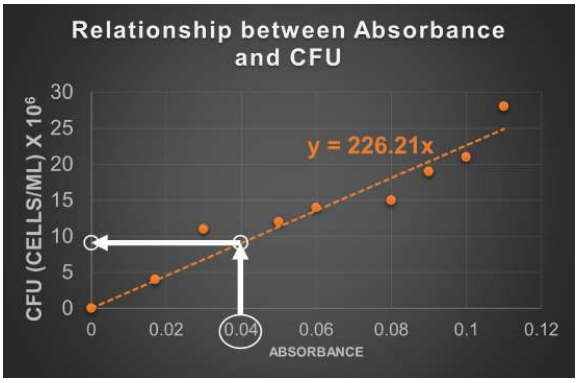
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2.58: Exercise 15.4

Answer

approximately 9 x 10^6 cells/mL

Solution



1. Find the number corresponding to the absorbance on the graph's axis (in this case 0.04 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 0.04 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 0.04 absorbance, the standard line is about at about 9, but the unit given on the graph indicates these numbers should be multiplied by 10^6. This is how 9 x 10^6 cells/mL was determined as the CFU at this absorbance.

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2.59: Exercise 15.5

Answer

70.1 x 10^6 cells/mL **or** 7.0 x 10^7 cells/mL

Solution

- **Equation for standard line:** y = 226.21x
- **The absorbance (which is x):** 0.31
- **Set up the equation:** y = (226.21) x (0.31)
- **Calculate y:** 70.125
- **Recall that the graph axis indicates y is x 10^6, so the final answer is:** 70.1 x 10^6 cells/mL **or** 7.0 x 10^7 cells/mL

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2.60: Exercise 15.6

Answer

40.7×10^6 cells/mL **or** 4.1×10^7 cells/mL

Solution

- **Equation for standard line:** y = 226.21x
- **The absorbance (which is x):** 0.18
- **Set up the equation:** y = (226.21) x (0.18)
- **Calculate y:** 40.718
- **Recall that the graph axis indicates y is x 10^6, so the final answer is:** 40.7×10^6 cells/mL **or** 4.1×10^7 cells/mL

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2.61: Exercise 22.1

Answers

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

Solution

- **Gas was produced.** The upside-down tiny tube inside the test tube (the Durham tube) is not completely filled with liquid medium. It has some gas in it. This indicates the bacterial species produced gas during fermentation and it was trapped inside of the Durham tube.
- **Acid was produced.** The medium is yellow. When the medium was inoculated it was red. When the pH of this medium becomes acidic, a pH indicator will change from red to yellow. Since the medium is yellow, it indicates that acid was produced to reduce the pH.
- **Fermentation occurred.** If either acid or gas is produced or if acid and gas are produced then fermentation has occurred.

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2.62: Exercise 22.2

Answers

- Did this bacterial species produce gas? **No**
- Did this bacterial species produce acid? **No**
- Did fermentation occur? **No**

Solution

- **Gas was not produced.** The upside-down tiny tube inside the test tube (the Durham tube) is completely filled with liquid medium. The Durham tube therefore does not have any gas in it (no bubble). If the bacterial species produced gas during fermentation it would have been trapped inside of the Durham tube.
- **Acid was not produced.** The medium is red. When the medium was inoculated it was red. Since the medium is still red, no acid was produced. In this medium, when acid is produced, a pH indicator changes the medium to yellow.
- **Fermentation did not occur.** If neither acid or gas are produced, this indicates fermentation did not occur.

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Identification of two Bacterial Species in a Mixed Culture Using Traditional Microbiological Identification Approaches

Written By: Rosanna Hartline

Introduction

Bacterial Diversity & Importance of Species Identification

Bacteria are among the most diverse types of life on Earth (Locey and Lennon, 2016). There are billions, perhaps trillions, of bacterial species occupying habitats that are living, such as animals, and non-living habitats with a vast variety of temperatures, pH, and salinities (Parker *et al*., 2022). The metabolic and physical and adaptations enabling bacterial survival is wide-ranging, particularly given that these microscopic organisms are only a single cell big (Parker *et al.*, 2022). The impacts bacteria have on other living things and the environment are extensive. Bacteria are responsible for critical nutrient cycling activities in environmental systems that would not occur without them (Parker *et al.*, 2022). Without bacteria, nutrient availability on Earth would be highly limited and greatly reduce the amount of life that could exist on this planet (Parker *et al.*, 2022).

Bacteria live symbiotically with other living things in parasitic, commensal, and mutualistic ways (Parker *et al.*, 2022). In fact, living in and on a single human, there are more than 100 trillion bacterial cells (Locey and Lennon, 2016). To understand the interactions of each bacterial species and their impacts on the environment and other living things, bacterial species must be identified and characterized. The implications of the types of bacterial species living in different habitats and environments ranges from destructive and lethal effects of some bacterial species to bacterial roles and activities that are crucial for the existence of life. In this study, the goal was to apply traditional microbiological approaches to successfully identify bacterial species of unknown identifies in a mixed culture. To accomplish this, the following approaches were used: quadrant streak plate, Gram stain, starch hydrolysis test, H_2S production test, oxidase test, and lactose fermentation test.

Quadrant Streak Plate

To identify bacterial species using traditional microbiological methods, isolating a single bacterial species from other microbes is crucial. Subsequent microbiological tests will have indeterminant results if there are multiple microbial species mixed, since there is no way of knowing which species is producing any positive test result. To isolate bacterial species, a commonly used approach is the quadrant streak plate technique (Raymond *et al.*, 2022). This approach utilizes a petri plate with nutrient-rich media and four streak zones streaked in a way such that there are successively less bacteria in each zone with isolated colonies in the fourth quadrant (Raymond *et al.*, 2022). A bacterial colony will develop from a single cell as the cells divide by binary fission, and thereby produce an isolated colony that will contain only one bacterial species (Klamm, 2019). These isolated colonies can then be characterized, aiding in species identification, and then may be transferred to a separate culture to create a monoculture for subsequent identification tests. Qualities such as colony color, form, size, and appearance are useful information to distinguish between different species of bacteria. These can be determined by careful examination of isolated colonies on a petri plate.

Gram Stain

The diversity of bacterial species in the world is subdivided into two major groups based on their cell wall structures and are designated as either Gram-positive or Gram-negative based on the results of the Gram stain (Raymond *et al*., 2022). The Gram stain will stain Gram-positive bacteria purple since the purple-colored crystal violet stain is not removed from the Gram-positive cell wall during the decolorization step of the Gram stain due to the thick peptidoglycan layer in the cell wall outside of the plasma membrane (Parker *et al.*, 2022). The Gram-positive bacterial cell wall also contains teichoic acid and does not have an outer membrane (Hartline, 2022). For Gram-negative bacteria, the purple-colored crystal violet stain is readily removed from its thin peptidoglycan layer during the decolorization step of the Gram stain and will appear pink due to the counterstain safranin used after the decolorization step (Raymond *et al.*, 2022). Most species that stain Gram-negative have a thin peptidoglycan layer outside the

of plasma membrane with an outer membrane outside of the peptidoglycan layer that has embedded lipopolysaccharide (also known as endotoxin) (Hartline, 2022; Parker *et al.*, 2022).

Starch Hydrolysis Test

One of the many microbiological tests used to characterize bacterial species and aid in species identification is the starch hydrolysis test. Starch is a polysaccharide, and therefore is composed of many sugars that bacteria can use as an energy source and a carbon source (Parker *et al.*, 2022). If a bacterial species has the amylase gene, it can produce the enzyme amylase enabling it to hydrolyze (break down) starch and utilize this rich energy and carbon source (Hartline, 2022). To determine if bacteria hydrolyze starch, bacteria are grown on a starch plate and following incubation, iodine is added to the plate. Iodine reacts with starch to produce a blue-black color enabling visualization of starch and where it is and is not present in the petri plate agar (Pakpour and Horgan 2021). If starch has been broken down by the bacteria, the agar will lack the blue-black color around the bacterial growth indicating that starch has been hydrolyzed, since starch is absent near the bacteria (Pakpour and Horgan 2021).

