WPUNJ Microbiology Laboratory Manual

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Contents

1. Microscopy

A basic definition of a microorganism is a living organism that cannot be seen with the naked eye but can be seen through a microscope. Microorganisms include bacteria, viruses, protists (algae and protozoa), fungus, and even some helminths. In this course, microscopes will be used primarily to view bacteria. Microorganisms are everywhere, and can be considered the good, the bad, and the ugly. There are not many that fall into the category the bad.

There are different types of microscopes based on how the microscope produces the image. Some microscopes use light whereas others use a beam of electrons. Light microscopes can use visible light or fluorescent light. Which type of microscope that is used depends on the goal of the scientist. There are different types of light microscopes: brightfield, darkfield, and phase contrast. The students in this course will only be using brightfield, parfocal, compound microscopes.

CARE OF THE LIGHT MICROSCOPE

It is important to treat the microscopes with care with every use. This way each microscope is prepared and ready for the next user without the risk of damage.

Transport: The microscope should be carried with two hands. One hand should grasp the arm of the microscope while the other should be under the base for support.

Electric Cord: 1) The power cord unplugs from the back of the base of the scope. Make sure it is plugged in in order to have light. 2) Keep excess cord off the lab bench for safety. 3) When returning the microscope to the cabinet, hang the cord around the ocular lenses.

Lens Cleaning: Clean the ocular and objective lenses with lens paper. Not Kim wipes or paper towels. These items can scratch the lenses. Clean the lenses before viewing your specimen.

The last thing you do before putting your microscope away is cleaning the lenses. Leaving oil on the objectives can limit the resolution thus making it unable to view bacterial specimens. Depending on your lab instructor, you may be instructed to use Windex. This helps cut the oil on the lenses and clean the lenses better. But it can leave residue on the lens if not completely remove with lens paper. The condenser lens may also need to be cleaned with lens paper due to too much oil being used.

COMPONENTS OF THE LIGHT MICROSCOPE

The following are the general parts and functions of the typical compound microscope (Figure 1.1):

Framework: This is the basic frame structure that consists of the arm and the base. All other parts are attached to this framework. The **arm** supports the tube that connects the eyepiece to the objective lenses. The **base** is the bottom of the microscope that supports the whole microscope.

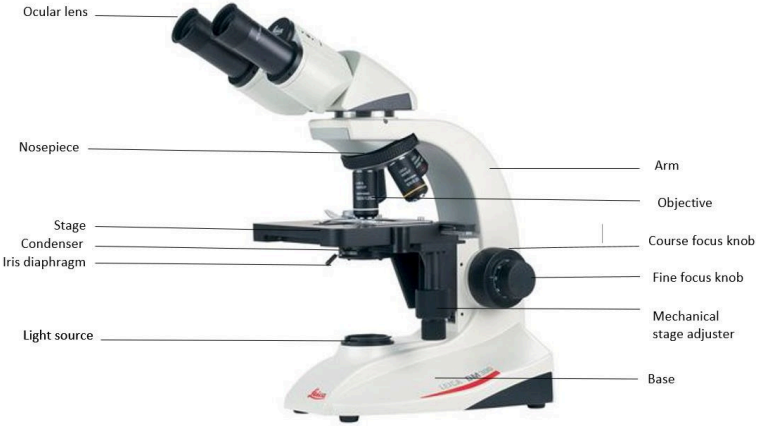


Figure 1.1: Components of a typical compound microscope.

Stage: The flat platform that supports the microscope slides is the **stage**. **Stage clips** hold the slide so that the **mechanical stage adjuster** (which has two knobs) can move the slide. One knob moves the slide left and right and the other up and down.

Light source: A lamp is in the base of the microscope that

serves the purpose of a **light source** to illuminate the specimen. The intensity of the light can be adjusted by rotating the knob/ wheel on the base. (The power switch for the light source is on the back on the scope at the base of the arm.)

Lens system: Compound microscopes are three lens systems: the oculars, the objectives, and the condenser. The **ocular lenses**, or eyepieces, are the lenses that are located at the top of the microscope closest to the eyes. They usually have a magnification of 10x, and most microscopes have two ocular lenses. This is known as binocular. The **objective lenses** are the lenses closest to the object which you are trying to view. In most microscopes, there are 4 objectives. The objectives are attached to the nosepiece in order to be able to change which objective you are using. Most commonly they have the magnification of 4x, 10x, 40x, and 100x, but we have some scopes that have 50x objectives. 4x is known as a scanning objective, 10x is a low power objective, 40x is a high dry objective, and 50x and 100x are both oil immersion objectives. The **condenser lens** is located under the stage. It collects and focuses the light from the light source through the slide being viewed. It does not have a magnifying power like the ocular and objective lenses. The condenser can be moved up and down by a knob, but its best position is just a below the highest point, a few mm below the slide. How much light the condenser transmits is controlled by the **iris diaphragm**. It is an adjustable disc with a hole in the center. This disc is within the condenser and controlled by a lever or knob outside of the condenser.

Focusing Knobs: There are two concentric focusing knobs on each side of the microscope: course adjustment knob and fine adjustment knob. The large outer knob is the **course adjustment knob**. It moves the stage up and down visibly, bringing the slide closer and farther from the objective. The smaller inner knob is the **fine adjustment knob**. The course adjustment knob is only used at the lowest magnification. The

smaller inner knob is used to bring the specimen into sharp focus.

THEORY

There are two concepts of microscopy: magnification and resolution. **Magnification** is simply the ability to make small objects appear larger whereas **resolution** is the ability to distinguish two objects from each other. An easier definition of resolution would be the clarity of an image, or the ability to show detail.

Let's take a closer look into the concept of magnification. In a compound microscope, the pathway of light is through the three-lens system of the condenser, objective, and the ocular. The condenser focuses the light onto the specimen. The light refracts, or bends, as it passes through the objective and the ocular lenses. The brain interprets what the eye is seeing through the ocular lens. (Figure 1.2.) The objective and ocular lens each have differing magnifying power. The determination of the total magnification of the specimen is by multiplying the magnification of the ocular and the magnification of the objective being used.

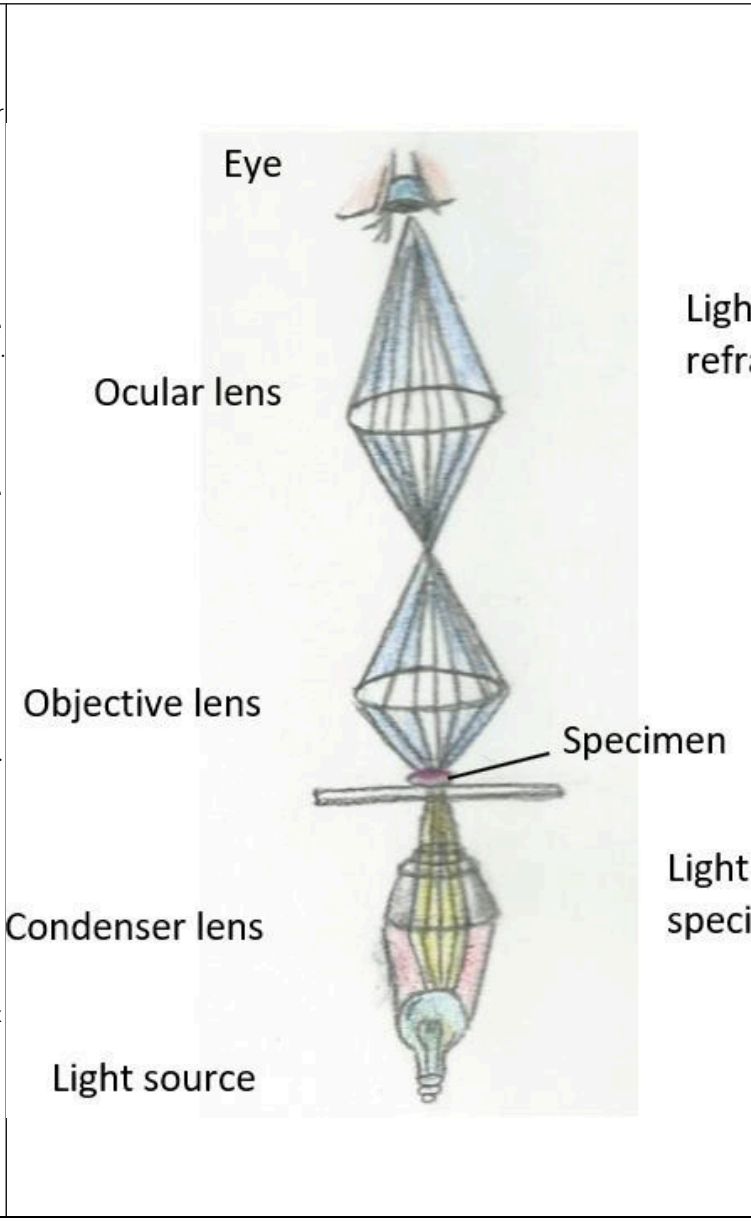


Figure 1.2: This drawing shows how the condenser focuses the light onto the specimen. After which, light is refracted by the objective lens, increasing the magnification. From there, light is then refracted by the ocular lens, increasing the magnification of the specimen.

Note: One effect of this arrangement is that the virtual image is effectively a meter or so from your eye. If you find yourself tiring your eyes, you may be trying to use your eye muscles to focus the image. The appropriate use of your eyes is to relax and look into the middle distance. Pretend you are watching TV and focus out past the microscope.

As magnification increases, there is a greater need for higher

resolution, making a sharper image. Two factors affect resolution: the wavelength of light and numerical aperture. The shorter wavelengths of light result in higher resolution. **Numerical aperture** is a property of a lens. It is the measure of the lens's ability to gather light. The higher the numerical aperture of a lens, the higher resolution of a specimen with that lens. Now let's tie those two factors mathematically together as resolving power, which measures the ability of a lens systems to resolve detail and is defined as the smallest distance between two points that can still be distinguished as two separate entities. The resolving power can be calculated with the following equation:

$$R = \frac{0.61\lambda}{NA}$$

R = resolving power, λ = wavelength of light being used, and NA = numerical aperture of the objective being used

Numerical aperture is a number written on the objective next to the magnifying power. It can be 0.4 (low power) to 1.25 (oil immersion). All in all, for any brightfield light microscope, by using the above equation, the limit of resolution is about 0.2 µm. This means that 2 objects closer than 0.2 µm would not be seen as 2 different objects.

To improve resolution by maximizing the numerical aperture and reducing light refraction, the use of immersion oil is done on the 50x and 100x objectives. (Figure 1.3)

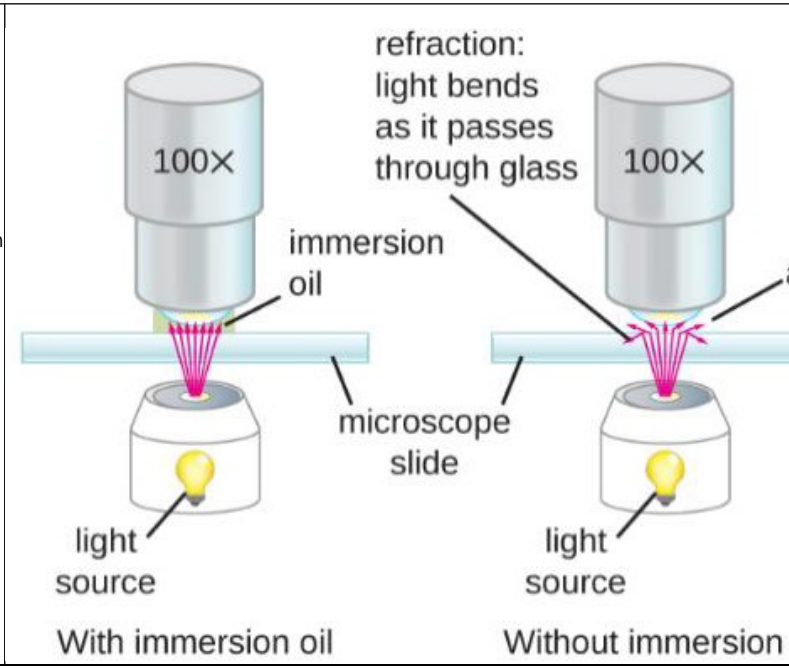


Figure 1.3: Immersion oil prevents the loss of light rays, which improves resolution. (Image from Openstax)

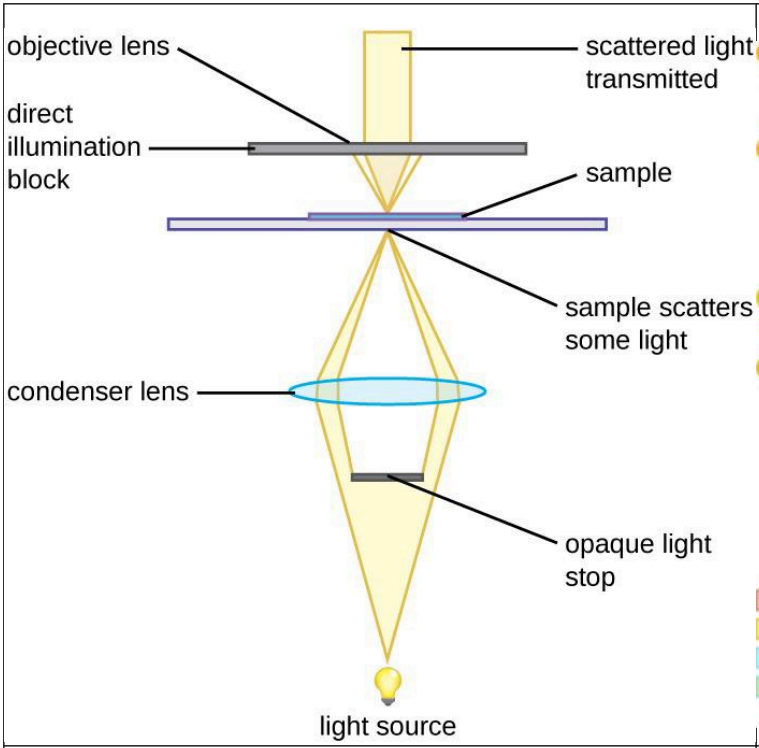
DEMONSTRATION OF OTHER LIGHT MICROSCOPES

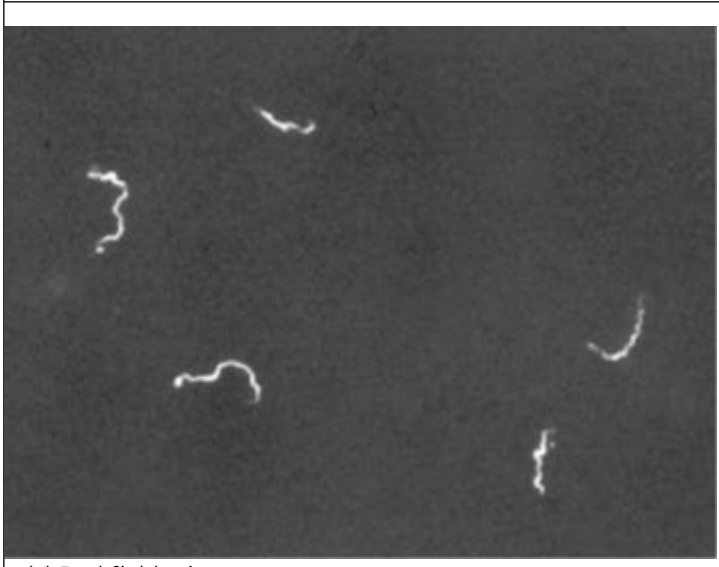
As previously stated, in this course, brightfield microscopes are going to be used. It allows light rays to pass directly to the eye without being deflected and transmitted through a specimen. In general, in order to view the specimen, a process called staining often needs to be done. It increases the contrast between the cell and its surroundings. However, a disadvantage is that staining results in cell death, which restricts the ability to observe living cells and their activities. You will be learning several staining techniques through the term.