H_2S Production

Use of SIM deep medium can be used to determine multiple characteristics of a bacterial species in a single test tube, including determining if bacteria can produce H_2S (Pakpour and Horgan 2021). Production of H_2S can indicate that either a bacterial species can convert cysteine (an amino acid) to pyruvate or that a bacterial species conducts a form of anaerobic respiration where H_2S is produced when a sulfur-containing compound is used as a final electron acceptor (Hartline, 2022). The SIM medium contains ferrous sulfide and if bacteria produce H_2S, the ferrous sulfide will react with H_2S to produce a black precipitate, indicating the bacterial species is H_2S-positive (Pakpour and Horgan 2021). The absence of black precipitate characterizes the bacterial species as H_2S-negative, since it does not produce H_2S gas.

Oxidase Test

Cytochrome *c* oxidase is an enzyme that some bacteria use as part of their electron transport chain in aerobic respiration (Raymond *et al.*, 2022). Only some bacterial species have the cytochrome *c* oxidase gene and therefore produce this enzyme, making it a useful characteristic for differentiating bacterial species in the identification process (Raymond *et al.*, 2022). Some bacterial species do not have the cytochrome *c* oxidase enzyme, but they can still conduct aerobic respiration using different enzymes (Hartline, 2022). Testing for the presence or absence of this enzyme is not an indicator of whether a species conducts aerobic respiration or not, just whether the species uses the cytochrome *c* oxidase as the enzyme that transfers electrons from the electron transport chain to oxygen, the final electron acceptor in this process (Hartline, 2022). The oxidase test determines whether a bacterial species has the cytochrome *c* oxidase enzyme. This test uses a chemical called tetramethyl-*p*-phenylenediamine and if cytochrome *c* oxidase is present in the bacteria, it will remove electrons from this chemical and the product molecule is a blueish or purplish color (Klamm, 2019). Therefore, oxidase positive bacteria produce a blueish or purplish color in the first 30 seconds of mixing the chemical with live bacteria and oxidase negative bacteria do not produce this color change.

Lactose Fermentation Test

Some bacterial species can use fermentation as a method of producing ATP and some bacterial species are not. Further, the types of sugars that different bacterial species can ferment also differs based on the genes (and therefore enzymes) that the species have or do not have (Hartline, 2022). The lactose fermentation test determines whether bacterial species are capable of utilizing lactose, a type of sugar, to conduct fermentation and will indicate whether a bacterial species will make acid, gas, or both as byproducts of the fermentation process (Lee 2021). Bacterial species that metabolize lactose have the gene for the enzyme beta-galactosidase enabling the breakdown lactose, a disaccharide, into monosaccharides that can enter catabolic pathways such as fermentation (Hartline 2022). To conduct this test, a lactose-containing medium with a pH indicator and Durham tube is inoculated with the bacteria being tested. If acid is produced by the fermentation process, the medium will change from red to yellow (Lee, 2021). If gas is produced by the fermentation process, a pocket of gas will form inside of the Durham tube. If no lactose fermentation occurs, no acid or gas will be produced.

Experimental Overview

Bacterial identification is an important skill for people in a vast range of professions where bacteria can impact their work including healthcare, food safety, and water safety. This experiment aimed to separate the bacterial species in a mixed culture and utilized traditional microbiological identification approaches to identify the bacterial species present in that culture. These

techniques resulted in successful identification of *Bacillus subtilis* as one of the species in that mixed culture. The other species in the culture was *Escherichia coli* but was misidentified in this process as *Serratia marcescens*.

Methods

Quadrant Streak Plate

From a TSB culture containing a mixture of two unknown bacterial species, a loopful of culture was aseptically transferred to a single streak along one side of a petri plate. The loop was flamed and cooled before an edge of the initial streak was dragged into a second streak on the petri plate (Hartline, 2022). The loop was again flamed, cooled, and part of the second streak was dragged into the third streak (Hartline, 2022). This was repeated one more time to complete a fourth streak (Hartline, 2022). This process was repeated eight times to produce a total of eight quadrant streak plates. The plates were inverted and incubated at 30 °C for 48 hours.

Gram Stain

Bacterial smears were prepared by either by aseptically transferring several loops of liquid culture to a microscope slide or aseptically transferring a bacterial colony using a loop from a petri plate to a drop of saline on a microscope slide. The slide was placed onto a slide warmer until dry and then heat-fixed by passing the slide through a Bunsen burner flame three times. Crystal violet was applied to stain the smear for one minute, rinsed with deionized water, covered with Gram's iodine for one minute, rinsed with deionized water, decolorized with ethanol for five to fifteen seconds, rinsed with deionized water, counterstained with safranin for one minute, and rinsed with deionized water (Hartline, 2022). Following this staining procedure, the slide was blotted with bibulous paper and examined with a light microscope.

Starch Hydrolysis Test

A loop was used to aseptically transfer isolated bacteria from a TSA slant culture to create a single-line streak on the surface of a petri plate with starch agar medium. The plate was inverted and incubated at 37 °C for 48 hours. The plate was then flooded with iodine to visualize presence and absence of starch in the agar.

H_2S Production

An inoculation needle was used to aseptically transfer bacteria from a TSA slant to a SIM deep culture and incubated at 37 °C for 48 hours.

Oxidase Test

A sterile plastic loop was used to aseptically transfer bacteria from the SIM deep culture to a moistened oxidase test strip. Results of the test were interpreted within the first 30 seconds. This test was repeated a total of three times to ensure that results were accurate.

Lactose Fermentation Test

Bacteria were aseptically transferred from a TSA slant to a test tube with phenol red lactose medium containing a Durham tube. This culture was incubated at 37 °C for 12 days.

Results & Discussion

Isolation of Bacterial Species

As a result of the quadrant streak plate procedure, isolated colonies were successfully produced. There were two distinctly different types of colonies that were differentiated by shape, color, and size. These differences in colony characteristics suggest that these are different microbial species (**Figure 2**).



Figure 2: The quadrant streak plate from the mixed culture of unknown bacterial designed 'H.'

One of the colony types, hereafter designed as bacterial specie 'alpha,' were a bright ivory color and shiny (Hartline, 2022). The colonies measured between 1 mm and 3 mm in diameter and had a circular form with sharp edges and raised elevation.

The other colony type, designated as 'beta,' produced dry and dull looking colonies with a milky-yellow coloration, an irregular form, flat elevation, and measured between 3 mm and 5 mm in diameter (Hartline, 2022). These colonies had distinct color variations that occurred as irregular, wavy rings within the colonies.

Identification of Bacterial Species Alpha

Gram Stain

A Gram stain conducted on the unknown isolated bacterial species Alpha revealed that this species has rod-shaped cells, or bacilli, and the cells appeared pink after the Gram stain (**Figure 3**).

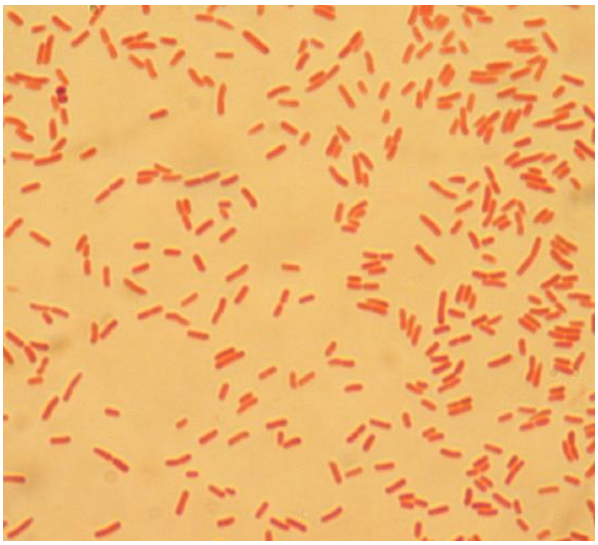


Figure 3: Isolated unknown bacterial species Alpha after Gram stain at 1000x magnification.

Since unknown bacterial species Alpha produced pink-colored cells in the Gram stain, this indicates it is a Gram-negative species (Hartline, 2022). This species likely has a cell wall with a thin layer of peptidoglycan outside of the plasma membrane and an outer membrane containing lipopolysaccharide (Hartline, 2022; Parker *et al.*, 2022).