Other types of light microscopes are darkfield and phase contrast. To achieve the darkfield effect, special condenser is used that contains an opaque disk that allows only oblique rays to strike the specimen. The result is a brightly lit, transparent specimen against a black background (Figure 1.4 a). Phase

contrast microscopes enhance the contrast between a cell and its surrounding by shifting the light that does not interact with the specimen down a ¼ wavelength. This is called a phase shift. (Figure 1.4 b). How this is done is by objective and condenser containing phase rings. The result is a darker background compared to the specimen. (Figure 1.4 c). Living cells can be seen in action in both darkfield and phase contrast microscopes.

There may be special microscopes available in the lab that can compare brightfield, darkfield, and phase contrast microscopy using a water sample.





(a) Darkfield microscopy

(b) Phase co

Figure 1.4: (a) This diagram depicts how the condenser in a darkfield microscopes has an opaque disk that only allows oblique light rays to strike the specimen, resulting in transparent, illuminated specimens. (b) The illustration shows how the annular ring below the condenser and the phase plate in the objective cause a phase shift of light. (c) The photo on the left is a simple squamous epithelial cell in brightfield whereas the photo on the right is the same cell in phase contrast. More details can be seen using phase contrast. (Images from Openstax.)

MATERIALS

Each student should have:

Microscope
Blue rack with immersion oil
Lens paper
Windex (depends on instructor)
Prepared slides. Slide used to teach how to focus on the microscope that your instructor chooses.

PROCEDURE

1. Carefully carry your microscope to your lab bench.
2. Plug the scope into an outlet and turn it on by pressing the power switch to the “on” position on the back on the microscope. If no light comes on, make sure the power cord is pressed all the way into the microscope next to the power switch. Also, check the light intensity wheel/ knob at the base of the microscope, and increase it so that you can visibly see the light with the naked eye. Don’t go to full intensity, that is for the highest magnifications.
3. Adjust your ocular lenses to fit your interpupillary distance. This is simply the distance between your eyes to be able to see through both oculars as one field of view. With your eyes a few cm from the oculars, you can align the dots of light.

4. Place a slide on the stage, held by the stage clips. Use the mechanical stage adjusters to position the slide so the beam of light is centered on the stained material. (You can use the beam of light to find the specimen on the slide.)
5. Open the iris diaphragm halfway and adjust the condenser to half a cm below the slide.
6. Rotate the nosepiece so that the lowest power objective (4x) is down.
7. Using the coarse adjustment knob, raise the stage all the way to the highest position, close to the objective.
8. Looking through the ocular lens, lower the stage slowly with the coarse adjustment knob until you see your specimen. It will be blurry at first.
9. Use the fine adjustment knob to bring the bacterial cell into sharp focus.
10. Adjust the iris diaphragm to narrow the beam and sharpen the focus, but don't narrow it so much that you lose the color and begin to see halos on objects.
11. After focusing on the bacterial cells, you can increase the magnification by rotating the nosepiece to the 10x objective. With the 4x objective bacteria are just a sand-paper texture, not individual objects, with the 10x, slightly larger.
    a. Most microscopes are **parfocal**. This means that the image will remain in focus when changing from a lower power objective to a higher power objective.
    b. Only minimal focusing should be necessary with the fine focus adjustment. Do not touch the coarse adjustment knob.
12. The next two objectives, 50x and 100x, will need the help of immersion oil in order to improve resolution.
    a. Rotate the objectives half way before getting to the 50x (not all instructors use the 50x so you can go directly to 100x objective). Place a drop of oil directly on the slide, and rotate the immersion oil objective into the oil.

b. If viewed and focused through the 50x, you can move onto the 100x objective. You may or may not need to add more immersion oil.

13. Once the specimen is in sharp focus, use the mechanical stage adjusters to explore the slide and find a field of view where the cells are not over-crowded and their shape and size can be easily seen. Show this to your instructor.
14. To remove the slide, first rotate the nose-piece so that the objective is away from the slide. *If you don't do this, you may scrape and damage the lens*.
15. After learning how to focus your first slide, now take the time to view the other 4 prepared slides on your own. Make sure to show your instructor at the total magnification of 1000x to illustrate your microscope skills. Viewing the other 4 slides is also exposing you to the 3 basic bacterial cell shapes: **coccus** (circle), **bacillus** (rod), and **spirillum** (spiral).
16. When you are finished using the microscope, clean off the immersion oil from the lenses on the microscope using lens paper and clean the oil from the prepared slides using Kim wipes.
17. Return the microscope to the cabinet with the electric cord looped over the ocular lenses.
18. Return all items to their location and place the prepared slides to properly labeled tray on the side bench.

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2. Aseptic Technique

Microbes exist ubiquitously in all types of environments: ponds, ocean, soil, air, and they are even in and on humans. Some types of microbes, called Archaea, thrive in extreme environments such as hot springs, volcanoes, and in the bottom of the ocean in white smoker hydrothermal vents. Microbes are on objects that you are in contact with in daily life. In all of these environments, it is not one microbe but several to hundreds living together. But, when we study microbial species in the lab, we rely on **pure culture** to understand the biology of the organism. Pure culture is created by inoculating media with a single strain with negligible genetic diversity. If a **mixed culture** (more than one bacterial species in one medium) or contamination occurs, you would not know which bacterial cell is doing what metabolically.

In order to work with pure culture in scientific labs, aseptic technique, or sterile technique, is used for various procedures such as transferring microbes (this is also known as **inoculation**), isolation of pure culture, and for performing metabolic tests. The principle is to prevent contamination as you work with microbes: before, during, and after. There are steps to ensure not to introduce microbes from the work area, the tools that you use, and the surrounding air into the culture of bacteria that you are using, and steps to ensure that you do not leave any microbes when you are finished.

You will be provided with pure cultures of bacteria and want to avoid any contamination. Possible contaminants can be from the lab bench, the air, the inoculating loop, and even yourself. Keeping bacterial cultures pure is key because this allows for the strain to be properly characterized.

MATERIALS

Each student should have:

Blue rack
2 sterile TSA slant (Trypticase Soy Agar)
1 sterile TSB tube (Trypticase Soy Broth)
3 test tube labels
Inoculating loop
Bunsen Burner
Striker
1 culture of either *Escherichia coli* or *Staphylococcus epidermidis*

PROCEDURE

GENERAL INFORMATION:

- Ethanol the lab bench prior to and after any laboratory work.
- Always carry the blue rack to collect your culture tubes and any other tubes. Caps are not sealed on. They can easily slide off. Always carry the tubes by the glass portion and not the caps.
- How to use the Bunsen Burner:
    - Attach the rubber hose of the Bunsen Burner to the gas jet, and then turn the handle in-line with the hose and jet. This is the “on” position.
    - Use the striker to light the Bunsen Burner.
    - Adjust the Bunsen Burner flame by altering the horizontal knob at the base of the burner until you see an inner and outer blue flame. The hottest part of the flame is the topmost section of the inner flame. This is where inoculating loops and needles should be sterilized. (Figure 2.1)
    - Bunsen Burners must be TURNED OFF WHEN NOT IN USE.
    - When finished using the Bunsen Burner for the day, turn off the gas, and make sure ALL FOUR gas jets handles are turned in the same direction to prevent any gas leaks.

- Do not place any test tube cap down on the lab bench or the blue rack. Putting it down raises the chance for contamination.

Figure 2.1 Adjust the Bunsen Burner until there is an inner and outer blue flame. At the top of the inner blue flame is the hottest portion of the flame which is to be used to sterilize the inoculating loop and needle

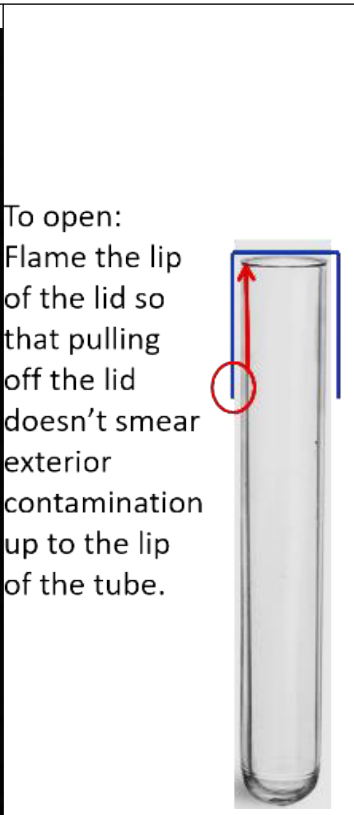

Figure 2.2 With repeated remova
important to keep the part of the
alternative method is to flame th
opening and closing.

HOW TO INOCULATE:

Slant to Broth:

1. Before beginning, unscrew the slant but leave the cap loosely on the tube. This will make it easier during the transferring process.
2. Flame the inoculating loop. (Slide the bright orange/ yellow zone slowly along the wire.)
3. Remove the cap from the slant of bacterial culture. **Do not set the lid down.** Hold it with the hand that is holding the loop (in hook of your little finger).
4. Insert the inoculating loop into the bacterial culture slant, and carefully collect a loop of bacterial culture from the surface of the slant agar.
5. Flame the opening of the tube and recap the tube. (Keep an eye on the loop with the bacteria.)
6. Now flame and remove the cap from the sterile broth. **Do not set the cap down.**
7. Insert the loopful of bacteria into the sterile broth.
8. Remove the inoculating loop, flame the opening of the tube, and recap the tube.
9. Flame the inoculating loop to sterilize it before you set it down.

Slant to Slant:

1. Before beginning, unscrew the slant but leave the cap loosely on the tube. This will make it easier during the transferring process.
2. Flame the inoculating loop.
3. Remove the cap from the slant of bacterial culture. **Do not set the lid down.** Hold it with the hand that is holding the loop (in hook of your little finger).
4. Insert the inoculating loop into the slant of bacterial culture, and carefully remove a loop of bacteria from the tube.

5. Flame the opening of the tube and recap the tube.
6. Flame the lip of the slant-tube lid.
7. Now remove the loosened screw cap from the slant, by grasping with little finger and twisting the tube with the tube-hand. **Do not set the lid down.** (Hook it with the little finger.)
8. Keep the tube tilted and insert the loopful of bacteria but travel along the top of the tube opposite of the agar until you reach the bottom of the slant (not the tube).
9. Gently touch the loop onto the surface of the agar slant and move the loop slowly as you withdraw from the tube. Be careful not to gouge the agar.
10. Flame the opening of the tube and recap the tube by screwing the cap back on.
11. Flame the inoculating loop to sterilize it.

Control:

The last sterile slant serves as a control for the aseptic technique. You are testing to see if you are flaming the inoculating loop efficiently. There is no bacterial culture use for this tube.

1. Unscrew the slant but leave the cap loose on the tube.
2. Flame the inoculating loop.
3. Remove the cap from the sterile tube and flame the opening of the tube.
4. Insert the inoculating loop into the sterile tube and gently touch the loop onto the surface of the agar slant and move the loop slowly as you withdraw from the tube. Be careful not to gouge the agar.
5. Flame the opening of the tube and recap the tube.
6. Flame the inoculating loop to sterilize it.

Store all tubes in the red racks in your lab section's bench area at room temperature.

One or more interactive elements has been excluded from this version of the text. You can view them online here: https://press.wpunj.edu/microbiologylabmanual/?p=80#oembed-1

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3. Streak Plate Method

You have learned that microbes are found everywhere and coexist with each other in mixed populations as well as with larger organisms such as you. To observe and study bacteria, you have to isolate individual bacterial species into pure cultures. A **pure culture** contains only a single kind of species whereas a **mixed culture** contains more than one species in one medium. (Figure 3.1). A **contaminated culture** contains the desired species but also unwanted species. In a contaminated culture it is hard to tell which bacteria are performing which function. These contaminants can outgrow the bacteria you are interested in studying.

Several methods of achieving pure culturing are used in the microbiology lab. Two commonly used ones in our lab are the streak plate and the pour plate methods. These involve diluting bacterial cells in a sample down to an end point of a single genotype dividing and giving rise to a single pure colony. A **colony** is a collection of identical cells of the same bacterial strain, growing on or within a solid medium. A streak plate is the most economical in time and materials: one medium plate and a quick inoculation. However, streaking is a procedure that takes practice to reliably large numbers of well-separated, individual colonies. For the **streak plate** method, one loopful of bacterial culture is diluted by streaking it onto the surface of an agar plate, making sure the cells are spread out. The pour plate method consumes more time and more materials. A standard **pour plate** method is a serial dilution of one loopful of a bacterial culture being diluted in a series of tubes of liquefied agar. After the dilution step is finished, each liquefied agar tube is poured into a sterile empty petri plate. Bacterial colonies will grow on and within the agar after it solidifies. These other methods have the advantage of quantifying the original

culture density. For this exercise, you will do the streak plate. There are several methods of performing the streak plate. Each student will do 2 T streak method plates.

MATERIALS

Each student should have:

Blue rack

4 sterile TSA plates (Trypticase Soy Agar)

Inoculating loop

Wax pencil

Bunsen Burner

Striker

1 culture of *Staphylococcus epidermidis*

1 culture of *Escherichia coli*

Figure 3.1: Streak plate method can create isolated colonies needed for a pure culture. Dark colony (A) can be streaked on another plate to produce pure culture without the light-colored contaminant (B).

PROCEDURE

T Streak: (Figure 3.2)

1. Label the bottom of the TSA plate along the edge of the plate with your wax pencil: your name, bacteria name, and date.

2. Create a large “T” along the whole bottom of the plate. This divides the plate into three sections: 1, 2, and 3.
3. Using aseptic technique, transfer one loopful of bacteria onto section 1 (above the “T”) by streaking onto the surface of the agar plate.
4. Flame the loop, and then stab into a sterile area of the plate to cool.
5. Streak from section 1 into section 2. Do not backtrack.
6. Flame the loop, and then stab into a sterile area of the plate to cool.
7. Streak from section 2 into section 3. Do not backtrack.
8. Flame the loop to sterilize when finished.
9. Store plates upside down in your lab section’s rack at room temperature.

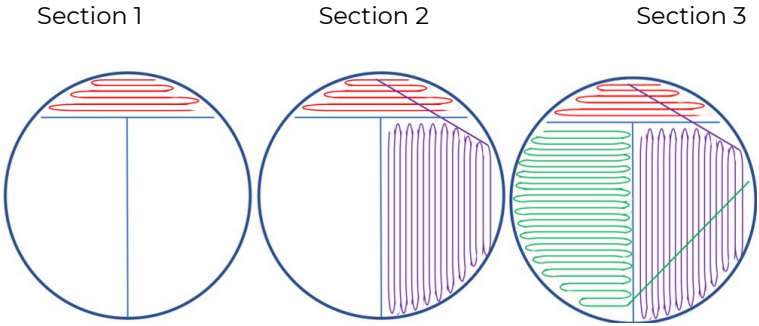

Figure 3.2: T streak method is literally drawing the letter “T” on the bottom of the plate to make it clear where to drag the loop. Use a single loopful of bacteria in section 1 and dilute down into the section following. Make sure to flame the inoculating loop between each streak. It helps to rotate the petri plate counterclockwise while you are doing the streak plate method. You can improve your ability to get sufficient bacteria into a new section by pulling bacteria from the first stroke in the previous section. Pull across your previous strokes by using the

light reflecting from the agar surface to visualize your previous strokes.

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4. Smear Preparation & Simple Stain

As we discussed in learning about brightfield microscopy, the cells must be stained to be viewed. Otherwise, they would be clear against a bright light, and you would not be able to see them. Staining allows for contrast, and allows the bacteria to be studied with respect for its cell shape, size, and arrangement.

The **smear preparation** is what causes the cells to adhere to the microscope slide so that they are not washed off during the staining process. Heat needs to be applied in order for this adherence to occur. It is this heat that causes cell death during the staining process. A goal in preparing a smear is a thin smear because the thickness can affect several factors. It can determine if you can view individual cells that aren't stacked on each other. A proper smear can show cell shape, the arrangement of the cells, or any other details regarding the microstructures associated with the cells. Overly thick smears also can affect the staining process by entrapping the stain, keeping it from being removed through the process of decolorizing or rinsing, leading to erroneous results.

Once the smear preparation is finished, it is time to stain. A common staining technique to observe cell shape, arrangement and size is a **simple stain** using only one stain to colorize the bacteria. Simple stain most often uses a basic stain. A **basic stain** is a stain that contains color-bearing ions called **chromophores** that are positively charged. Bacteria themselves have a slightly negative surface. Owing to opposite charges attracting one another, the stain binds to the bacterial cell, causing the bacteria to be colored against a transparent background. Another name for the basic stain is **positive stain** due to the chromophores having the cationic charge.