H_2S Test

Since unknown bacterial species Alpha was Gram-negative, the next indicated test to conduct was to assess whether this species produces H_2S gas or not. After inoculation and incubation of a SIM deep, the medium did not produce a black coloration (**Figure 4**).

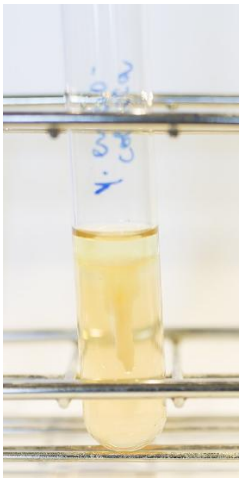


Figure 4: SIM deep culture with unknown bacterial species Alpha.

Since the SIM deep did not produce a black coloration, this means that no H_2S was produced in the culture to interact with the ferrous sulfide in the medium (Pakpour and Horgan 2021). Therefore, unknown bacterial species Alpha does not produce H_2S and is H_2S-negative. This result reveals that unknown bacterial species Alpha does not produce pyruvate from cysteine and does not conduct the form of anaerobic respiration that produces H_2S gas as a byproduct (Hartline, 2022).

Although not directly a part of this identification protocol, the presence or absence of motility can be determined using this SIM deep culture (Pakpour and Horgan 2021). Growth migrated away from the stab line in the SIM deep suggesting unknown bacterial species Alpha is motility positive and therefore has one or more flagella enabling movement in this bacterial species (Hartline, 2022; Pakpour and Horgan 2021).

Oxidase Test

Due to the H_2S negative test result, the identification protocol next indicates the oxidase test. The oxidase test did not produce a purple or blue color in the first 30 seconds (**Figure 5**). The test was repeated a total of three times to ensure the validity of this result and the results did not change.



Figure 5: One of the test strips produced from the oxidase test of unknown bacterial species Alpha.

The lack of purple or blue color in under 30 seconds means that unknown bacterial species Alpha is oxidase negative. These results indicate that unknown bacterial species Alpha does not have the cytochrome *c* oxidase enzyme or the gene that codes for cytochrome *c* oxidase (Raymond *et al.*, 2022). Cytochrome *c* oxidase is a key enzyme in the electron transport chain of many species capable of aerobic respiration. This enzyme transfers low energy electrons from the electron transport chain to the final electron acceptor, O_2, to remove it from the chain. While this result does not indicate whether this bacterial species can conduct aerobic respiration or not, it does indicate that it does not use the cytochrome *c* oxidase enzyme to transfer electrons from the electron transport chain to O_2 (Hartline, 2022). If this species does conduct aerobic respiration, it uses a different enzyme for this process at the end of the electron transport chain (Hartline, 2022).

Lactose Fermentation Test

Since the oxidase test produced a negative result for unknown bacterial species Alpha, the next indicated identification test in the protocol was the lactose fermentation test. After inoculation and incubation, the phenol red lactose medium maintained a red color and the Durham tube was still filled with the liquid medium and lacked a gas pocket inside of it (**Figure 6**).



Figure 6: Lactose fermentation culture of unknown bacterial species Alpha.

Since the phenol red lactose medium did not become yellow, this indicates that acid was not produced by the bacteria. No air pocket in the Durham tube in this culture indicates that no gas was produced by the bacteria in this culture. Since neither gas nor acid were produced, this indicates that bacterial species Alpha is lactose fermentation negative. Fermentation is a metabolic process conducted by some bacterial species as a way of producing ATP for the cell's energy needs. This process breaks down a sugar such as glucose or lactose to pyruvate generating 2 net ATP. The pyruvate is then fermented where an organic molecule is used as the final electron acceptor and produces byproducts such as acid and/or gas. This result suggests this bacterial species either does not conduct fermentation, does not have the beta-galactosidase gene for breaking down lactose, or both (Hartline, 2022).

Based on this result, unknown bacterial species Alpha was identified using this laboratory protocol as *Serratia marcescens*.

Identification of Bacterial Species Beta

Gram Stain

The isolated bacterial species designated as Beta was Gram-stained and results show the cells appear as purple rods (**Figure 7**).

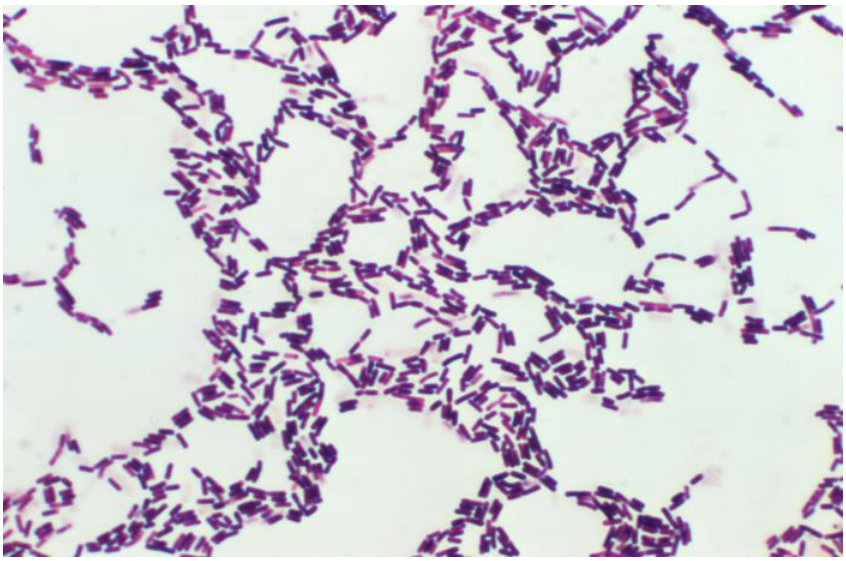


Figure 7: Gram stain of unknown bacterial species Beta magnified at 1000x.

The purple coloration of this bacterial species after the Gram stain indicates this species is Gram-positive. This result indicates this species has a thick layer of peptidoglycan in its cell wall that retains the purple coloration of the crystal violet stain during the Gram stain protocol (Parker *et al.*, 2022). The rod-shaped cells indicate this species have cells that are Gram-positive bacilli.

Starch Hydrolysis Test

Since unknown bacterial species Beta stained Gram-positive, the next indicated test in the identification protocol was the starch hydrolysis test. Following incubation of the unknown bacteria on a starch plate and staining the starch plate with iodine, a clear zone surrounded the bacterial growth on the petri plate (**Figure 8**).

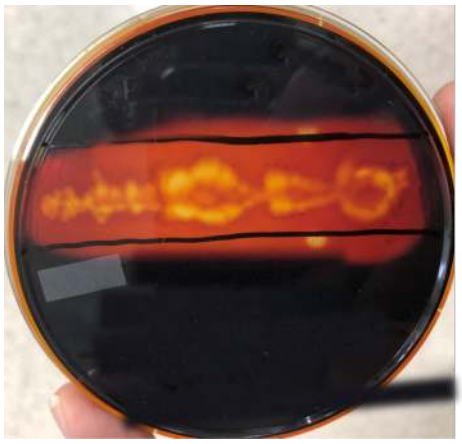


Figure 8: Starch hydrolysis plate flooded with iodine with growth of unknown bacterial species Beta.

The starch hydrolysis test showed a clear zone surrounding the bacterial growth indicating that starch was absent in the agar surrounding unknown bacterial species Beta (Pakpour and Horgan 2021). This result reveals that Beta is starch hydrolysis positive and therefore must have the amylase gene to produce the amylase enzyme that breaks down starch (Hartline, 2022). Starch hydrolysis is a characteristic which enables some species of bacteria to access a wealth of energy in starch molecules. Starch is a polysaccharide and, if bacteria can break down starch with the amylase enzyme, the bacteria can utilize the sugars in starch as a source rich in energy. The fact that this species can break down starch means this species has an additional way to survive that other bacteria may not have.

Since the starch hydrolysis test was positive for unknown bacterial species Beta, the identification protocol indicates that this species is *Bacillus subtilis*.

Conclusion

Unknown bacterial species Alpha was identified as *Serratia marcescens* using this identification protocol using traditional microbiological techniques. In fact, this species was not *S. marcescens*, but was *Escherichia coli* indicating one or more errors occurred resulting in incorrect identification of this bacterial species. There are two plausible explanations for this misidentification. First, it is possible when unknown bacterial species Alpha was aseptically collected from the TSA slant that the loop was too hot, and any bacteria collected from the slant were killed by the heat of the loop. As a result, the lactose fermentation tube may not have been successfully inoculated, resulting in the lactose fermentation negative result. The other possible explanation for incorrect identification is that a contaminant colony on the quadrat streak plate was collected and tested throughout this identification process instead of the actual unknown bacterial species that was targeted.

Unknown bacterial species Beta was identified as *Bacillus subtilis* in this experiment. The Gram-positive bacterial species in unknown culture ‘H’ was indeed *B. subtilis* and therefore identification of this species was successful. This result was likely due to several factors including careful aseptic technique to prevent contamination, thoughtful examination and documentation of each laboratory result throughout the process, and careful execution of all the laboratory protocols to ensure accurate experimental results.

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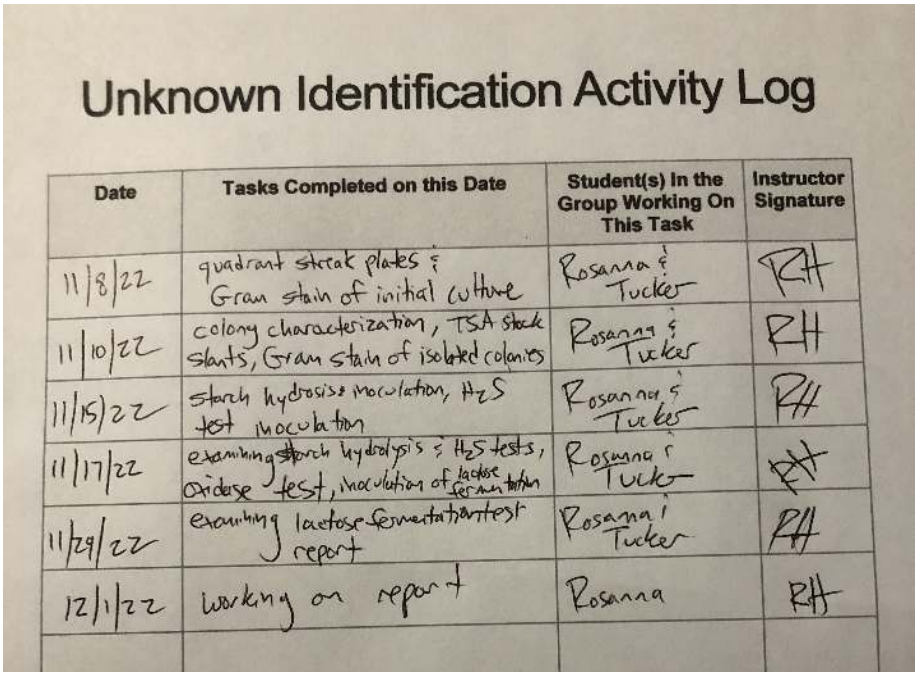
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Activity Log



Unknown Identification Activity Log

Date	Tasks Completed on this Date	Student(s) In the Group Working On This Task	Instructor Signature
11/8/22	quadrant streak plates & Gram stain of initial culture	Rosanna & Tucker	RH
11/10/22	colony characterization, TSA stock slants, Gram stain of isolated colonies	Rosanna & Tucker	RH
11/15/22	starch hydrosis inoculation, H_2S test inoculation	Rosanna & Tucker	RH
11/17/22	examining starch hydrolysis & H_2S tests, oxidase test, inoculation of lactose fermentation	Rosanna & Tucker	RH
11/29/22	examining lactose fermentation test report	Rosanna & Tucker	RH
12/1/22	working on report	Rosanna	RH

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- Yersinia enterocolitica in SIM Agar1 125.jpg by A doubt is licensed under CC BY-SA 4.0

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3.1: Myth of Spontaneous Generation

Materials

Student Preparation of TSB Medium

- TSB medium powder
- stir plates (1 per group)
- magnetic stir bars (1 per group)
- 50 or 100 mL graduated cylinders (1 per group)
- 250 mL or 300 mL beakers for making TSB medium (1 per group)
- balances
- weigh papers or boats
- weigh utensils
- distilled or deionized water

Re-creation of Spallanzani's Experiment

- test tube racks (1 per group)
- test tubes (2 per group)
- test tube caps (1 per group)
- 5 mL or 10 mL pipet (1 per group)
- pipet bulbs or pipet pumps (1 per group)
- 10-20 mL student-prepared TSB (before autoclaving)
- labeling tape and markers

Re-creation of Pasteur's Experiment

- 125 mL Erlenmeyer flasks (2 per group)
- neoprene stoppers with one hole, size 5 (2 per group)
- 5 mm flint glass tubing, prepped as straight glass tubes (1 per group)
- 5 mm flint glass tubing, prepped as swan-neck glass tubes (1 per group)
- 50 mL or 100 mL graduated cylinder (1 per group)
- 100 mL student-prepared TSB (before autoclaving)
- labeling tape and markers

Preparations

Re-creation of Pasteur's Experiment

Straight Glass Tubes

1. Wear safety goggles.
2. Use a file to score around the flint glass tubing to cut lengths of glass tubing about 6-8 cm in length.
3. Put on protective gloves (not lab gloves; use thicker gloves such as gardening gloves or autoclave gloves).
4. Snap the tubing at the score site.
5. Repeat until you have enough straight-glass tubes for class (1 per group).

Swan-Neck Tubes

1. Wear safety goggles.
2. Use a file to score around the flint glass tubing to cut lengths of glass tubing about 20-25 cm in length.
3. Put on protective gloves (not lab gloves; use thicker gloves such as gardening gloves or autoclave gloves).
4. Snap the tubing at the score site.
5. Use a Bunsen burner to heat the glass tube to bend the tube into a swan-neck shape. This will require you to heat the glass tube at two different locations where one site is bent downward and the other site is bent upward. Make sure that there is enough of a straight section before the swan-neck curves so the straight section can pass through the one-hole neoprene stopper.

6. Repeat until you have enough swan-neck glass tubes for class (1 per group).

Put Glass Tubes through Stoppers

1. Carefully push a straight glass tube through the hole in one of the neoprene stoppers.
2. Carefully push the straight region of the swan-neck class tube through the hole in one of the neoprene stoppers.
3. Repeat for each group.

After Lab Class

1. Autoclave the test tubes and flasks prepared by students for the Spallanzani and Pasteur experiment re-creations.
2. Incubate the test tubes and flasks prepared by students for the Spallanzani and Pasteur experiment re-creations in a 30°C or 37°C incubator or leave out on a bench.
3. When growth is observed in the uncovered test tube from the Spallanzani experiment have students examine results from the Spallanzani experiment re-creation.
4. When growth is observed in the flask with the straight glass tube from the Pasteur experiment have students examine results from the Pasteur experiment re-creation.

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3.2: Get to Know the Microscope and Microbes

Materials

- light microscopes (1 per student or 1 per pair of students)
- slides: letter 'e'
- prepared slides of your choosing:
  - a helminth species
  - a fungal species
  - a protozoan species
  - bacterial species

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3.3: Molecules of Life

Burn Test

> **Note**
>
> There are multiple ways this test can be run with a class:
>
> **Option 1:** Have all student groups test all samples.
>
> **Option 2:** Have all student groups test one sample (recommended sample: sugar) and have the other samples pre-prepared and students interpret the pre-prepared results.
>
> **Option 3:** Instructor demonstrates one sample (recommended sample: sugar) and all other samples are pre-prepared and students interpret the results.
>
> **Option 4:** All samples are pre-prepared and students interpret the results.
>
> Consider which option you are choosing when determining the quantities of materials you will need below.