MATERIALS

Each student should have:

Blue rack
2 glass slides
Stain bottle rack: Methylene blue (or crystal violet)
Lens paper
Windex (depends on instructor)
Inoculating loop
Wax pencil
Metal slide clip
Bunsen Burner
Striker
Stain tray
1 culture of either *Escherichia coli* or *Staphylococcus epidermidis*
Microscope

SMEAR PREPARATION PROCEDURE

From a Slant culture:

MAKE 2 SMEAR PREPS. ONE WILL BE USED AS A BACKUP.

1. Draw a circle with the wax pencil on a slide.
2. Flip the slide over. Write “Up”.
3. Place a small drop of distilled water onto the slide within the wax circle.
4. Using aseptic technique, remove a small amount of bacteria from a slant or an isolated colony on a streak plate, and transfer onto the slide, spreading within the wax circle. (Make sure not to take too much bacteria. You want thin smears.)
5. Air dry the slide. Do not apply heat to a wet slide. Do not blow on the slide.
6. After all water has evaporated and smear is completely dry, place the slide in a metal slide clip and pass it multiple times through the Bunsen Burner flame as demonstrated

by your instructor. This is called **heat-fixing** a smear. It adheres the bacterial cells to the slide. Avoid prolonged heating of the slide as it can distort the cell shape and size.

PROCEDURE OF SIMPLE STAIN

STAIN ONLY ONE SLIDE. You may not need the second slide. This serves as a backup in case you have error in your staining procedure. It saves you time.

1. Place one heat-fixed smear prep on a stain tray.
2. Stain slide with methylene blue or crystal violet for 60 seconds.
3. Rinse slide with distilled water until clear.
4. Blot dry with a paper towel.
5. Examine under the microscope and show your instructor at 1000x magnification, oil immersion.
6. After your instructor's approval of your staining technique, dispose of your slides in the plastic beaker labeled "Self prepped" in the Discard Area.

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5. Negative Stain

Most the stains we use are basic stains with positively charged chromophores that colorize the bacteria. **Acidic stains** with negatively charged chromophores are also used to provide contrast to view bacteria. The anionic charged chromophores are repelled by the similarly negatively charged bacterial cells, resulting in transparent cells and a dark background. Acidic stains are more commonly named **negative stains**. Fewer negative stains exists. These are nigrosin stain, india ink, and Congo red.

An advantage of using negative staining is that heat fixation is not done, which allows more accurate determination of cell shape and size. It can also be used to see special structures, such as capsules, and is useful in observing spirochetes due to being very thin and small.

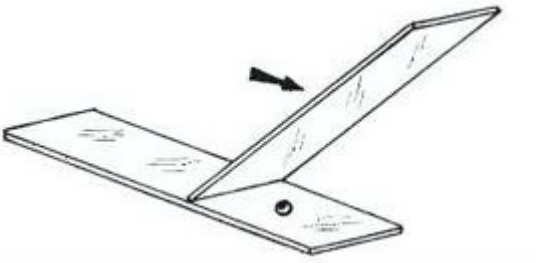
MATERIALS

Each student should have:

Blue rack
2 glass slides
Nigrosin stain
Lens paper
Windex (depends on instructor)
Inoculating loop
Bunsen Burner
Striker
1 culture of either *Escherichia coli* or *Staphylococcus epidermidis*
Microscope

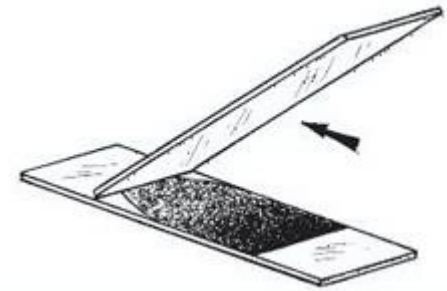
PROCEDURE OF NEGATIVE STAINING

1. Place a small drop of Nigrosin stain of the end of the slide.
2. Using aseptic technique, transfer one loopful of bacteria into the drop of stain. Do not spread the stain and bacteria, but contain it as a drop at the end of the slide.



3. Place the edge of the second slide (the spreading slide) into the drop of bacteria and stain until it spreads along the entire edge.

4. Push the spreading slide along the bottom slide, dragging the stain and bacteria evenly.



5. Air dry.
6. Examine under the microscope, and show your instructor at 1000x magnification.
7. After your instructor's approval of your staining technique, dispose of your slides in the plastic beaker labeled "Self prepped" in the Discard Area.

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6. Gram Stain

Differential staining techniques provide additional information on bacterial strains that a simple stain cannot. Differential staining uses two stains sequentially. A standard process uses **primary stain, mordant, decolorizer, and counterstain**. The **mordant** helps ensure that the primary stain remains bound to the positive bacteria. The **decolorizer** removes the primary stain from the negative bacteria. Differential staining distinguishes bacteria according to whether they can retain the first, or **primary stain** during the decolorization stage. This is informative because the ability to retain the primary stain is based on structural differences. The result for a bacterial strain may be **positive** for cells retaining the primary stain. Or the result may be **negative** for cells that lose the primary stain and are visible only with the secondary or **counterstain**.

The most important, and most widely used, differential stain is the Gram stain. With a few exceptions, the most bacteria are divided into the categories Gram positive or Gram negative. This staining technique was developed by the physician Hans Christian Gram in 1884 while working with infected lung tissue.

The structure of the cell envelope of the bacterial cell is what determines whether the bacteria cell is gram positive or gram negative. The major component is the thickness of the peptidoglycan in the cell wall. **Gram positive** bacteria have a thicker layer whereas **Gram negative** bacteria have a much thinner layer of the peptidoglycan. (Figure 6.1) The large number of layers of peptidoglycan in a Gram-positive cell wall are permeable because of water between the layers. The ethanol used for decolorization draws the water out, trapping the stain. Gram positives also have teichoic and lipoteichoic acids in the cell wall, which stabilizes the primary stain with the

mordant. This tight cell wall in the presence of the decolorizer causes the primary stain to remain. The outer membrane also plays a role in gram negative bacteria. This covers the thinner layer of peptidoglycan. It is made up of lipids, lipopolysaccharides and lipoproteins. During the decolorization step, the ethanol solubilizes these hydrophobic compounds and the bacteria to become more porous. This allows the primary stain to leach from gram negative bacteria, and they become transparent. The counterstain makes them visible.

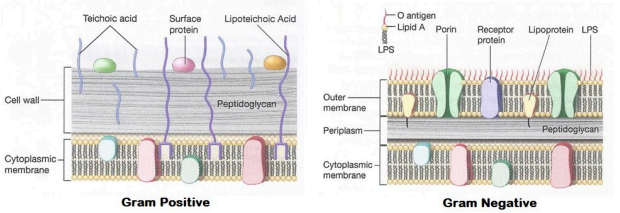


Figure 6.1 The difference between cell envelope structure of Gram positive and Gram negative bacteria. Image from https://laboratoryinfo.com/gram-staining-principle-procedure-interpretation-and-animation/

MATERIALS

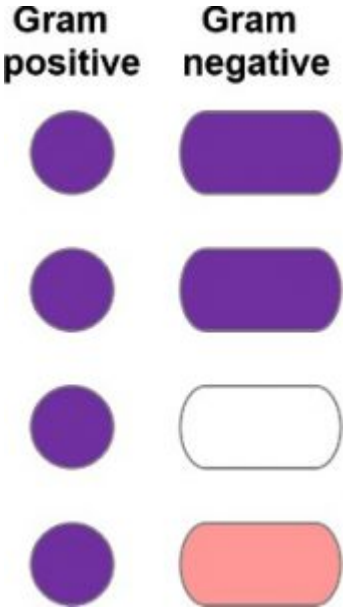
Each student should have:

Blue rack
2 glass slides
Stain bottle rack: crystal violet, Gram's iodine, 95% ethanol, and safranin
Lens paper
Windex (depends on instructor)
Inoculating loop
Wax pencil
Metal slide clip
Bunsen Burner and Striker
1 culture of *Escherichia coli*
1 culture of *Staphylococcusepidermidis*
Microscope

PROCEDURE OF SMEAR PREPARATION
MAKE 2 SMEAR PREPS. ONE WILL BE USED AS A BACKUP.

1. Draw a circle with the wax pencil on a slide.
2. Flip the slide over. Write “Up”.
3. Add one drop of distilled water within the target circle.
4. Using aseptic technique, transfer one loopful of <u>each</u> bacterial species onto a single location on the slide, mix and spread the 2 species of bacteria within the wax circle.
5. Air dry the slide. Do not apply heat to a wet slide. Do not blow on the slide.
6. Heat fix the slide.

PROCEDURE OF GRAM STAIN



STAIN ONLY ONE SLIDE. You may not need the second slide.

This serves as a backup in case you have error in your staining procedure. It saves you time.

1. Place one smear prep on a stain tray.
2. Stain slide with the primary stain **crystal violet** for 60 seconds.
3. Rinse slide with distilled water until clear.
4. Add the mordant **Gram's iodine** onto the slide for 60 seconds.
5. Decolorize with **95% ethanol** for 8-12 seconds.
6. Stop decolorization by rinsing with distilled water.
7. Counterstain by adding **safranin** to the slide for 60 seconds.
8. Rinse with distilled water.
9. Blot dry with a paper towel.
10. Examine under the microscope, and show your instructor at 1000x magnification.
11. After your instructor's approval of your staining technique, dispose of your slides in the plastic beaker labeled "Self prepped" in the Discard Area.

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Brown, A. E. (2009). Benson's Microbiological Applications: Laboratory Manual in General Microbiology. New York: McGraw Hill.

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7. Acid Fast Stain

The acid-fast stain is a differential stain specifically used to detect bacteria in the genera *Mycobacterium* and some *Nocardia*. They contain a waxy material in the cell wall called **mycolic acid**. This prevents the bacteria from being stained by different stains. (Figure 7.1) The primary stain of carbol fuchsin contains phenol, which penetrates the waxy cell wall. As with other differential stains, a positive retains the primary stain.

There are two acid-fast techniques: Ziehl-Neelsen Method and Kinyoun Method. How they differ is based on the mordant. In the Ziehl-Neelsen method procedure, the primary stain is applied over a steaming hot water bath. The steam is the mordant. By heating the mycolic acid, it further makes it more porous, letting the stain penetrate into the cell wall. The Kinyoun method does not use steam as the mordant, but an increased concentration of phenol in the carbol fuchsin. This increased amount is adequate to allow penetration of the stain into the bacterial cells, and the carbol fuchsin is not removed during the decolorizing step. This is safer since it does not release the levels of noxious phenol fumes being heat-vaporized in the Ziehl-Neelsen method, but the challenge is which technique stains more effectively than the other.

This staining technique has an important purpose. The acid-fast stain is used as a diagnostic tool in the identification of *Mycobacterium tuberculosis* and *Mycobacterium leprae*, which are the pathogens that cause tuberculosis and leprosy, respectively.

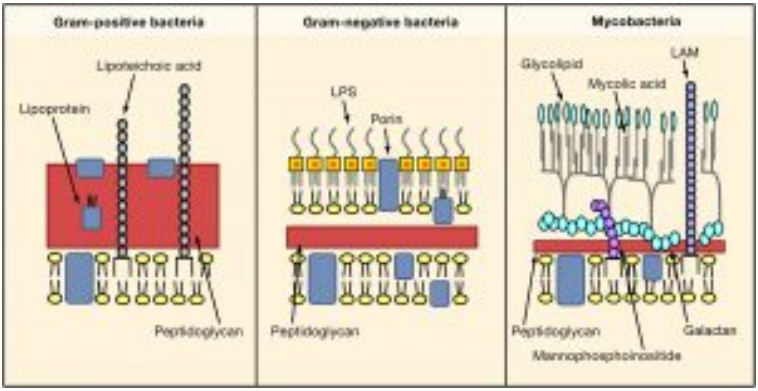


Figure 7.1 Mycolic acid is attached to the cell wall. Acid fast positive bacteria still contain peptidoglycan but less in amount compared to Gram positive and Gram negative bacteria. Image from https://www.cell.com/fulltext/S0092-8674(06)00190-5

MATERIALS

Each student should have:

Blue rack
2 glass slides
Carbol fuchsin
Acid Alcohol
Stain bottle rack: methylene blue
Lens paper
Windex (depends on instructor)
Inoculating loop
Wax pencil
Metal slide clip
Bunsen Burner
Striker
1 slant culture of *Mycobacterium vaccae*
1 culture of *Staphylococcus epidermidis*
Microscope

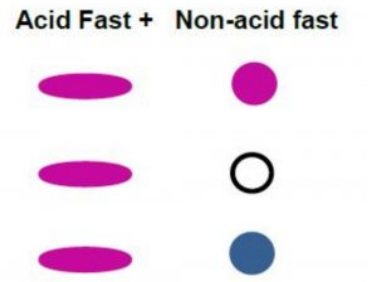
Share cultures between students.

PROCEDURE OF SMEAR PREPARATION
MAKE 2 SMEAR PREPS. ONE WILL BE USED AS A BACKUP.

1. Draw a circle with the wax pencil on a slide.
2. Flip the slide over. Write “Up”.
3. Add one drop of distilled water onto the slide within the target circle.
4. Using aseptic technique, transfer 1 loopful of *Staphylococcus epidermidis* first onto the slide, and try NOT to spread within the wax circle yet.
5. Transfer small amount of *Mycobacterium vaccae* into the *Staphylococcus epidermidis*, spreading within the wax circle. Break apart clumps of *Mycobacterium*
6. Air dry the slide. Do not apply heat to a wet slide. Do not blow on the slide.
7. Heat fix the slide.

PROCEDURE OF ACID-FAST STAIN (KINYOUN METHOD)

STAIN ONLY ONE SLIDE. You may not need the second slide. This serves as a backup in case you have error in your staining procedure. It saves you time.



1. Place one smear prep on a stain tray.
2. Saturate the slide with **carbol fuchsin** for 15 minutes.
3. Rinse slide with distilled water until clear over stain tray.
4. Decolorize with **acid alcohol** for 7-10 seconds.
5. Stop decolorization by rinsing with distilled water.

6. Counterstain with **methylene blue** for 60 seconds.
7. Rinse with distilled water.
8. Blot dry with a paper towel.
9. Examine under the microscope and show your instructor at 1000x magnification.
10. After your instructor's approval of your staining technique, dispose of your slides in the plastic beaker labeled "Self prepped" in the Discard Area.

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Brown, A. E. (2009). Benson's Microbiological Applications: Laboratory Manual in General Microbiology. New York: McGraw Hill.

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8. Endospore Stain

The endospore stain differentiates between developmental stages of bacteria. There are two genera of bacteria, *Clostridium* and *Bacillus*, that make up most of the Firmicutes phylum. They have two stages of development: vegetative stage and sporulation stage. The **vegetative stage** is when the bacterial cell is the normal actively growing **vegetative cell**. It is capable of metabolism, enzymatic activity, and has the normal water content. Due to actively reproducing, its DNA is exposed, and also has sensitivities to extreme temperatures, radiation, and chemicals. The **sporulation stage** is when the vegetative cell is forming endospores as insurance for when essential nutrients may be depleted, especially when carbon and nitrogen become unavailable. **Endospores** are a resting, or dormant, stage that protect DNA and proteins from unfavorable conditions. They are dehydrated structures that do not grow or carry out metabolism, so they are insensitive to antibiotics. They have many layers surrounding the core, which contains the DNA. The outermost layer is made up of a thick protein coat of **exosporium** that forms a protective barrier. (Figure 8.1). Endospores are resistant to heat, radiation, acids, and many chemicals due to the composition of these layers.