Preparation

- If you choose one of Options 2, 3, or 4 above, prepare the burn test as follows:
  - Place a small amount of each substance in its own separate test tube (baking soda, hair, salt, starch, sugar, vegetable oil, water), preferably a screw-top test tube that can be re-used semester after semester.
  - Wear safety goggles. Without a cap or top on the test tube, place the bottom of the test tube in the flame of a Bunsen burner while the opening of the test tube is facing away from you (you only need to do this for the organic samples since the inorganic samples will not burn). You may want to do this in a fume hood.
  - Continue burning until the organic samples turn black (this can take a while for oil - just persist).
  - Once cool, add caps or tops to the test tubes

Materials

- If you choose option 1 or 2, make sure to have the following on hand:
  - test tubes (enough for each sample students will test per group)
  - Bunsen burners
  - test tube holders
  - safety goggles or safety glasses
  - the substances that will be tested: baking soda, hair, salt, starch, sugar, vegetable oil, water
  - droppers to transfer liquid samples
  - spoons or other utensils to transfer solid samples
  - test tube racks (enough for 1 per group)

Building Monomers of Biological Molecules

Materials

- organic molecular modeling sets (enough for each student to have one or to share with another student)

Foods & their Biological Molecules

Preparation

The day of or the night before the lab, create food item suspensions as follows:

1. add pieces of green banana to a blender and add DI or distilled water
2. puree until homogenous
3. transfer solution to storage container
4. clean blender

5. repeat steps 2-5 using black banana, chicken meat (muscle), and egg

Materials

- blended suspensions of green banana, black banana, chicken meat, egg
- five test tubes per group
- labeling tape
- labeling markers
- safety goggles or safety glasses (1 per student)
- test tube rack (1 per group)
- droppers (2-3 per food suspension)
- DI or distilled water
- biuret reagent in dropper bottles (1 for every two groups - groups can share)
- Benedict's solution in dropper bottles (1 for every two groups - groups can share)
- hot plates with beakers containing boiling water or almost boiling water (1 for every two groups - groups can share)
- iodine in dropper bottles (1 for every two groups - groups can share)
- brown paper or brown paper towels
- chemical waste container (biuret and Benedict's waste - can be disposed of in the same container)

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3.4: Aseptic Technique

Preparation

- 2 test tubes with sterile TSB per group

Materials

- 2 test tubes with sterile TSB per group
- test tube rack (1 per group)
- Bunsen burner (1 per group)
- inoculating loop (1 per group)
- labeling tape
- marker

After Lab Class

1. Incubate test tubes of (hopefully still) sterile TSB at 37°C until the next lab class.
2. Next lab class have students examine test tubes for growth and discuss possible sources of contamination (particularly for groups that had TSB showing growth).

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3.5: Plating on Petri Plates for Isolation

Preparation

- sterile TSB test tubes (2 to grow cultures and another 3, 6, 9, or 12 [depending on how many cultures you want groups to share])
- 3 TSA Petri plates per group
- Inoculate TSB test tube with *Escherichia coli* and incubate at 37°C 24 hours before lab class
- Inoculate TSB test tube with pigmented strain of *Serratia marcescens* and incubate at 30°C 24 hours before lab class
- Just before lab class, aseptically transfer 200 µm of *Escherichia coli* culture to sterile TSB test tube(s) - add to 1-4 sterile TSB test tubes (*E. coli* cultures for students to use)
- Just before lab class, aseptically transfer 200 µm of *Serratia marcescens* culture to sterile TSB test tube(s) - add to 1-4 sterile TSB test tubes (*S. marcescens* cultures for students to use)
- Just before lab class, aseptically transfer 100-200 µm of *Escherichia coli* culture to sterile TSB test tube(s) and 100-200 µm of *Serratia marcescens* culture to the same test tube(s) - add to 1-4 sterile TSB test tubes (*E. coli* + *S. marcescens* cultures for students to use)

Materials

- 3 TSA plates per group
- test tube racks (1 per group)
- Bunsen burner (1 per group)
- inoculating loop (1 per group)
- markers
- culture of *E. coli*
- culture of *S. marcescens* (pigmented strain)
- culture of *E. coli* + *S. marcescens* (pigmented strain)

After Lab Class

1. Incubate quadrant streak plates at 30°C for 24-48 hours.
2. Refrigerate streak plates to prevent overgrowth if the next lab class occurs after 24-48 hours.
3. Have students examine streak plates and characterize colonies next lab class.

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3.6: Simple Stain

Preparation

1. Have test tubes with sterile TSB on hand.
2. 24 hours before the laboratory, inoculate test tubes of sterile TSB with a bacterial species of your choice (*Escherichia coli* or *Staphylococcus aureus* are good options) and incubate at the appropriate temperature for that species (37 °C for the *E. coli* and *S. aureus*).

> Remember that you will have multiple groups needing these cultures so make enough cultures so each group has their own or enough so each group only shares the culture with another group.

> **Note**
>
> You can use multiple bacterial species to have students compare different cell morphologies and arrangements. The Results & Questions section of this lab will require editing to provide questions for multiple species.

Materials

- 24-hour bacterial cultures of choice in TSB - see **Preparation** section above
- plain microscope slides (at least 1 per group)
- Bunsen burners (1 per group)
- inoculating loops (1 per group)
- (if using bacteria on solid medium rather than TSB) droppers with saline, DI water, or distilled water
- slide warmer
- test tube racks (1 per group plus one or more racks to hold cultures)
- wood test tube clamps/holders (1 per group)
- small chemical waste containers (1 per group)
- methylene blue in a dropper bottle (1 per group or enough for two groups to share one bottle)
- distilled or DI water in squirt bottles (1 per group)
- bibulous paper (enough for each groups to blot their slides)
- light microscopes (1 per group)
- colored pencils
- (optional, but recommended) Wax pencils (1 per group)
- (if using oil immersion) immersion oil dropper bottles
- (if using oil immersion) lens paper

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3.7: Gram Stain

Preparation

1. Have test tubes with sterile TSB on hand.
2. 12-18 hours before the laboratory, inoculate test tubes of sterile TSB with a mixture of *Escherichia coli* and *Staphylococcus aureus* and incubate at 37 °C .

> **Note**
>
> Remember that you will have multiple groups needing these cultures so make enough cultures so each group has their own or enough so each group only shares the culture with another group.

Materials

- 12-18 hour cultures of *E. coli* and *S. aureus* mixture - see **Preparation** section above
- plain microscope slides (at least 1 per group)
- Bunsen burners (1 per group)
- inoculating loops (1 per group)
- (if using bacteria on solid medium rather than TSB) droppers with saline, DI water, or distilled water
- slide warmer
- test tube racks (1 per group plus one or more racks to hold cultures)
- wood test tube clamps/holders (1 per group)
- small chemical waste containers (1 per group)
- crystal violet in a dropper bottle (1 per group or enough for two groups to share one bottle)
- Gram's iodine in a dropper bottle (1 per group or enough for two groups to share one bottle)
- 95% ethanol in a dropper bottle (1 per group or enough for two groups to share one bottle)
- safranin in a dropper bottle (1 per group or enough for two groups to share one bottle)
- distilled or DI water in squirt bottles (1 per group)
- bibulous paper (enough for each groups to blot their slides)
- light microscopes (1 per group)
- colored pencils
- (optional, but recommended) Wax pencils (1 per group)
- (if using oil immersion) immersion oil dropper bottles
- (if using oil immersion) lens paper

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3.8: Endospore Stain

Preparation

1. Have test tubes with sterile TSB on hand.
2. 36-48 hours before the laboratory, inoculate test tubes of sterile TSB with *Bacillus subtilis* and incubate at 37 °C .

> **Note**
>
> Remember that you will have multiple groups needing these cultures so make enough cultures so each group has their own or enough so each group only shares the culture with another group.