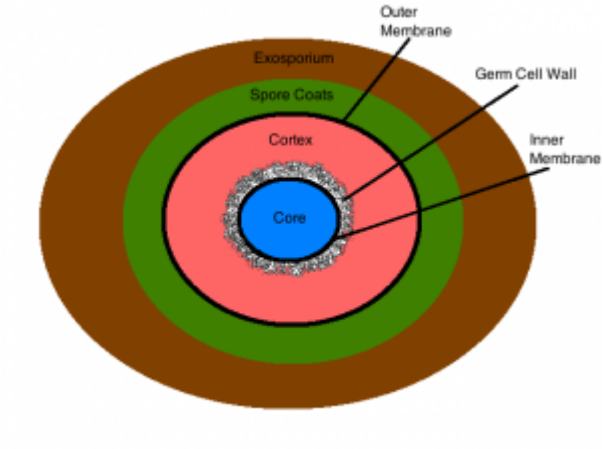


Figure 8.1 This drawing illustrates the structure of an endospore and its layers of protection.

https://microbewiki.kenyon.edu/index.php/File:Figure_3_endospore_structure.png

MATERIALS

Each student should have:

Blue rack
2 glass slides
Malachite green stain
Stain bottle rack: safranin
Lens paper
Windex (depends on instructor)
Inoculating loop
Wax pencil
Metal slide clip
Bunsen Burner
Striker
1 slant culture of *Bacillus subtilis*
Microscope

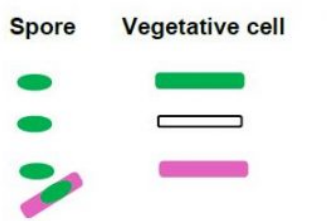
PROCEDURE OF SMEAR PREPARATION

MAKE 2 SMEAR PREPS. ONE WILL BE USED AS A BACKUP.

1. Draw a circle with the wax pencil on a slide.
2. Flip the slide over. Write “Up”.
3. Place a small drop of distilled water onto the slide within the wax circle.
4. Using aseptic technique, remove a small amount of bacteria from a slant
5. Air dry the slide. Do not apply heat to a wet slide. Do not blow on the slide.
6. Heat fix the slide.

PROCEDURE OF ENDOSPORE STAIN

STAIN ONLY ONE SLIDE. You may not need the second slide. This serves as a backup in case you have error in your staining procedure. It saves you time.



1. Place one smear prep on a boiling hot water bath.
2. Place a piece of paper towel over smear.
3. Saturate towel & slide with **malachite green** for 5 minutes.
4. Decolorize slide with **distilled water** until clear over stain tray.
5. Counterstain with **safranin** for 60 seconds.
6. Rinse with distilled water.
7. Blot dry with a paper towel.
8. Examine under the microscope, and show your instructor at 1000x magnification.
9. After your instructor’s approval of your staining technique, dispose of your slides in the plastic beaker labeled “Self prepped” in the Discard Area.

REFERENCES

Brown, A. E. (2009). Benson's Microbiological Applications: Laboratory Manual in General Microbiology. New York: McGraw Hill.

Chess, B. (2015). Laboratory Applications in Microbiology: A Case Study Approach. New York: McGraw Hill. Microbe Wiki. (2012, December 16). Retrieved from Bacterial Endospores:

https://microbewiki.kenyon.edu/index.php/Bacterial_endospores

OpenStax. (2016, November 1). Microbiology. Retrieved from https://legacy.cnx.org/content/col12087/1.4

9. Standard Plate Count

In certain circumstances, we need to know the concentration of bacteria in a sample. For instance, the count of bacteria might indicate the extent of infection occurring, such as in a bladder infection. Quality control has to depend on the numbers of bacteria present in items like drinking water, food, medication, cosmetics and even swimming areas. Different methods of determining the number of bacteria are used, but each method has its own advantages and disadvantages. We are going to use the standard plate count method. It is a direct method of counting viable, or live, cells, which is its advantage. The **standard plate count** is where a sample is diluted in a series of dilution blanks, and then aliquots (measured amounts) of the dilutions are plated using either the pour plate method or spread plate method. (See Figure 9.1). The expected result is a single bacterial cell gives rise to a single bacterial colony. However, this is not true for bacteria that do not immediately separate after dividing, so multiple cells could form a well-isolated colony. A more accurate unit for counting is a **colony forming unit (cfu)**. The number of bacterial cfu in the original sample is determined by multiplying the plate count by the dilution factor of the plate that is being counted. To be statistically accurate and effective, only numbers between 30 and 300 cfus are considered. Fewer than 30 cfus makes the statistics invalid due to a random noise associated with the probability a cfu will end up on a plate. At higher numbers, this noise is a small percentage of the count. Greater than 300 cfus could be inaccurate due to overcrowding inhibiting the growth of possible colonies and imprecision of counting.

Disadvantages of standard plate count are 1) the time it takes to obtain results due to the incubation period for the colony forming units to grow and 2) the limitation of what bacteria can be analyzed. The method uses specific media and conditions. Pour plates do not limit oxygen diffusion significantly, but the method may be beneficial in cases of bacteria species that grow poorly in air (microaerophiles). For aerobes, the minor oxygen limitation from metabolic activity slows growth and keeps colonies small. Selective medium is used when determining bacterial number for a urinary tract infection.

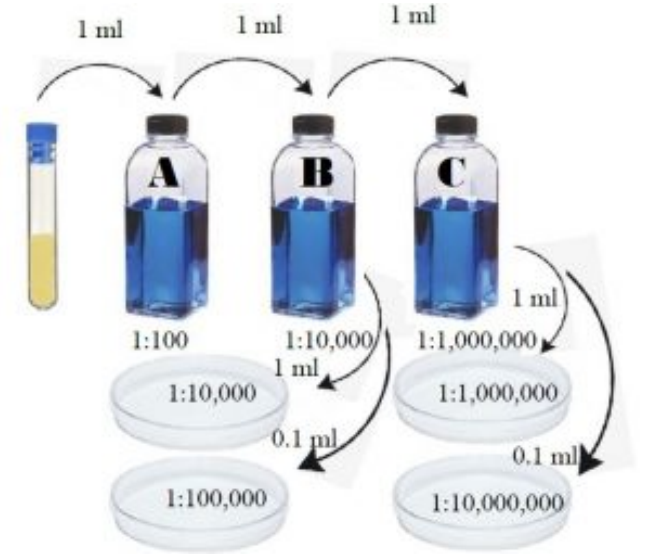


Figure 9.1 Quantitative plate procedure of standard plate count.

MATERIALS

Per pair of students should have:

Blue rack
Wax pencil
3 sterile 99 ml water blanks
7 sterile 1 ml serological pipettes
2- 1 ml blue pi-pumps
1 broth culture of *E. coli*
4 empty sterile petri plates
4 pour tubes (remain in hot water bath until ready to use)
(OPTIONAL: Bunsen Burner & Striker)

PROCEDURE OF SERIAL DILUTION AND PLATING

1. Label three sterile 99 ml water blanks A, B, and C.
2. Label the bottom of the four sterile petri plates 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. On each plate also include your names, bacteria name, and date.
3. Gently flick the culture tube to disperse the bacteria.
4. Transfer 1 ml of *E.coli* from the culture tube into blank A using a sterile 1 ml pipette. Place the used pipette into the small biohazard bag on the lab bench.
5. Shake blank A 25 times in an arc of 1 foot in order to distribute the bacteria evenly. Do not let the bottle go upside down. (Suggestion: place a paper towel over the lid of the water blank while shaking. If any leakage onto the paper towel, place that paper towel into the biohazard bag near the prep room door.)
6. Using a different sterile 1 ml pipette, transfer 1 ml from blank A into blank B.
7. Shake blank B 25 times in an arc of 1 foot in the same manner.
8. Using a different sterile 1 ml pipette, transfer 1 ml from blank B into blank C.
9. Shake blank C 25 times in the same manner.
10. Using a different sterile 1 ml pipette, transfer 1 ml from blank B into plate 10^{-4} plate.

11. Using a different sterile 1 ml pipette, transfer 0.1 ml from blank B into plate 10^{-5} plate.
12. Using a different sterile 1 ml pipette, transfer 1 ml from blank C into plate 10^{-6} plate.
13. Using a different sterile 1 ml pipette, transfer 0.1 ml from blank C into plate 10^{-7} plate.

Dilution scheme

99 mL +	1 mL from	To plate 10^{-4}	To plate 10^{-5}	To plate 10^{-6}	To plate 10^{-7}
Blank A	Original				
Blank B	Blank A	1 mL	0.1 mL		
Blank C	Blank B			1 mL	0.1 mL

14. Taking the blue rack with you, go to the water bath and remove 4 pour tubes, and bring back to your lab bench.
15. Pour one tube of liquefied Trypticase Soy Agar into each plate, cover with the plate lid, and swirl gently. Push the plates slowly and gently towards the center of the lab bench to solidify.
16. Under the instruction of your lab professor, you will move the plates at a later time during the lab session to your lab section's storage location to be stored at room temperature.
17. Place empty pour tubes, *E.coli* culture tube, and used blank bottles in the Discard Area. Return the blue pi-pumps back to its location. Please make sure the rubber pi-chuck is removed from used pipettes if it became removed from the blue pi-pump.

During next week's lab period, you will examine the results of these plates to determine how many bacterial cells are in one milliliter of the original culture of *E. coli*.

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OpenStax. (2016, November 1). Microbiology. Retrieved from https://legacy.cnx.org/content/col12087/1.4

10. Hand Washing

On each square centimeter of your skin, there are about 1,500 bacteria. Some bacteria have been recently acquired from the environment and belong to a group called **transient microbiota**. But most belong to what is called the **resident microbiota**. These microorganisms (not only bacteria) that constantly live on and in our bodies. **Transient microbiota** are generally easily removed by washing. If you are handling material that is human- contaminated, the transient microbiota may include pathogens. You can pick up transient microbiota from your daily environment, such as doorknobs, faucets, tables, other people you interact with. Your resident microbiota plays a role in protecting you against any that may be pathogenic.

The resident microbiota of the skin live under the stratum corneum (the outermost layer of the skin), can be found on the surface, and in glands and follicles. Those microorganisms have learned to adapt to the many niches that they live in and tend to remain stable over time. (See pages 909-910, OpenStax, appended below). Different types of bacteria dominate the dry, moist, and sebum-rich regions of the skin. While hands are mostly dry, we also use them to touch sebaceous regions such as the face, neck and hair. The sebaceous secretions are antimicrobial. *Staphylococcus*, like *S. epidermidis,* are more typical of moist areas, but are also found on the hands. This microbiome aids in host defense by producing antimicrobial peptides that target the pathogens, preventing their establishment on the skin.

Even with the protection of resident microbiota, pathogens can cause infection. Microorganisms and their diseases are transmitted through direct and indirect contact. A simple but important approach in preventing the spread of disease is

handwashing. The physicians by the names of Ignaz Semmelweis and Oliver Wendall Holmes are given the credit for the concept of disease prevention through hand washing. Semmelweis noticed a high death rate at child birth and puerperal infections when doctors did not wash their hands going from the autopsy room to delivery. He proposed the practice of washing with chlorinated lime solution. Holmes observed less infant deaths and puerperal infections when midwives carry out the deliveries compared to doctors. He proposed hand washing with calcium chloride. Both methods resulted in an increase of successful deliveries and decrease of infections.

In this experiment, you will be observing different types of hand washing and the effectiveness it has on removing both resident and transient microbiota from the skin. You will be comparing 4 scenarios: unwashed hands, vigorously washing with water, washing with soap (0.3% chloroxylenol), and washing with povidone iodine (13%) scrub.

The sebaceous secretions are an important consideration in this experiment. When we use a swab to transfer skin bacteria to a plate, we are also transferring antimicrobial sebaceous secretions. The bacteria are spread on a plate along with sebaceous secretions or other compounds such as found in hand moisturizer. These can inhibit microbial growth. Occasionally, we see larger number of colonies after hand-washing. This is part of the experiment and helps us understand the nature of the skin microbiome.

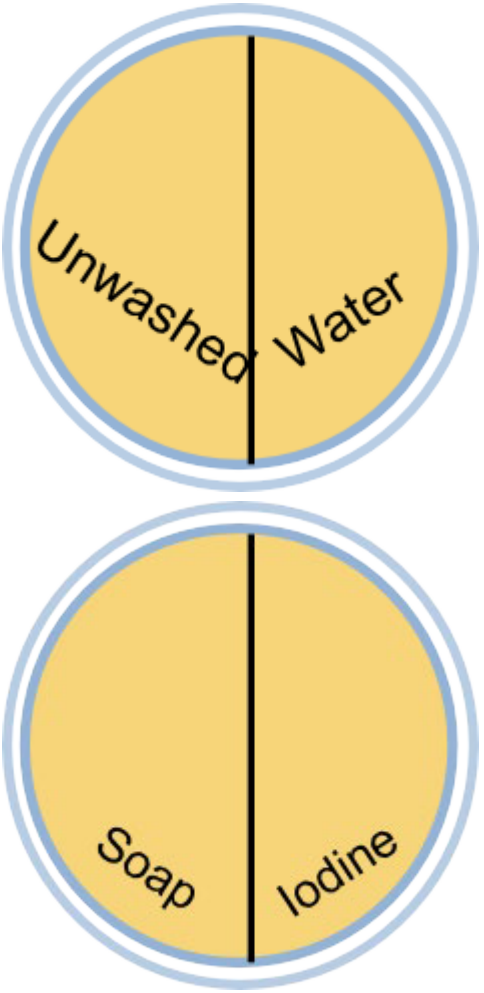
MATERIALS

Per pair of students should have:

Wax pencil
2 TSA plates (Trypticase Soy Agar)
4 Sterile swabs
1 Povidone iodine scrub brush

PROCEDURE OF HAND WASHING

1. Label the bottom of each plate with student names and date. On each plate with a wax pencil, draw a line down the middle of the plate, dividing it half. Label as follows:



2. Designate one partner as the one being washed and the other as the sample collector. The student who is being washed will not touch anything: not the sink handles, the soap dispenser, the distilled water bottle, the TSA plates, anything. Simply do the scrubbing. The sample collector will be the one to touch all that was mentioned previously and be the one to take the samples off of the hand of the one being washed.
3. Unwashed hands: Place water from a distilled water bottle onto a hand, and spread between the two hands. This moistens the skin so that bacteria can be removed easily.
4. Then rub a sterile swab along the surface of the skin. After sample is collected, gently rub the swab along the surface of the TSA plate labeled "Unwashed". (Note which side of the swab is used in the hand and use that side on the plate.)
5. Vigorously wash with water: Place hands and scrub together under running warm water for 60 seconds.
6. Then rub a sterile swab along the surface of the skin. After sample is collected, gently rub the swab along the surface of the TSA plate labeled "Water".
7. Washing with soap: Place soap onto hands and then scrub together for 60 seconds. Rinse all soap off, to avoid including the effect traces of soap.
8. Then rub a sterile swab along the surface of the skin. After sample is collected, gently rub the swab along the surface of the TSA plate labeled "Soap".
9. Wash with Iodine Scrub: Wet hands under running water. Then coat hands with sponge side of Povidone iodine scrub brush. Once well-coated, flip brush over and scrub with bristles for 60 seconds. Rinse all iodine off, completely.
10. Then rub a sterile swab along the surface of the skin. After sample is collected, gently rub the swab along the surface of the TSA plate labeled "Iodine".
11. All swabs and scrub brushes can be placed in the regular trash can.
12. Once all samples are collected, the plates can be stored in

your lab section's rack at temperature. You will examine your results next lab.

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From Openstax: p 908-910 Chapter 21: Skin and Eye Infections

21.1 Anatomy and Normal Microbiota of the Skin and Eyes

Learning Objectives

- Describe the major anatomical features of the skin and eyes
- Compare and contrast the microbiomes of various body sites, such as the hands, back, feet, andeyes
- Explain how microorganisms overcome defenses of skin and eyes in order to cause infection
- Describe general signs and symptoms of disease associated with infections of the skin and eyes

Human skin is an important part of the innate immune system. In addition to serving a wide range of other functions, the skin serves as an important barrier to microbial invasion. Not only is it a physical barrier to penetration of deeper tissues by potential pathogens, but it also provides an inhospitable environment for the growth of many pathogens. In this section, we will provide a brief overview of the anatomy and normal microbiota of the skin and eyes, along with general symptoms associated with skin and eye infections.

Layers of the Skin

Human skin is made up of several layers and sublayers. The two main layers are the **epidermis** and the **dermis**. These layers cover a third layer of tissue called the **hypodermis**, which consists of fibrous and adipose connective tissue (Figure 21.2).

The epidermis is the outermost layer of the skin, and it is relatively thin. The exterior surface of the epidermis, called the **stratum corneum**, primarily consists of dead skin cells. This layer of dead cells limits direct contact between the outside

world and live cells. The stratum corneum is rich in **keratin**, a tough, fibrous protein that is also found in hair and nails. Keratin helps make the outer surface of the skin relatively tough and waterproof. It also helps to keep the surface of the skin dry, which reduces microbial growth. However, some microbes are still able to live on the surface of the skin, and some of these can be shed with dead skin cells in the process of **desquamation**, which is the shedding and peeling of skin that occurs as a normal process but that may be accelerated when infection is present.

Beneath the epidermis lies a thicker skin layer called the dermis. The dermis contains connective tissue and embedded structures such as blood vessels, nerves, and muscles. Structures called **hair follicles** (from which hair grows) are located within the dermis, even though much of their structure consists of epidermal tissue. The dermis also contains the two major types of glands found in human skin: **sweat glands** (tubular glands that produce sweat) and **sebaceous glands** (which are associated with hair follicles and produce **sebum**, a lipid-rich substance containing proteins and minerals).

Clinical Focus

Part1

Sam, a college freshman with a bad habit of oversleeping, nicked himself shaving in a rush to get to class on time. At the time, he didn't think twice about it. But two days later, he noticed the cut was surrounded by a reddish area of skin that was warm to

the touch. When the wound started oozing pus, he decided he had better stop by the university's clinic. The doctor took a sample from the lesion and then cleaned the area.

- What type of microbe could be responsible for Sam's infection?

Jump to the next Clinical Focus box.

Perspiration (sweat) provides some moisture to the epidermis, which can increase the potential for microbial growth. For this reason, more microbes are found on the regions of the skin that produce the most sweat, such as the skin of the underarms and groin. However, in addition to water, sweat also contains substances that inhibit microbial growth, such as salts, lysozyme, and antimicrobial peptides. Sebum also serves to protect the skin and reduce water loss. Although some of the lipids and fatty acids in sebum inhibit microbial growth, sebum contains compounds that provide nutrition for certain microbes.

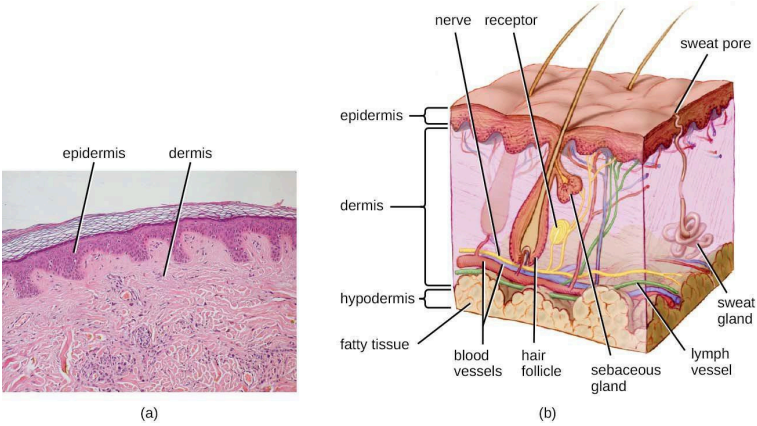

Figure 21.2 (a) A micrograph of a section through human skin shows the epidermis and dermis. (b) The major layers of human skin are the epidermis, dermis, and hypodermis. (credit b: modification of work by National Cancer Institute)

Check Your Understanding

- How does desquamation help with preventing infections?

Normal Microbiota of the Skin

The skin is home to a wide variety of normal microbiota, consisting of commensal organisms that derive nutrition from skin cells and secretions such as sweat and sebum. The normal microbiota of skin tends to inhibit transient- microbe colonization by producing antimicrobial substances and outcompeting other microbes that land on the surface of the skin. This helps to protect the skin from pathogenic infection.

The skin's properties differ from one region of the body to another, as does the composition of the skin's microbiota. The

availability of nutrients and moisture partly dictates which microorganisms will thrive in a particular region of the skin. Relatively moist skin, such as that of the nares (nostrils) and underarms, has a much different microbiota than the dryer skin on the arms, legs, hands, and top of the feet. Some areas of the skin have higher densities of sebaceous glands. These sebum-rich areas, which include the back, the folds at the side of the nose, and the back of the neck, harbor distinct microbial communities that are less diverse than those found on other parts of the body.

Different types of bacteria dominate the dry, moist, and sebum-rich regions of the skin. The most abundant microbes typically found in the dry and sebaceous regions are Betaproteobacteria and Propionibacteria, respectively. In the moist regions, *Corynebacterium*and *Staphylococcus*are most commonly found (Figure 21.3). Viruses and fungi are also found on the skin, with *Malassezia*being the most common type of fungus found as part of the normal microbiota. The role and populations of viruses in the microbiota, known as viromes, are still not well understood, and there are limitations to the techniques used to identify them. However, Circoviridae, Papillomaviridae, and Polyomaviridae appear to be the most common residents in the healthy skin virome.[1][2][3]

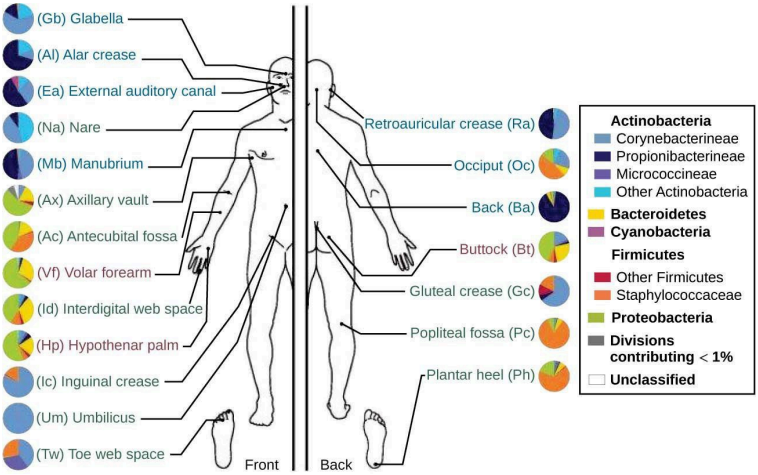

Figure 21.3 The normal microbiota varies on different regions of the skin, especially in dry versus moist areas. The figure shows the major organisms commonly found in different locations of a healthy individual's skin and external mucosa. Note that there is significant variation among individuals. (credit: modification of work by National Human Genome Research Institute)

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11. Antimicrobial Sensitivity Testing: Kirby Bauer Disk of Diffusion Method

A general term used is **antimicrobial**, which is a compound that inhibits or kills microorganisms. There are different types of antimicrobials. Examples are antibiotics, disinfectants, and antiseptics. **Antibiotics** are produced by microorganisms that inhibit or kill other microorganisms by targeting specific cellular processes. Antibiotics shut down specific cellular processes, so their mode of action affects specific kinds of cells. **Disinfectants and antiseptics** don't have that specificity. They destroy structures in a wide range of cells, including your own. Because of this, **Disinfectants** are used to destroy or inhibit microbial cells on inanimate objects. A subgroup of disinfectants, **antiseptics,** kill or prevent growth for microbes on human tissue. They are also not specific in what cells they affect, but they only affect your dead outer layer of cells.

A procedure named the Kirby Bauer Disk of Diffusion Method finds the suitability of specific antibiotics for specific infections. This method consists of growing a uniform layer of bacteria on a solid medium (known as a lawn). A small filter disk, saturated with a specific concentration of antibiotic, is place on that plate. During incubation, the antibiotic will diffuse from the disk into the agar, forming a concentration gradient. The level of sensitivity to the particular agent can be determined by measuring a clearing around the disk where the bacteria did not grow. This clearing is known as the **Zone of Inhibition (ZoI)**. Its size varies for the agent as a result of the resistance of

the bacteria. The size of the ZoI can be used to decide the effectiveness of the antibiotic. A larger ZoI indicates that the bacteria are more sensitive to the antibiotic. But the simple presence of a ZoI does not mean that the antibiotic will work for treating a patient. Kirby Bauer method is a standardized test, and has minimum ZoI diameters for each antibiotic that varies by bacterial type involved. These diameters are related to the probable clinical outcomes for patients. The different criteria for different bacterial types are primarily based on the efficiency of delivering the drug to the typical infection site for a bacterial type. For instance, a pill dissolves in the gut enters the blood and perfuses tissues from every accessible side. Skin and lung sites have no blood flow on the side away from the tissues. So, skin and lung infections will tend to have higher ZoI diameter criteria for sensitivity than gut infections, so that even with decreased perfusion the infection will still be successfully treated. Test results are reported as sensitive, resistant, or intermediate. (Table 11.1). If the zone of inhibition is greater than the standard size zone of inhibition, then the bacteria is sensitive to the antibiotic. If the zone of inhibition is smaller than a specific ZoI diameter, then the bacteria is resistant to the antibiotic, meaning that antibiotic is not effective on that bacteria. Physicians can isolate and identify the bacteria causing the infection in the patient and use the Kirby Bauer method to see which antibiotic would be most effective.

Using 95% ethanol in a glass petri dish

Part of the effectiveness of flame sterilization is the mix of free-radicals that aggressively react with the cells. Ethanol increases the range of free-radicals, allowing for lower-heat sterilization.

MATERIALS

Each pair of students should have:

Wax pencil

2 media plates: depending on availability:

MH plate (Mueller Hinton) and 1 TSA plate (Trypticase Soy Agar)
OR
2 TSA plates
2 Sterile swabs
Sterile forceps
Bunsen Burner
Striker

Disinfectants: hydrogen peroxide, 5% bleach, 5% formalin, Lysol

Antibiotics: Choose 3

a) Penicillin
b) Cephalothin
c) Chloramphenicol
d) Tetracycline
e) Erythromycin
f) Ampicillin

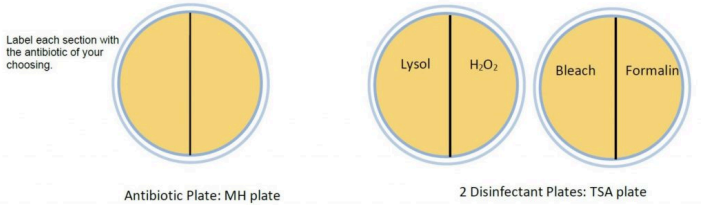
1 broth culture of bacteria of instructor's choosing for your pair for equal distribution:

Staphylococcus epidermidis
Klebsiella aerogenes
Escherichia coli

At your station: 95% ethanol in a glass petri dish
Sterile filter disks

PROCEDURE OF KIRBY BAUER DISK OF DIFFUSION METHOD

1. Label the bottom of each plate with student names and date. On the MH plate with a wax pencil, draw three lines to divide into thirds. On the TSA plate, draw two lines to make quadrants. Label as follows:



Antibiotic Plate: MH plate
2 Disinfectant Plates: TSA plate

2. Using aseptic technique, make a lawn of bacteria on each plate by inserting a sterile cotton swab into the broth

bacterial culture, absorbing excess fluid. Gently and evenly spread the moistened swab over the surface, making sure you cover the entire surface. In order to create thorough lawn, rotate the plate counterclockwise twice, continuing to spread the swab on the surface between each turn.

3. Use a separate sterile swab for each plate.
4. Allow the plates to dry for a few minutes before applying disks.
5. At each station, there are two glass petri dishes: a) 95% ethanol and b) sterile filter disks.
6. **Disinfectants**: Dispense disks as follows:
   a. Dip forceps into 95% ethanol and then place into outer flame of Bunsen Burner until red. Allow to cool for a few seconds by waving in the air.
   b. Carefully remove a sterile filter disk from the glass plate (do not put the lid down on the bench) with the forceps.
   c. Saturate the disk with one drop of 5% bleach.
   d. Place the disk in the center of the quadrant on the TSA plate labeled "5% bleach".
   e. Dip forceps into 95% ethanol and then place into outer flame of Bunsen Burner until red. Allow to cool for a few seconds by waving in the air.
   f. Repeat steps a through e for the next three disinfectants: hydrogen peroxide, 5% formalin, and Lysol.
7. **Antibiotics**: Dispense disks as follows:
   a. Dip forceps into 95% ethanol and then place into outer flame of Bunsen Burner for a few seconds. Allow to cool for a few seconds by waving in the air.
   b. Carefully push half of the antibiotic disk out of the cartridge with the forceps, and then once half is available, pull the disk out with the forceps.
   c. Place the disk in the center of a section labeled with the appropriate antibiotic.
   d. Dip forceps into 95% ethanol and then place into outer flame of Bunsen Burner until red. Allow to cool for a few

seconds by waving in the air.

e. Repeat steps a through d for the next two antibiotics of your choosing.

Incubate your plates at room temperature in your lab section's racks.

DETERMINING RESULTS (WEEK 2)

1. Measure the diameter of the zone of inhibition of each antimicrobial, specifically the antibiotics, using a small metric ruler in millimeters. Simply use the naked eye for the determination of the zone of inhibition.

2. You can determine if your bacteria is resistant (R), sensitive (S), or intermediate to the antibiotics by looking at Table 11.1. The zone of inhibition varies between different bacteria and different antibiotics.

Table 11.1: Zones of inhibition in the Kirby Bauer Disk of Diffusion Method of Antibiotic Sensitivity Testing.

Disk Diffusion Zone Diameter Chart (Table 1)

Antimicrobial Agent	Code	Potency	Resistant[b]	Intermediate[c]	Susceptible[d]
Amoxicillin/Clavulanic Acid[5,10,3,104,vi] (Augmentin)	AmC-30	20/10ug			
for Staphylococcus species[45,50,62]			≤19	-	≥20
for H. influenzae and parainfluenzae[86,91]			≤19	-	≥20
for Enterobacteriaceae			≤13	14--17	≥18
Ampicillin[9,13]	AM10	10ug			
for Enterobacteriaceae[14,17]			≤13	14--16	≥17
for Staphylococcus species[45,55,59,60]			≤28	-	≥29
for Enterococcus species[76,77,80,81]			≤16	-	≥17
for H. influenzae and parainfluenzae[85,87,91]			≤18	19--21	≥22
for beta-hemolytic streptococci[93,119,120]			-	-	≥24
for Vibrio cholerae[17]			≤13	14-16	≥17
for N. meningitidis[135]			-	-	-
Cephalothin[1,11,33]	CF-30	30ug			
for Staphylococcus species[45,50,62]			≤14	15--17	≥18
for Enterobacteriaceae[16,19,20]			≤14	15--17	≥18
for H. influenzae and parainfluenzae[8,5]			≤25	26--28	≥29
for S. pneumoniae			≤20	-	≥21
for Staphylococcus species[53]			≤12	13--17	≥18
for Enterococcus species			≤12	13--17	≥18
for Enterobacteriaceae[54]			≤12	13--17	≥18
for N. meningitides			≤19	20--25	≥26
for Streptococcus other than S. pneumoniae			≤17	18--20	≥21
for Vibrio cholerae[129]			≤12	13--17	≥18
Erythromycin[2,7,8]	E-15	15ug			
for S. pneumoniae			≤15	16--20	≥21
for Staphylococcus species			≤13	14--22	≥23
for Streptococcus other than S. pneumoniae[116]			≤15	16--20	≥21
for Enterococcus species			≤13	14--22	≥23
Penicillin[9,13,45,55,10,3,104,119,124,125,126,147,148]	P-10	10U			
for Staphylococcus species[56]			≤28	-	≥29
for N. gonorrhoeae[93,95,97,98]			≤26	27--46	≥47
for beta-hemolytic streptococci[3,106,107,108,109,120]			-	-	≥24
for Enterococcus species[76,77,80,81]			≤14	-	≥15
for N. meginitidis[139]			-	-	-
Tetracycline[2,5,6,146,150]	Te-30	30ug			
for H. influenzae and parainfluenzae[9,2]			≤25	26--28	≥29
for N. gonorrhoeae[92,99]			≤30	31--37	≥38
for Streptococcus other than S. pneumoniae[92]			≤18	19--22	≥23
for S. pneumoniae[9,2]			≤18	19--22	≥23
for Staphylococcus species[53]			≤14	15--18	≥19
for Enterococcus species			≤14	15--18	≥19
for Enterobacteriaceae			≤11	12--14	≥15
for Acinetobacter species			≤11	12--14	≥15
for Vibrio cholerae[128]			≤14	15--18	≥19

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12. Lethal Effects of Ultraviolet Light

Electromagnetic spectrum is the range of wavelengths of electromagnetic radiation (or energy), extending from the longest wavelength of radio waves to the shortest of gamma rays. The two forms of electromagnetic radiation that are mutagenic are ionizing radiation and nonionizing radiation. Ionizing radiation carries enough energy that electrons are ejected and ions are formed which breaks DNA strands and denature proteins. Examples of ionizing radiation are gamma rays and x rays. Nonionizing radiation just excites electrons to a higher energy state. This includes visible light. (Figure 12.1). Shorter wavelengths can be absorbed by molecular bonds, the way sunscreen or a black-light poster absorb UV. UV is absorbed by the ring-structure bonds of pyrimidines. The electrons are excited to a higher energy state and interact with excited electrons from the neighboring pyrimidine. Abnormal bonds are formed in DNA. Pyrimidine dimers occur when two adjacent pyrimidines (particularly thymine) form covalent bonds between their ring-structures. (Figure 12.2). An example of nonionizing radiation is **ultraviolet light**. The wavelength of ultraviolet spans between 4 and 400 nm, but the most germicidal wavelength is **260 nm** due to this being the wavelength that DNA maximally absorbs ultraviolet light, causing the pyrimidine dimer formation. (Figure 12.3). Genes with pyrimidine dimers are damaged and cannot be transcribed of replicated. This may be lethal.