Materials

- 36-48 hour cultures of *B. subtilis* - see **Preparation** section above
- plain microscope slides (at least 1 per group)
- Bunsen burners (1 per group)
- inoculating loops (1 per group)
- (if using bacteria on solid medium rather than TSB) droppers with saline, DI water, or distilled water
- slide warmer
- test tube racks (1 per group plus one or more racks to hold cultures)
- wood test tube clamps/holders (1 per group)
- small chemical waste containers (1 per group)
- malachite green in a dropper bottle (1 per group or enough for two groups to share one bottle)
- safranin in a dropper bottle (1 per group or enough for two groups to share one bottle)
- distilled or DI water in squirt bottles (1 per group)
- hot plates (1 per group or enough for two groups to share)
- large beakers - 1000 mL+ (1 per group or enough for two groups to share)
- wire racks (1 per group or enough for two groups to share)
- paper towels
- bibulous paper (enough for each groups to blot their slides)
- light microscopes (1 per group)
- colored pencils
- (optional, but recommended) wax pencils (1 per group)
- (if using oil immersion) immersion oil dropper bottles
- (if using oil immersion) lens paper

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3.9: Capsule Stain

Materials

- 12-18 hour bacterial culture in skim milk broth culture (*Enterobacter aerogenes* or *Serratia marcescens*)
- plain microscope slides (at least 1 per group)
- Bunsen burners (1 per group)
- inoculating loops (1 per group)
- test tube racks (1 per group plus one or more racks to hold cultures)
- wood test tube clamps/holders (1 per group)
- small chemical waste containers (1 per group)
- 1% crystal violet
- 20% copper sulfate
- light microscopes (1 per group)
- colored pencils
- (if using oil immersion) immersion oil dropper bottles
- (if using oil immersion) lens paper

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3.10: Acid-Fast Stain

Preparation

1. Have test tubes with sterile TSB on hand.
2. 12-18 hours before the laboratory, inoculate test tubes of sterile TSB with a mixture of *Staphylococcus epidermidis* and *Mycobacterium chelonae* and incubate at 37 °C .

Materials

- 12-18 hour cultures (enough for groups to share)
- plain microscope slides (at least 1 per group)
- Bunsen burners (1 per group)
- inoculating loops (1 per group)
- (if using bacteria on solid medium rather than TSB) droppers with saline, DI water, or distilled water
- slide warmer
- test tube racks (1 per group plus one or more racks to hold cultures)
- 400-500 mL beakers with ~200 mL of water each (1 per group)
- hot plate (1 per group)
- slide rack hat fits over beaker (1 per group)
- paper towels
- carbol fuchsin in dropper bottles (1 per group)
- acid-alcohol in dropper bottles (not ethanol from the Gram stain kit) (1 per group)
- methylene blue in dropper bottles (1 per group)
- wood test tube clamps/holders (1 per group)
- small chemical waste containers (1 per group)
- distilled or DI water in squirt bottles (1 per group)
- bibulous paper (enough for each groups to blot their slides)
- light microscopes (1 per group)
- colored pencils
- (optional, but recommended) Wax pencils (1 per group)
- (if using oil immersion) immersion oil dropper bottles
- (if using oil immersion) lens paper

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3.11: Determination of Bacterial Numbers

Plate Counts

Preparation

- sterile TSB in test tubes on hand for *E. coli* cultures (1 per group)
- TSA plates (4 per group)
- Sterilize:
  - microcentrifuge tubes (7 per group)
  - tips for pipettes (for pipettes that can dispense 990 µL and 100 µL)
- inoculate sterile TSB in test tubes with *E. coli* 8-16 hours before lab and incubate at 37 °C (1 per group)

Materials

- sterile microcentrifuge tubes (7 per group)
- sterile tips for pipettes (for pipettes that can dispense 990 µL and 100 µL)
- pipettes for dispensing 990 µL and 100 µL (enough for each group or for two groups to share)
- containers for used pipette tips
- 8-16 hour *E. coli* cultures in TSB (1 per group)
- TSA plates (4 per group)
- vortex (enough of them for groups to share)
- labeling marker (1 per group)
- spreader tools (1 per group)
- ethanol in squirt bottles
- empty petri plates (1/2 per group)
- Bunsen burners (1 per group)
- strikers (1 per group)

Absorbance Measurements

Preparation

- test tubes with 5 mL sterile TSB (4 per group)
- inoculate sterile TSB in test tubes with *E. coli* 8-16 hours before lab and incubate at 37 °C (1 per group) - if the plate counts are being done with the absorbance measurements, these are the same cultures used for the plate counts

Materials

- test tubes with 5 mL sterile TSB (4 per group)
- 8-16 hour *E. coli* cultures in TSB (1 per group)
- pipettes for transferring 5 mL (enough for each group to complete four transfers
- transfer pipettes (5 per group)
- cuvettes
- waste container for *E. coli* samples emptied from cuvettes
- spectrophotometer set to 600 nm (enough for two groups to share)
- labeling tape (1 per group)
- labeling marker (1 per group)

Creating a Standard Line between Plate Counts and Absorbance

Materials

- graph paper or computers with graphing software (1 per student)
- rulers (1 per student if using graph paper)

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3.12: Eukaryotic Cells

Materials

- eukaryotic cell models (1 per group)
- light microscopes (1 per student or 1 per pair of students)
- colored pencils
- prepared slides of your choosing:
  - a protozoan species
  - an algal species
  - a fungal species
  - a helminth species

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3.13: Starch Hydrolysis

Preparation

- 24 hour cultures of *E. coli*, *B. subtilis*, and *P. vulgaris* (enough for groups/individual students to share)
- starch agar plates (one per student or one per group)

Materials

- 24 hour cultures of *E. coli*, *B. subtilis*, and *P. vulgaris* (enough for groups/individual students to share)
- starch agar plates (one per student or one per group)
- test tube racks (1 per group/student)
- inoculating loops (1 per group/student)
- strikers (1 per group/student)
- Bunsen burners (1 per group/student)
- iodine (enough dropper bottles for groups or individual students to share)
- labeling markers (1 per group)

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3.14: Catalase Test

Preparation

- 24 hour cultures of *Staphylococcus aureus* and *Streptococcus pyogenes* (enough for groups to share)

Materials

- 24 hour cultures of *Staphylococcus aureus* and *Streptococcus pyogenes* (enough for groups to share)
- new microscope slides (one per group)
- inoculating loops (one per group)
- hydrogen peroxide (enough for groups to share)
- transfer pipettes or droppers

> **Note**
>
> The hydrogen peroxide cannot be old or have been exposed to light for prolonged periods of time. If this is the case, the reaction may not work. Be sure to use new or newer hydrogen peroxide in a light-proof container.

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3.15: Cytochrome C Oxidase

Preparation

- 24 hour petri plate cultures of *Pseudomonas aeruginosa* or *Alcaligenes faecalis* (oxidase positive) and *Escherichia coli or Proteus mirabilis* (oxidase negative) (enough for groups to share)

Materials

- 24 hour petri plate cultures of *Pseudomonas aeruginosa* or *Alcaligenes faecalis* (oxidase positive) and *Escherichia coli or Proteus mirabilis* (oxidase negative) (enough for groups to share)
- empty petri plates (enough for each group to have 1/2 of an empty petri plate)
- oxidase test strips (enough for each group to have 2)
- DI or distilled water in squirt bottles
- sterile transfer pipettes or sterile transfer swabs (enough for each group to have 2)

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3.16: Citrate Test

Preparation

- 24 hour cultures of citrate positive species (*Klebsiella*, *Enterobacter*, *Proteus*, *Serratia*, *Pseudomonas*, or *Salmonella*) and citrate negative species (*Escherichia coli* or *Shigella*) (enough for groups/individual students to share)
- Simmons' citrate agar slants (two per group)

Materials

- 24 hour cultures of citrate positive species (*Klebsiella*, *Enterobacter*, *Proteus*, *Serratia*, *Pseudomonas*, or *Salmonella*) and citrate negative species (*Escherichia coli* or *Shigella*) (enough for groups/individual students to share)
- Simmons' citrate agar slants (two per group)
- test tube racks (1 per group)
- inoculating loops (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- labeling tape (1 per group)
- labeling markers (1 per group)

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3.17: Bacterial Oxygen Requirements

Preparation

- 24 hour cultures of *Pseudomonas aeruginosa*, *Escherichia coli*, and a *Clostridium* species of choice (if using) - Clostridium species are obligate anaerobes so cultivation will require strict anaerobic conditions (enough for groups to share)
- thioglycollate tubes (enough for each group to have 2-3, depending on the number of species being tested)
- TSA plates (2 per group)

Materials

- 24 hour cultures of *Pseudomonas aeruginosa*, *Escherichia coli*, and a *Clostridium* species of choice (if using) - Clostridium species are obligate anaerobes so cultivation will require strict anaerobic conditions (enough for groups to share)
- thioglycollate tubes (enough for each group to have 2-3, depending on the number of species being tested)
- labeling tape (1 per group)
- labeling markers (1 per group)
- test tube racks (1 per group)
- inoculating loops (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- TSA plates (2 per group)
- anaerobic jar or bags with heat sealer
- GasPak anaerobic generators
- anaerobic indicators