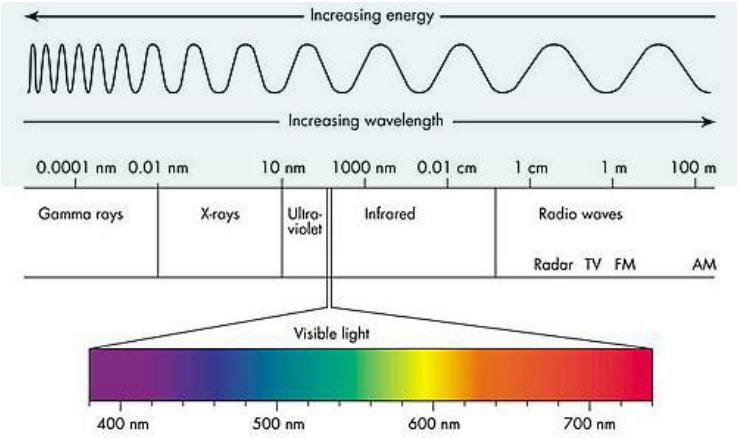

Figure 12.1 Electromagnetic spectrum illustrates how the shorter the wavelength, the more energy, therefore the more damaging to cells. (http://www.astro.wisc.edu/~kerry/pics/em.jpg)

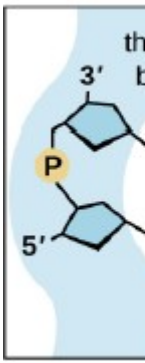

Figure 12.2 U
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Several elements affect the killing properties of UV light, including the time of exposure to the ultraviolet light and the presence of materials that will block the radiation from reaching the cells. An example of this is the plastic lid of the petri plate. Endospores are more resistant than vegetative cells to UV light. The DNA of endospores is protected by small, acid-soluble proteins that either absorb UV or promote a DNA conformation that avoids pyrimidine dimer formation.

Repair is very expensive, particularly at lower levels of light (Figure 12.4). When the cost of repair exceeds the available resources of the cell, then damage is not repaired and new enzymes for energy and repair cannot be produced if the relevant genes are damaged with pyrimidine dimers. “Death by UV equals debt by UV.”

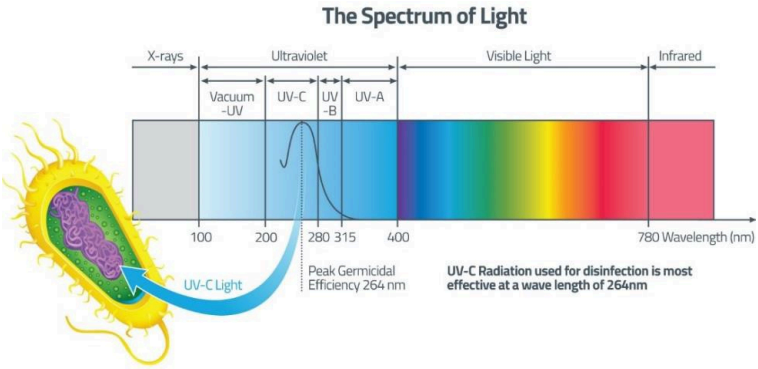


Figure 12.3 The most germicidal wavelength is 260 nm due DNA maximally absorbing UV light at this wavelength, causing pyrimidine dimers. (http://www.uvguard.com/wp- content/uploads/2015/09/uv-light- spectrum-dia.jpg)

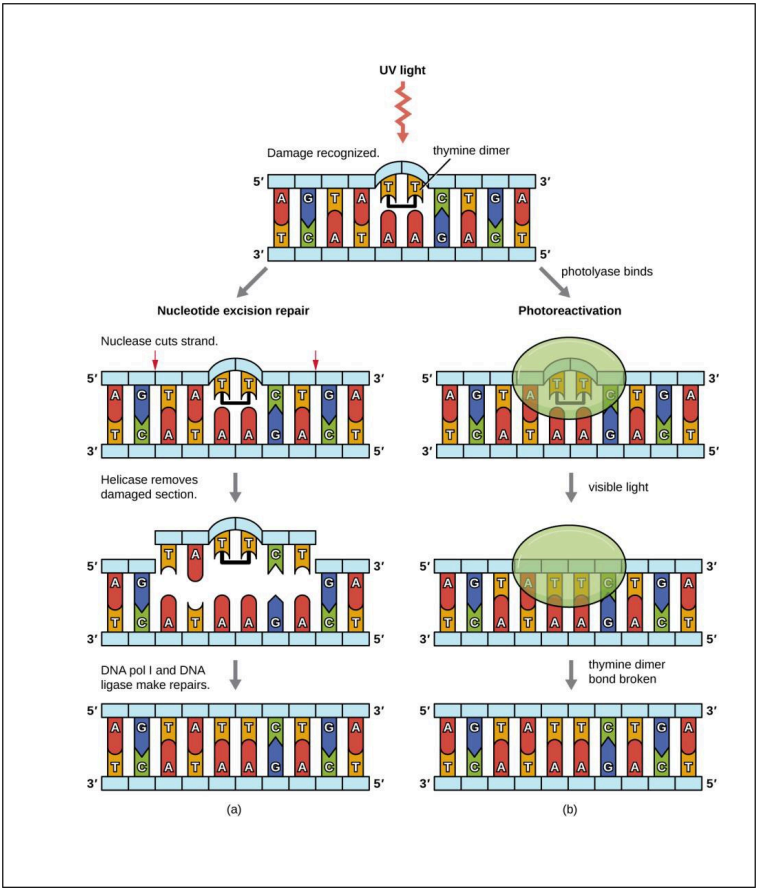


Figure 12.4 (OpenStaxMicrobiology, Figure 11.23) Bacteria have two mechanisms for repairing thymine dimers. (a) In nucleotide excision repair, an enzyme complex recognizes the distortion in the DNA complex around the thymine dimer and cuts and removes the damaged DNA strand. The correct nucleotides are replaced by DNA pol I and the nucleotide strand is sealed by DNA ligase. (b) In photoreactivation, the enzyme photolyase binds to the thymine dimer and, in the presence of visible light, breaks apart the dimer, restoring the base pairing of the thymines with complementary adenines on the opposite DNA strand.

This experiment is going to compare the germicidal effects of ultraviolet light on endospore former bacteria (*Bacillus subtilis*) to two non-endospore former bacteria (*Escherichia coli* and *Staphylococcus epidermidis*).

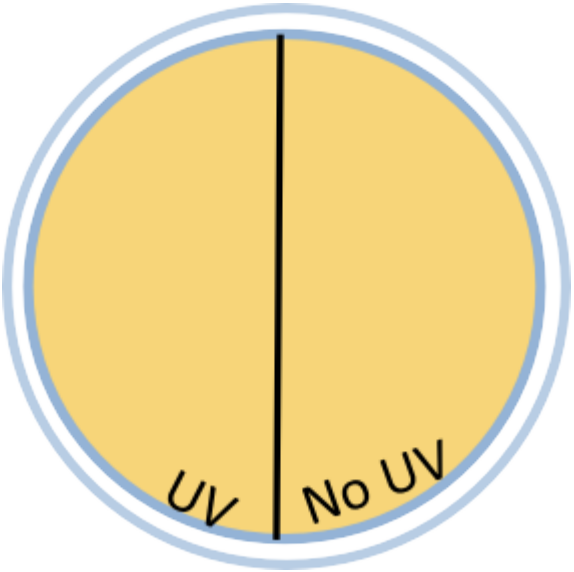
MATERIALS

Per student:

Blue rack
Sterile cotton swab
Wax pencil
Index card
1 TSA plate (Trypticase Soy Agar)
Safety glasses
Pair of nitrile gloves
1 broth bacterial culture of *Escherichia coli* **or** *Staphylococcus epidermidis* **or** *Bacillus subtilis* (resuspended from a slant)**
(Optional: Bunsen burner & striker)

PROCEDURE OF ULTRAVIOLET LIGHT LAB

1. The instructor will distribute one cultural tube per student, and assign an exposure time to each student.
2. Label the bottom of the TSA plate with your name, date, the bacterial culture, and exposure time in which you are working with.
3. On the bottom, draw a line down the middle of the plate, dividing it in half. Label one side “UV” and the other “No UV”.



4. Using aseptic technique, make a lawn of bacteria on each plate by inserting a sterile cotton swab into the broth bacterial culture, absorbing excess fluid. Gently and evenly spread the moistened swab over the surface, making sure you cover the entire surface. In order to create thorough lawn, rotate the plate counterclockwise twice, continuing to spread the swab on the surface between each turn.
   a. **Students who have a *Bacillus subtilis* slant will need to resuspend the colonies into a liquid: Using a sterile swab, swipe the culture off of the slant and put that swab into a sterile water bottle. Shake the swab inside the water until the bacteria is removed off of the swab. (Or add sterile water to the slant. Screw closed and vortex.)
   b. Otherwise follow your instructor's directions to resuspend.
5. Wearing safety glasses and nitrile gloves, bring your TSA plate and index card to the ultraviolet light station.
6. Remove the lid from the petri plate, and cover the half of the plate labeled "No UV" with the index card.
7. Expose your plate to the UV light for your assigned

exposure time. You can use the second hand on the clock or the timer on your cell phone to time the exposure.

8. Place your finished plate with its lid on upside down in a rack in your lab section's area.

For the instructor:

Once the entire class has completed the ultraviolet light exposure and has filled the plate rack, cover the rack with a box labeled "UV light" that is provided. The box will prevent light repair by photolyase. Store at room temperature in your lab section's bench area.

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13. Growth Requirements of Aerobes versus Anaerobes

Not all bacteria can tolerate oxygen, and many actually need to be in an environment without it in order to survive the oxidation. It is very useful to classify into groups based on their response to oxygen or of the lack of oxygen.

Bacteria that require oxygen for growth are called **aerobes**. They carry out respiratory metabolism, or aerobic respiration, where oxygen is the terminal electron acceptor. There are different types of aerobes: obligate aerobes and microaerophiles. **Obligate aerobes** absolutely require oxygen for growth for aerobic respiration to be carried out. By this process, they yield a great deal of energy compared to anaerobic bacteria. **Microaerophiles** are bacteria that live at lower concentrations of oxygen than atmospheric concentration, such as in the soil, water, or the human vagina. They prefer approximately 10% oxygen concentration rather than 20%. They have a limited capacity to neutralize reactive oxygen species (toxic byproducts of oxygen and oxidation). Examples of the kinds of enzymes they might lack are peroxidase, catalase, and superoxidase dismutase.

Bacteria that are sensitive to oxygen are called anaerobes. Some are more sensitive than others. The types of anaerobes are: facultative anaerobes, aerotolerant anaerobes, and obligate anaerobes. **Facultative anaerobes** can carry out fermentation or anaerobic respiration if oxygen is not present. But it can also carry out aerobic respiration if oxygen is present.

The reason for this is that these bacteria possess the detoxifying enzymes mentioned previously. **Aerotolerant anaerobes** are a subgroup of facultative anaerobe. They can carry out only fermentation if oxygen is not present. They do not carry out aerobic respiration if oxygen is present, but they are not harmed by its presence in the environment. Instead of possessing the detoxifying enzymes, aerotolerant anaerobes contain metallic ions that have the same function. **Obligate anaerobes** are bacteria that cannot tolerate oxygen due to having the sensitivity to the byproducts of oxygen, such peroxides and superoxides. They do not possess the detoxifying enzymes. They carry out fermentation or anaerobic respiration.
Because of their sensitivity to the byproducts of oxygen, anaerobes have to be grown in an oxygen-free environment. Different methods have been developed, but we will be using either one of two ways to do so: fluid thioglycollate medium or a GasPak anaerobic jar.

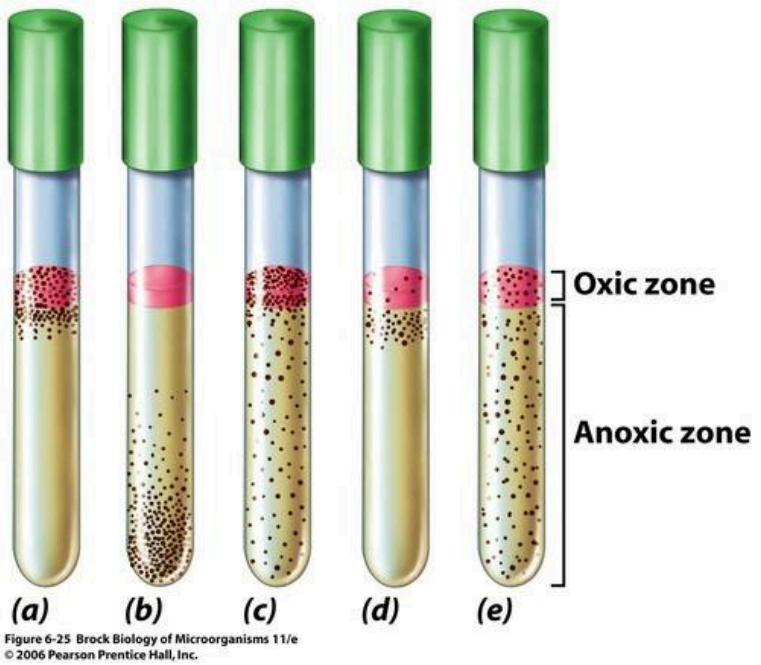

Figure 13.1 (a) Obligate aerobe (b) Obligate anaerobe (c) Facultative anaerobe (d) Microaerophile (e) Aerotolerant anaerobe

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Fluid thioglycollate medium is a liquid medium that supports growth of aerobes and anaerobes. (Figure 13.1). It contains sodium thioglycolate, which chemically combines with dissolved oxygen to deplete the oxygen in the medium. It also prevents the accumulation of peroxides. It contains an oxidation-reduction (redox) indicator named resazurin. It turns pink when oxidized, and is colorless when reduced. A thin band is more desirable because it is fresher. There is a small amount of agar in the medium that assists in the maintenance of low

redox potential: it keeps the anaerobes at the bottom of the tube.

An anaerobic jar becomes an anaerobic environment by using a GasPak sachet. The GasPak contains inorganic carbon, activated carbon, and ascorbic acid. Once the sachet becomes activated by simply opening it, it creates an oxygen-free and carbon dioxide-rich environment inside the sealed anaerobic jar. (Figure 13.2).

Figure 13.2 The GasPak sachet contains chemicals to release carbon dioxide and hydrogen gas. The hydrogen gas binds with free oxygen in the jar, producing water. This can be seen by the condensation in the inside of the jar

This lab will focus on examples of obligate aerobe, facultative anaerobe, and obligate anaerobe.