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3.18: Fermentation

Preparation

- 24 hour cultures of *Escherichia coli*, *Bacillus subtilis*, and *Proteus vulgaris* (enough for groups to share)
- phenol red glucose medium in test tubes with Durham tubes (3 per group)
- phenol red lactose medium in test tubes with Durham tubes (3 per group)

Materials

- 24 hour cultures of *Escherichia coli*, *Bacillus subtilis*, and *Proteus vulgaris* (enough for groups to share)
- phenol red glucose medium in test tubes with Durham tubes (3 per group)
- phenol red lactose medium in test tubes with Durham tubes (3 per group)
- test tube racks (1 per group)
- inoculating loops (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- labeling tape (1 per group)
- labeling markers (1 per group)

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3.19: SIM Deep Tests

Preparation

- 24 hour cultures of *Escherichia coli*, *Proteus vulgaris*, and *Staphylococcus aureus* (enough for groups to share)
- SIM deeps (3 per group)

Materials

- 24 hour cultures of *Escherichia coli*, *Proteus vulgaris*, and *Staphylococcus aureus* (enough for groups to share)
- SIM deeps (3 per group)
- test tube racks (1 per group)
- inoculating needles (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- labeling tape (1 per group)
- labeling markers (1 per group)
- Kovac's reagent (enough for groups to share)

Expected Test Results

	H_2S production	indole production	motility
Escherichia coli	-	+	+
Proteus vulgaris	+	-	+
Staphylococcus aureus	-	-	-

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3.20: Coagulase Test

Preparation

- inoculate petri plates with *Staphylococcus aureus* and *Staphylococcus epidermidis* 24 hours before class (enough from groups to share)

Materials

- petri plates with *Staphylococcus aureus* and *Staphylococcus epidermidis* 24 hours before class (enough from groups to share)
- transfer pipet
- rabbit plasma (suitable for coagulase test)
- slides (slide test)
- test tubes (test tube test)
- sterile plastic inoculation loops
- tape
- markers

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3.21: Gelatin Hydrolysis

Preparation

- 24 hour cultures of one gelatin hydrolysis positive species and one gelatin hydrolysis negative species (enough for groups to share):
  - **gelatin hydrolysis positive species:** *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*
  - **gelatin hydrolysis negative species:** *Staphylococcus epidermidis*, *Escherichia coli*
- gelatin hydrolysis medium in test tubes (2 per group)

Materials

- 24 hour cultures (enough for groups to share)
- gelatin hydrolysis medium in test tubes (2 per group)
- test tube racks (1 per group)
- inoculating needles (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- labeling tape (1 per group)
- labeling markers (1 per group)

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3.22: Nitrate Reduction

Preparation

- 24 hour cultures of *Escherichia coli*, *Alcaligenes faecalis*, and *Pseudomonas aeruginosa* (enough for groups to share)
- nitrate broth medium in test tubes with Durham tubes (3 per group)

Materials

- 24 hour cultures of *Escherichia coli*, *Alcaligenes faecalis*, and *Pseudomonas aeruginosa* (enough for groups to share)
- nitrate broth medium in test tubes with Durham tubes (3 per group)
- 0.8% sulfanilic acid in dropper bottles (enough for groups to share)
- 0.6% N, N-Dimethyl-alpha-naphthylamine (enough for groups to share)
- zinc powder (enough for groups to share)
- wooden applicator sticks for zinc powder
- test tube racks (1 per group)
- inoculating loops (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- labeling tape (1 per group)
- labeling markers (1 per group)

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3.23: MR-VP Tests

Preparation

- 24 hour cultures of *Escherichia coli* and *Enterobacter aerogenes*
- MR-VP broth in test tubes (two per group)

Materials

- 24 hour cultures of of *Escherichia coli* and *Enterobacter aerogenes*
- MR-VP broth in test tubes (two per group)
- test tube racks (1 per group)
- inoculating loops (1 per group)
- strikers (1 per group)
- Bunsen burners (1 per group)
- labeling tape (1 per group)
- labeling markers (1 per group)
- empty test tubes (two per group)
- transfer pipets (2 per group)
- methyl red reagent in dropper bottles (enough for groups to share)
- Barritt's reagent A in dropper bottles (enough for groups to share)
- Barritt's reagent B in dropper bottles (enough for groups to share)

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3.24: EMB Agar

Preparation

- 24 hour cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (enough for groups/individual students to share)
- EMB agar plates (three per student or three per group)

Materials

- 24 hour cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (enough for groups/individual students to share)
- EMB agar plates (three per student or three per group)
- test tube racks (1 per group/student)
- inoculating loops (1 per group/student)
- strikers (1 per group/student)
- Bunsen burners (1 per group/student)
- labeling markers (1 per group)

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3.25: Mannitol Salt Agar

Preparation

- 24 hour cultures of *Staphylococcus aureus* and *Staphylococcus epidermidis* (enough for groups/individual students to share)
- mannitol salts agar plates (one per student or one per group)

Materials

- 24 hour cultures of *Staphylococcus aureus* and *Staphylococcus epidermidis* (enough for groups/individual students to share)
- mannitol salts agar plates (one per student or one per group)
- test tube racks (1 per group/student)
- inoculating loops (1 per group/student)
- strikers (1 per group/student)
- Bunsen burners (1 per group/student)
- labeling markers (1 per group)

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3.26: DNA, RNA, and DNA Replication

Materials

- DNA puzzle kits (puzzle kits available here through Carolina Biological) (one per student or one per pair of students to share)

Instruction Instructions

This activity works best when students are stepped through the activity by their instructor. Walk through the instructions one step at a time and keep students together on that same step. Walk around to make sure students are following instructions accurately. Correct students who deviate from instructions or who skip ahead.

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3.27: PCR

Preparation & Materials

It is recommended to choose a PCR kit provided by a science education supplier. Follow the materials and preparation instructions for the kit you choose. You will also need to provide students with detailed laboratory instructions since kit instructions differ. Here are a couple examples of kits:

- Ward's® DNA Amplification by PCR Lab Activity
- Lambda PCR Lab Activity
- Water Analysis by PCR: What's in my Water?
- Amplification of Lambda DNA by PCR Kit
- BioBuilder® Golden Bread PCR
- DNA Barcoding Kits

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3.28: DNA Fingerprinting

Preparation

- 1X TAE buffer (enough for the electrophoresis chambers you are using and for making the gels)
- electrophoresis gels (enough for electrophoresis chambers you are using - one for the class or one for each group)
  - 1% agarose heated in 1X TAE buffer
  - at least 3 wells are needed per group (4 if you are using a ladder)

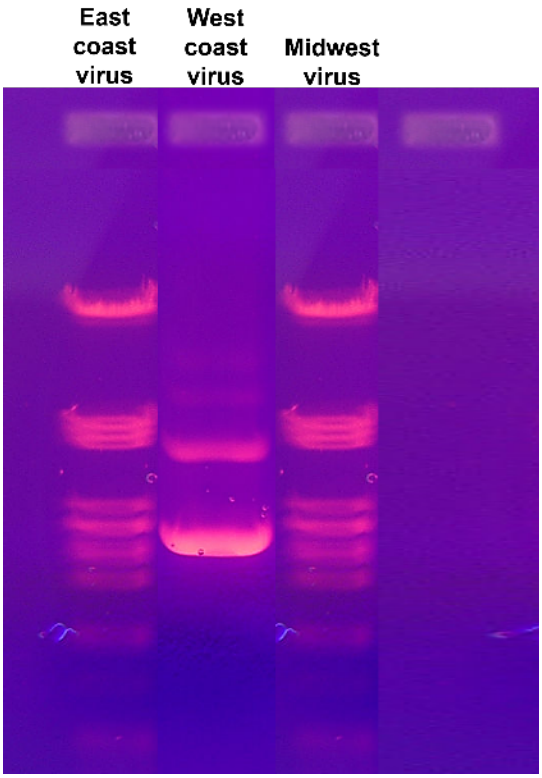
Materials

- 1X TAE buffer (enough for the electrophoresis chambers you are using and for making the gels)
- electrophoresis gels (enough for electrophoresis chambers you are using - one for the class or one for each group)
- power supplies (enough for the electrophoresis chambers you are using)
- DNA samples:
  - option 1: have students load loading dye into the wells, but have loading dye in different microcentrifuge tubes labeled as "East coast virus," "West coast virus," and "Midwest virus." Use this option if you don't have enough class time for letting the gel fully run and stain. If you are using this option, print out copies of one of the gel images below (1 per student) so students can analyze the results
  - option 2: purchase a virus DNA fingerprinting kit through a biology education supplier that has a quick-stain approach. You may wish to include in this option a DNA ladder.
- sterile micropipette tips to dispense 20 µL
- micropipettes to dispense 20 µL
- rulers