2 options of the experiment based on availability of materials: Fluid thioglycollate Medium Or GasPak Anaerobic Jar

For Fluid Thioglycollate Medium Experiment:

MATERIALS

Per group of 3 students should have:

3 test tubes of fluid thioglycollate medium
3 test tubes (one per above test tube)
Wax pencil or their own pen or pencil
3 Inoculating loops (one per student)
Bunsen Burner
Striker
Example set of species:
Clostridium sporogenes
Escherichia coli
Micrococcus luteus

PROCEDURE OF FLUID THIOGLYCOLLATE MEDIUM

1. Each student is responsible for one bacterial culture being transferred.
2. Label each fluid thioglycollate medium tube with your name, date, and the bacterial culture in which you are working with.
3. Using aseptic technique, transfer 3 loopfuls of the bacterial culture to a properly labeled sterile fluid thioglycollate medium tube.
4. Place the 3 freshly inoculated tubes into your lab section's red racks at room temperature.

For GasPak Anaerobic Jar Experiment:

MATERIALS

Per pair of students should have:

2 TSA plates (Trypticase Soy Agar)
2 Inoculating loops (one per student)
2 wax pencils
Bunsen Burner
Striker
Example set of species:
Clostridium sporogenes
Escherichia coli
Micrococcus luteus

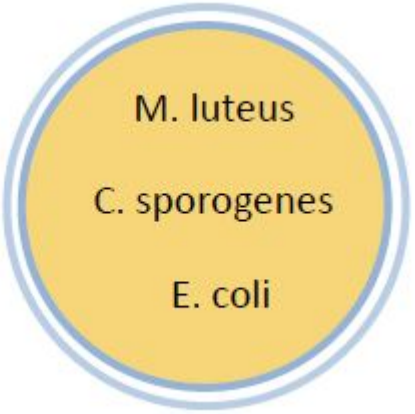
Per class:

One Gas Pak sachet
One Anaerobic Jar
One Regular Plate Rack

PROCEDURE OF GASPAK ANAEROBIC JAR

Each student is responsible for one plate.

1. Label the bottom of each TSA plate with your names and date. Label as follows:



2. Using aseptic technique, use a loop to inoculate each plate with a single streak of each bacterial directly under their label.

3. Place one plate in the anaerobic jar upside down and the other in the regular rack upside down to be incubated at the normal oxygen concentration.

For the instructor:

1. Once all plates have been collected for the class, rip open the GasPak, and remove it from the silver envelope and place it inside the anaerobic jar.
2. Screw the anaerobic jar shut.
3. The jar will be oxygen free within 2½ hours and greater than 15% carbon dioxide within 24 hours.
4. Place both the anaerobic jar and the regular plate rack in your lab section's area on the lab bench at room temperature in order to observe results next week.

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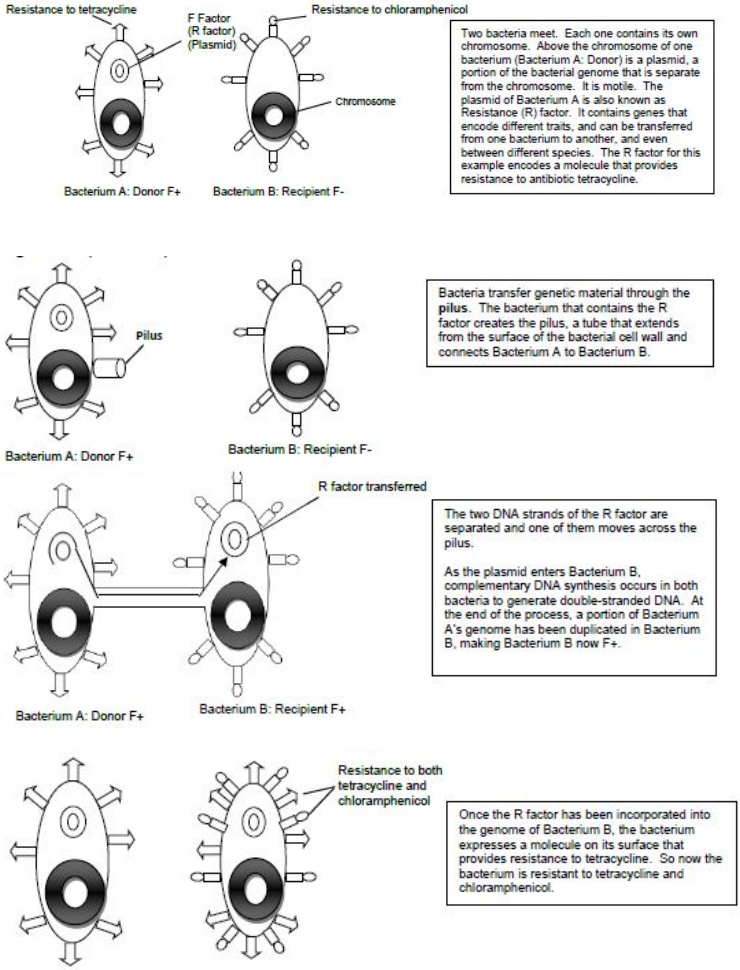
14. Bacterial Conjugation

Conjugation is commonly known as bacterial “sex.” It is a mode of genetic exchange in which a plasmid or other genetic material is transferred by a donor bacterial cell to a recipient bacterial cell via a direct connection. Both Gram positive and Gram-negative bacteria cells can conjugate. The donor has a plasmid, known as F factor (F = fertility), that allows the synthesis of a conjugative pilus. The recipient cell has a recognition site on its surface for the pilus. The pilus is a tube that extends from the surface of the bacterial cell wall and connects two bacteria, therefore making a direct connection. The bacteria involved in conjugation are denoted by either F+ or F-. F+ is for the donor cell that has the F plasmid, and F- is for the recipient cell that lacks it. After the two bacteria are attached by the pilus, one strand of DNA of the F plasmid moves across the pilus from the F+ cell to the F- cell, and DNA synthesis occurs in both bacterial cells to generate double-stranded DNA. At the end of the process, the recipient bacterial cell has a portion of the donor bacterial cell’s genome and can express traits that were on that plasmid in the donor cell.

Examples of conjugation are antibiotic resistance of bacteria, chemical tolerance, and ability to use new metabolites. Special resistance (R) plasmids, or factors, that bear genes for resisting antibiotics and other drugs are shared among bacteria through conjugation. This course will be carrying out an experiment of the example of antibiotic resistance by demonstrating the transfer of the R factor which has resistance to the antibiotic tetracycline between two subspecies of E. coli, Top10F and JW0157. The donor F+ cell (Top10F) is resistant to tetracycline, which is found on a F factor plasmid, and the

recipient F- cell (JW0157) is resistant to chloramphenicol. (Figure 14.1). Ahead of class time, both subspecies were grown separately overnight in media enriched with the appropriate antibiotic at 37°C. The following day each culture was diluted by 1:100 dilution into media without any antibiotics and placed into a dry shaker at 37°C, where they were vigorously shaken until they reached the middle of exponential growth phase.

Figure 14.1 Schematic drawing of bacterial conjugation.



MATERIALS

Optional for instructor:

4-5 ml serological pipettes

Green pi-pump

Per group of three students should have:

Wax pencil

3 LB + tetracycline (10 µg/ml) & chloramphenicol (25 µg/ml) plates

(LB = Luria-Bertani)

3 Sterile cotton swabs

PROCEDURE OF BACTERIAL CONJUGATION

The following procedure should be performed by the instructor:

1. Fill three Petri plates with each of the cultures as follows:
   a. Donor only
   b. Recipient only
   c. Donor + recipient (equal volumes of each)*

   *Note: The volume of each culture depends on the size of the petri plate. The volume should be enough so that entire bottom of Petri plate is covered and liquid culture will agitate when the plate is placed onto a platform shaker.
2. Agitate cultures on a platform shaker (not too fast) at room temperature between 30-90 minutes.

To be performed by the students in groups of 3:

1. Label the bottom of the LB + tetracycline & chloramphenicol plates with student names and date. Label one plate with one cultural scenario: Donor Only, Recipient Only, and Donor + Recipient.
2. Each student is responsible for one scenario: Using a sterile cotton swab, make a lawn of bacteria from each broth culture onto a LB + tetracycline & chloramphenicol plate.

Incubate at room temperature in your lab section's racks.

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15. Metabolic Activities A: Inoculation of Media

When microbiologists or doctors come across a microbe that may be causing an infection, they need to be able to isolate, characterize, and identify this unknown microbe in order to treat the patient, develop further antimicrobials, or simply learn.

The process of identifying involves analyzing morphology, cultural characteristics, and physiological characteristics. **Morphology** includes viewing the cell shape, colony color, and results of specific stains, particularly Gram stain. "Cultural characteristics" refers to how the bacteria grows on various liquid and solid media, and how it consumes oxygen (obligate aerobe vs. facultative anaerobe vs. obligate anaerobe and so on). Physiological characteristics are determined through a series of biochemical assays. A **biochemical assay** is testing for aspects of microbial metabolism, which is the means of a microbe getting its energy and nutrients (carbon source) to live and reproduce. Essentially these assays, or tests, are detecting which enzymes are being producing by the bacterium. Bacteria can be differentiated by which specific enzymes are present or absent.

You will be learning the procedure of how to inoculate and perform the metabolic tests with a "known" bacterium for a two-week period. You will be working in pairs. Afterwards, you will be working individually for the next two-week period identifying an unknown bacterium.

For identifying the unknown bacterial strain, you will perform a Gram stain to determine if the strain is gram positive or gram negative and observe the cell shape. You will also decide what the colony color is by examining the original culture given to

you on a TSA plate. These three traits are considered most important when you are using a key to identify a stain. Unfortunately, while the colony color and other visual characteristic are very powerful, they also require extensive experience to use reliably. Only a few colony colors are easily interpreted, but when you have a distinctive color that characteristic should be given a rank just after Gram stain and cell shape. The metabolic tests are a "best-fit" system which works well even if some tests fail. Not every strain of a bacterial species may produce an expected result. A positive result simply means that the metabolism of interest occurred. Species range from mostly positive to mostly negative results and this helps make identification possible. You have to look at all results as a whole and figure out which bacterium matches closest to a bacterium in the Key, or "Unknown Booklet". The "Unknown Booklet" is a list of bacteria with morphological and cultural characteristics described and results of the biochemical assays. Your unknown will be one of these bacteria.

MATERIALS

*Per pair of students for "Knowns" ***

2 Blue racks
2 Inoculating loops
1 Inoculating needle
Wax pencil
2 Bunsen Burners
2 Strikers
9 test tube labels
Coffee can
1 of each type of medium:

TSA agar	(agar plate, Trypticase Soy)	Nitrate broth	(yellow cap tube)
Milk agar	(agar plate, opaque white)	MR-VP broth	(black cap tube)
Starch agar	(agar plate, black line on lid)	PR Sucrose	(blue cap tube)
Simmon's Citrate agar	(screw-top agar tube)	PR Dextrose	(green cap tube)
SIM medium agar	(clear cap tube)	PR Mannitol	(red cap tube)
Urea broth	(white cap tube)	PR Lactose	(yellow cap tube)

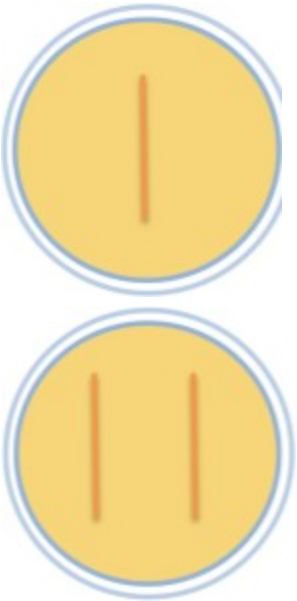
***Per student for "Unknowns":*

1 Blue rack
1 Inoculating loop
1 Inoculating needle
Wax pencil
Bunsen Burner
Striker
9 test tube labels
Coffee can
1 of each type of medium listed above

PROCEDURE OF METABOLIC ACTIVITIES: INOCULATION

1. The instructor will distribute one cultural plate per pair of students to share for the learning process ("Knowns"), but for identifying the Unknown Bacteria, students will work individually.
2. Inoculation of plates:
   a. Label the bottom of the plates with names, date, medium name, and bacterial label (name, letter, or number).
   b. Using aseptic technique, you will inoculate the medium with a single streak of bacteria on the milk and starch

plates. You will inoculate the TSA plate with two streaks of bacteria. See below. **When you remove bacteria from streak plate, you take from an isolated colony.**



Milk & Starch Strike Plate TSA Strike Plate

3. Simmon's Citrate Agar (screw cap):
   a. Using aseptic technique, you will transfer bacteria from an isolated colony using an inoculating loop from the bacterial plate onto a slant surface.
   b. Using a test tube label, label the tube "Citrate".
4. SIM Medium (clear cap):
   a. Using aseptic technique, you will transfer bacteria from an isolated colony an inoculating needle from the bacterial plate, and then stab the needle all the way to the bottom on the medium.
   b. Using a test tube label, label the tube "SIM".
5. Inoculation of all other broths (one tube of each):
   a. Using aseptic technique, you will transfer bacteria from

an isolated colony using an inoculating from the bacterial plate into a broth. This only has to be done once per tube.
b. The tubes are pre-labeled by their cap-color which indicates the medium:

MR-VP broth	(black cap broth tube)	**Phenol Red (PR) tubes have Durham tubes inside**	
Nitrate broth	(yellow cap broth tube)	PR Sucrose	(blue cap broth tube)
Simmon's Citrate agar	(screw-top agar slant)	PR Dextrose	(green cap broth tube)
SIM medium agar	(clear cap agar tube)	PR Mannitol	(red cap broth tube)
Urea broth	(white cap broth tube)	PR Lactose	(yellow cap broth tube)

6. Place all test tubes into a coffee can. Put lab tape onto the outside of the coffee can. Label the coffee can with your name(s), bacterial label, and date. Place in your lab section's incubator and correctly labeled shelf.
7. Store your 4 plates (this includes the original bacterial culture plate) in a rack upside down in your lab section's lab bench area at room temperature. We do not incubate the plates for a week due to dehydration of the medium.

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Brown, A. E. (2009). Benson's Microbiological Applications: Laboratory Manual in General Microbiology. New York: McGraw Hill.

Chess, B. (2015). Laboratory Applications in Microbiology: A Case Study Approach. New York: McGraw Hill. Cowan, M.K. (2015). Microbiology: A Systems Approach (4th edition). New York: McGraw Hill.

16. Metabolic Activities B: Physiological Characteristics Results & Interpretation

Now that your bacterial species has had the time to grow and metabolize on several types of media, it is time to collect the results of the biochemical assays to determine physiological characteristics. When performing the Unknown procedure, you will be able to interpret all data, and identify the bacterium.

Some biochemical test results are simply interpretation of appearance due to the medium having a pH indicator. For other tests, chemical reagents must be added to the inoculated medium so that the results can be seen. The procedure of this exercise will go through the steps that need to be done in order to receive results of the biochemical assays for interpretation along with an explanation of the actual metabolism that is being carried out by the bacteria. Photos of positive and negative results are available.

MATERIALS

Per pair of students for "Knowns"

Blue rack and empty 20 mm test tube

Coffee can and wax pencil

Green pi-pump and 5 ml serological pipette

Your inoculated tubes and plates from previous lab:

TSA agar	(agar plate, Trypticase Soy)	Nitrate broth	(yellow cap broth tube)
Milk agar	(agar plate, opaque white)	MR-VP broth	(black cap broth tube)
Starch agar	(agar plate, black line on lid)	PR Sucrose	(blue cap broth tube)
Simmon's Citrate agar	(screw-top agar tube)	PR Dextrose	(green cap broth tube)
SIM medium agar	(clear cap agar tube)	PR Mannitol	(red cap broth tube)
Urea broth	(white cap broth tube)	PR Lactose	(yellow cap broth tube)

This first stage is the "*Knowns*" run-through the tests will show how to interpret the tests. Students will do this work in pairs.

The second stage is the "*Unknowns*" where the actual identification of an unknown culture occurs. In that stage, each student will perform these procedures, independently.