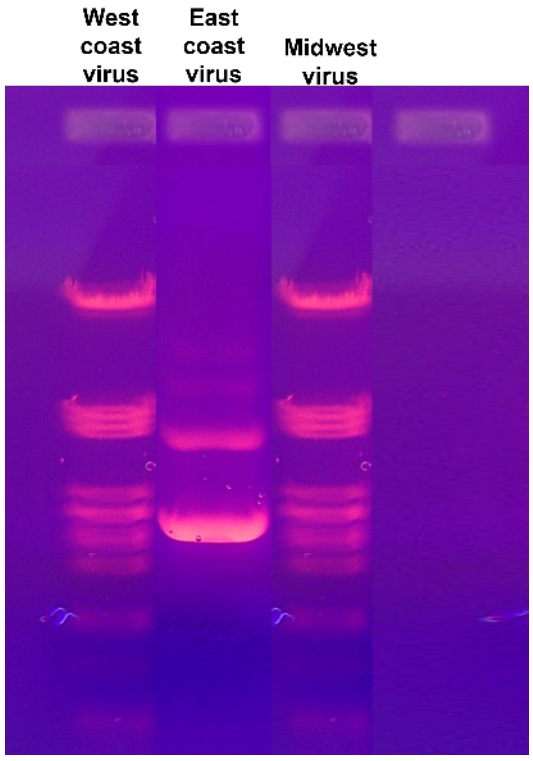
Gel Result Printouts

Choose one of the gel images below if you are using option 1 described above. Print out enough of the gel of your choice for each student to have one printout.

Midwest Virus is the East Coast Virus



Midwest Virus is the West Coast Virus



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- Gel electrophoresis 2.jpg by Mnolf is licensed under GNU Free Documentation License

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3.29: Bacterial Transformation

Materials

- bacterial transformation kit(s) (enough for entire class) - examples of kits are linked below
  - Ward's Bacterial Transformation with GFP
  - Edvotek Transformation with GFP
  - Green Gene Transformation
  - Exploring Gene Expression through Transformation
  - pGLO Bacterial Transformation
- bacterial transformation protocol handouts (1 per student)
- additional materials may be required by the kit you choose

Preparation

- follow the specific preparation instructions in the bacterial transformation kit you choose

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3.30: Protozoan Parasites

Materials

- prepared microscope slides of *Plasmodium* sp. (enough for the class to share)
- prepared microscope slides of *Trypanosoma cruzi* (enough for the class to share)
- prepared microscope slides of *Trichomonas vaginalis* (enough for the class to share)
- (if using 1000x) immersion oil (enough for the class to share)
- light microscopes (1 per student or 1 per pair of students)
- colored pencils

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3.31: Helminth Parasites

Materials

- prepared microscope slides of *Enterobius vermicularis* eggs and adult worms (enough for the class to share)
- prepared microscope slides of *Dipylidium caninum* eggs and adult worm compsite (enough for the class to share)
- prepared microscope slides of *Schistosoma* sp. eggs and adult worms (enough for the class to share)
- (if using 1000x) immersion oil (enough for the class to share)
- light microscopes (1 per student or 1 per pair of students)
- colored pencils

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3.32: Fungal Parasites

Materials

- *Aspergillus* slides (1 per student or 1 per pair of students)
- microscopes (1 per student or 1 per pair of students)
- colored pencils

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3.33: Viral Epidemic

Materials

- Epidemic Simulation Kit

Setup Instructions

- Follow setup instructions in the kit.

Activity Instructions

- There will need to be an even number of people in the "population," so if your class has an odd number of students, the instructor can join the simulation to make the numbers even.
- Watch students carefully when they add their sample into the well plates to make sure they are putting them in the correct locations.
- Use a random group generator to randomize student pairs (otherwise, groups of students who are in the same social circle will end up sharing their drops with each other and you will be less likely to get good results). You can find a free random group generator at ClassTools.Net

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3.34: Virus Bioassay

Materials

- plant grow lights
- tomato or bean seeds
- rolling tobacco (3 different brands mixed together) (1 gram per group)
- Tobacco Mosaic Virus Inoculation Kit (1 per class)*
- 50 mL beakers (1 per group)
- mortar and pestle (enough for groups to share)
- balances (enough for groups to share)
- weigh papers/boats (1 per group)

> **Note**
>
> *I have found the beans they provide in this kit difficult to grow. Unless you are good at growing these types of beans, recommend to use seed packets for tomato or bean plants and follow the instructions on the packet.

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3.35: Control of Microbial Growth

Preparation

- prepare 3 TSA petri plates per student
- spreadsheet with formulas preprared to calculate t-test results for the class results

Materials

- 3 TSA petri plates per student
- sterile cotton swabs (3 per student)
- sterile distilled or DI water in beakers, containers or cups (1 per group)
- soap in squeeze bottles (1 per group)
- disinfectant spray (1 per group)
- markers
- graph paper and rulers or computers for graphing (1 per student)
- spreadsheet prepared to calculate t-test results for the class results

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3.36: Bacterial Susceptibility to Antibiotics (Kirby-Bauer Test)

Preparation

- TSA petri plates (enough for 3 per group)
- 18-24 hour TSB cultures of *Staphylococcus aureus* (one per group or enough for groups to share)

Materials

- TSA petri plates (enough for 3 per group)
- 18-24 hour TSB cultures of *Staphylococcus aureus* (one per group or enough for groups to share)
- test tube racks (1 per group)
- Bunsen burners (1 per group)
- strikers (1 per group)
- sterile cotton swabs (3 per group)
- waste containers with disinfectant for used cotton swabs (1 per group)
- tweezers (1 per antibiotic type - label tweezers so they do not get mixed up)
- antibiotic disks (one of each type per group):
  - erythromycin (15 µg)
  - penicillin G (10 units)
  - streptomycin (10 µg)
  - tetracycline (30 µg)

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3.37: Human Microbiome

Preparation

- 2 TSA plates per student

Materials

- 2 TSA plates per student
- sterile cotton swabs (2 per student)
- DI or distilled water
- markers
- materials for Gram staining
  - plain microscope slides (at least 1 per group)
  - Bunsen burners (1 per group)
  - inoculating loops (1 per group)
  - droppers with saline, DI water, or distilled water
  - slide warmer
  - wood test tube clamps/holders (1 per group)
  - small chemical waste containers (1 per group)
  - crystal violet in a dropper bottle (1 per group or enough for two groups to share one bottle)
  - Gram's iodine in a dropper bottle (1 per group or enough for two groups to share one bottle)
  - 95% ethanol in a dropper bottle (1 per group or enough for two groups to share one bottle)
  - safranin in a dropper bottle (1 per group or enough for two groups to share one bottle)
  - distilled or DI water in squirt bottles (1 per group)
  - bibulous paper (enough for each groups to blot their slides)
  - light microscopes (1 per group)
  - (optional, but recommended) Wax pencils (1 per group)
  - (if using oil immersion) immersion oil dropper bottles
  - (if using oil immersion) lens paper

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3.38: Unknown Bacteria Identification Project

Preparation

- 24 hour TSB cultures, each with one Gram-negative species listed on the project flow chart and one Gram-positive species listed on the project flow chart (enough for one per pair of students)
- See Instructor Setup sections for the following chapters:
  - Plating on Petri Plates for Isolation
  - Gram Stain
  - Starch Hydrolysis
  - SIM Deep Tests
  - Cytochrome c Oxidase
  - Coagulase Test
  - Fermentation Test
- An assignment in your learning management system (such as Canvas) where students can upload their project reports and have them checked with TurnItIn. Be sure to include your grading rubric in TurnItIn and Canvas.

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