PROCEDURES FOR METABOLIC ACTIVITY TESTS:

1. Collect your test tubes and plates from the incubator. Remove your test tubes from the coffee can, and place them in a blue rack.
2. Two tests take longer to produce accurate results (Voges-Proskauer and Oxidase). Start them early, to save time. These two tests each use a medium that also covers another test. Start with:
   a. MR-VP broth (Voges-Proskauer test and Methyl Red test)
   b. TSA plate (Oxidase test and Catalase test)

a. MR-VP Broth (black cap). Used for two tests:

1. Using a 5 ml serological pipette and a green pi-pump, remove 2 ml from the MR-VP tube, and transfer aseptically into an empty 20 mm test tube.
2. Label the 2 ml tube VP with the wax pencil.

1. Voges-Proskauer test

- Wear gloves. Add 10 drops of each: Barritt's reagents A & B into the 2 ml.
- Shake tube left to right to oxygenate bacteria for 1 to 2 minutes.
- Set aside for at least 30 minutes. Do not read immediately. Then observe.
- Label the remaining 8 ml in the black cap tube MR.

2. Methyl Red test

- Add ¼ dropper full of methyl red reagent and shake. Then observe color.

MR-VP broth contains a large quantity of glucose. Some bacteria are able to ferment glucose through different pathways, such as butylene glycol pathway and mixed acid pathway. The butylene glycol pathway is where acid products (pyruvate) are quickly converted to the alcohols acetoin and 2,3-butanediol. This is what the Voges-Proskauer test is testing for by adding Barritt's A and B. The reagents oxidize the acetoin, if present, producing a red to burgundy color for a positive result. (Figure 16.1).

The mixed acid pathway is when glucose is fermented to produce several acids, such as lactic, acetic, and formic acids, that are fairly stable, lowering the pH of the medium. Methyl red pH indicator is added to the medium. The change to a red color, showing a low pH of at least 4.4, is a positive result. (Figure 16.2).

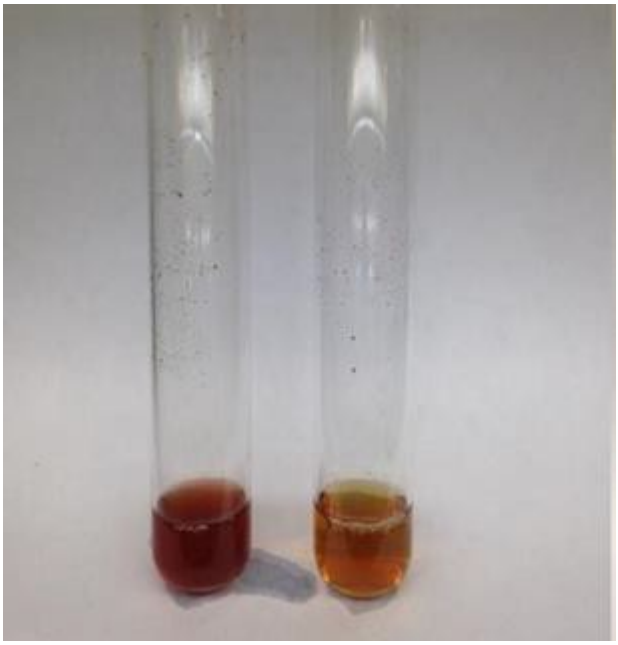


Figure 16.1: Voges Proskauer test: The tube on the left is positive (red/ burgundy color) while the tube on the right is negative.

Figure 16.2: Methyl red te
positive (red color) while
negative. This test is base
not acidic.

b. TSA Plate: Used for two tests

1. Oxidase test

This test identifies bacteria that produce the enzyme **cytochrome oxidase**.

Cytochrome oxidase participates in the electron transport chain of aerobic respiration, transferring electrons to oxygen and producing water. In aerobic respiration, oxygen is the terminal electron acceptor. This enzyme is detected by an oxy-swab which contains the oxidase reagent of an artificial electron acceptor. A positive for cytochrome oxidase is a purple color. (Figure 16.3).

- Add a drop of distilled water directly onto an oxy-swab.
- Using one streak on the TSA plate, roll the oxy-swab along the outside edge of bacterial streak.
- Press swab against inside of petri plate lid to saturate bacteria with reagent and get past the glycocalyx.
- Wait at least 1 minute before looking at result.

2. Catalase test

The catalase test is used to differentiate bacteria that are aerobes or facultative anaerobes from bacteria that are obligate anaerobes, microaerophiles or aerotolerant anaerobes. During aerobic respiration, oxygen is used as a terminal electron acceptor, forming water. However, hydrogen peroxide is a byproduct, which is a reactive oxygen species which can react with parts of the cell and cause damage. Aerobes and facultative anaerobes can use enzymes to detoxify oxygen. For example, the enzyme **catalase** breaks down

hydrogen peroxide into water and harmless oxygen gas. To detect for the enzyme catalase, apply hydrogen peroxide to a bacterial streak. A positive result is bubbles because this is the oxygen being released from the bacteria. (Figure 16.4).

- Add a dropper full of hydrogen peroxide directly to the other streak on the TSA plate (not used for the oxidase test) and observe.

Figure 16.3: Oxidase test: The swab on the left is positive (dark purple) while the swab on the right is negative (no color change).

Figure 16.4: C
while the pla

c. Starch Hydrolysis

The differential medium starch agar (black line on lid) is used to distinguish bacteria that can hydrolyze, or break down, starch into mono and disaccharides from bacteria that cannot. Bacteria produce the exoenzyme **amylase**, which breaks the bonds apart in the starch. Iodine must be added to the plate to detect that the reaction occurred. Iodine complexes with starch to give a black or dark purple color, but if the starch has been broken down into simple sugars, a yellow to brown color will be present. (Figure 16.5). Amylase is an exoenzyme, so the cases where the culture itself is blocking the iodine from entering the agar do not count as a sign of amylase activity.

- Add iodine directly to the surface of the plate. Completely cover the plate.
- Observe color change.

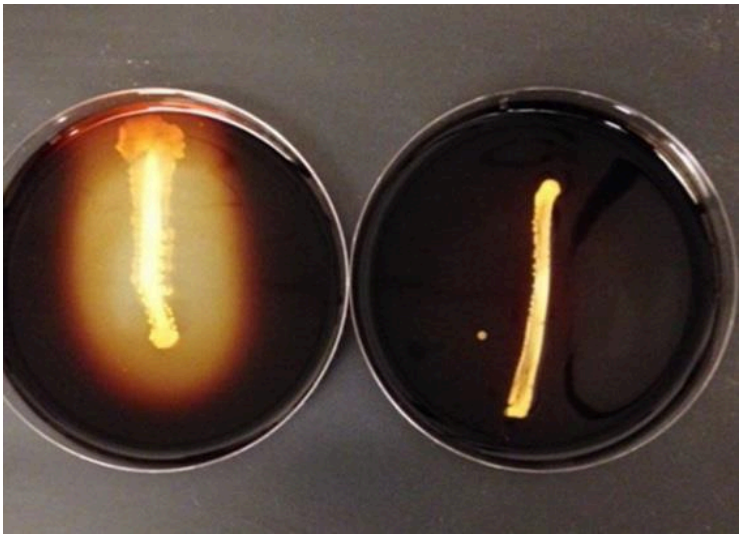


Figure 16.5: Starch hydrolysis: The plate on the left is positive (clearing) while the plate on the right is negative (no clearing).

Figure 16.6: C
(clearing) wh

d. Casein Hydrolysis

The differential medium milk agar is used to distinguish bacteria that can hydrolyze the protein casein into amino acids from bacteria that cannot. Casein is the major protein in milk, giving it its white color. Bacteria hydrolyze casein by producing the exoenzyme **caseinase**, which breaks down the peptide bonds between the amino acids. If casein is broken down in the milk agar, the opaque white color of the milk now appears as a clearing due to being amino acids and no longer a polypeptide (protein). (Figure 16.6).

- Observe plate for clearing.

e. Urea Hydrolysis

Urea is a waste product of animal metabolism that is broken down by a number of bacteria. Urea broth is a differential medium that contains a pH indicator that tests the ability of bacteria to produce the exoenzyme **urease** that hydrolyzes urea into ammonia and carbon dioxide. This enzyme is produced by some bacteria.

Ammonia takes up H+ to become NH4+ and increases the pH so in effect the phenol red pH indicator changes from yellow to a bright pink as a positive result. (Figure 16.7).

- Observe color of medium.

Figure 16.7: Urea Hydrolysis: The tube on the left is positive (hot pink color) while the tube on the right is negative (no color change/ orange).

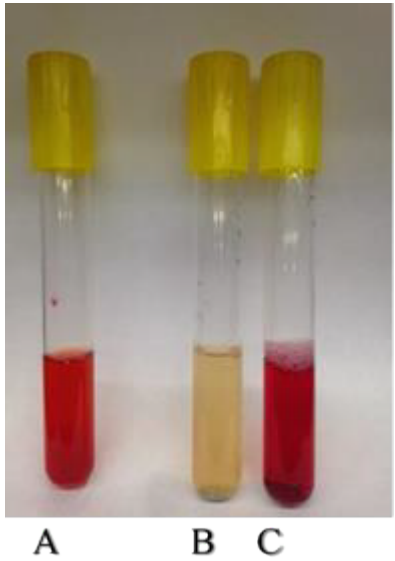


Figure 16.8: Nitrate Reduction: Tube A is positive (red color). Tube B and C both have Zinc granules added. Tube B is positive (yellow color). Tube C is negative (red color).

f. Nitrate Reduction

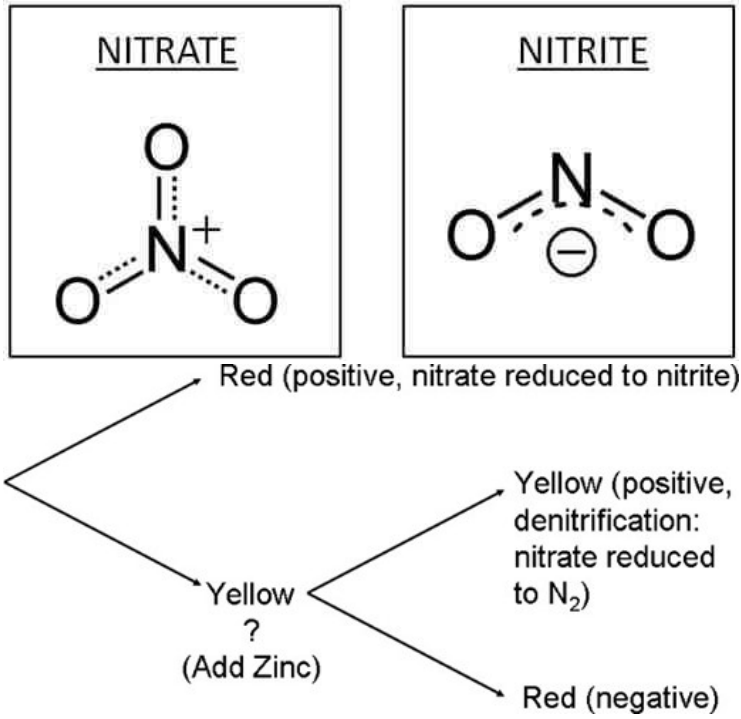
Some fermenters can reduce nitrate (NO3-) to nitrite (NO2-) in order to regenerating NAD+ from NADH. As fermenters, they get very little energy from their metabolism and die from their

waste. These bacteria will leave behind a lot of NO2-. This is called **dissimilatory nitrate reduction**.

Some facultative anaerobes and obligate anaerobes use nitrate, not oxygen, as a terminal electron acceptor during anaerobic respiration. This type of anaerobic respiration is called **denitrification**. These bacteria have enzymes, including nitrate reductase, that transfer electrons from the electron transport chain to NO3-, partially reducing it to NO2- and then eventually reduce it to nitrous oxide (N2O) or nitrogen gas (N2). Neither NO3- nor NO2- remain, because of the much greater efficiency of respiration.

- Wearing gloves, Add 10 drops of each: Nitrate Reagents 1 & 2.
- Gently shake the tube from left to right.
- If a red color is produced, then the bacterium is positive for nitrate reductase. (Figure 16.8).
- If a yellow color remains, another reagent has to be added to confirm a negative result.
- Add Zinc granules and shake. It may take time to see a red color. (You do **not** add Zinc granules to an already red positive tube.)

Nitrate reagents 1 & 2 react with nitrite to make a red color. Zinc is a catalyst for reducing nitrate to nitrite, similar to dissimilatory nitrate reduction. Zinc checks for the original nitrate.



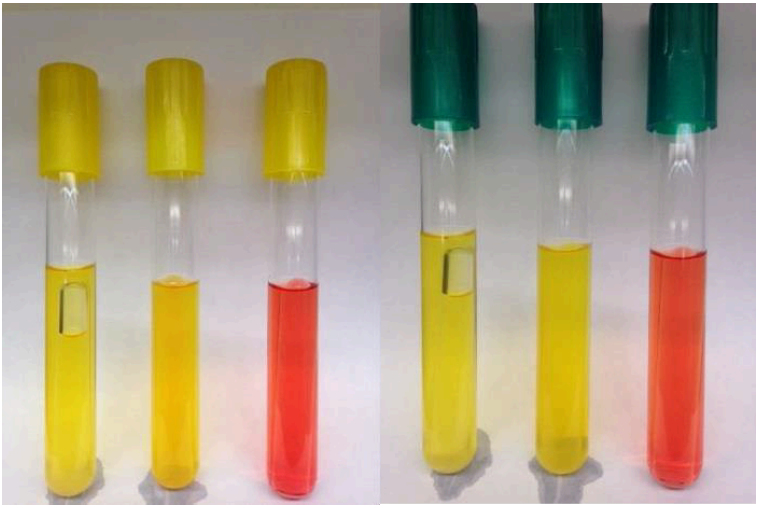
g. Phenol Red Carbohydrate Broth

Phenol red carbohydrate broth is a differential medium used to detect for fermentation of specific carbohydrates, most commonly lactose, dextrose (glucose), mannitol, and sucrose. The ability of bacteria to ferment a specific sugar may be used to differentiate between genera and species. The medium contains the pH indicator phenol red, which turns yellow in the presence of **acid** (as low as pH 6.8), and a small inverted tube

called the Durham tube to collect any **gas** that may be released during **fermentation**. (Figure 16.9)

The number of carbons in the acid produced determines gas production. For instance acetic acid production (2C) would be associated with gas production while lactic acid production (3C the same as pyruvate) would produce no gas.

- Observe color of media in the 4 tubes.
- Observe Durham tube, looking for gas in the Durham tube.



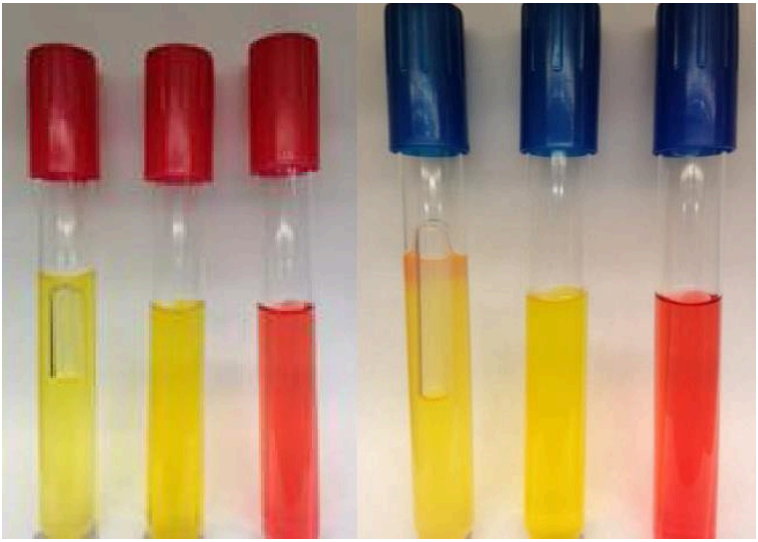


Figure 16.9: Phenol Red Carbohydrate Broth: For each set of three tubes, the tube on the left is positive for fermentation due to acid and gas (yellow and bubble in Durham tube) while the tube in the middle is only positive for acid (yellow). The tube on the right is negative for fermentation (red and no bubble). Color cap system: Yellow cap = lactose, Green cap = dextrose, Red cap = mannitol, Blue cap = sucrose.

h. SIM Medium. Used for 2 tests

SIM medium is a semisolid differential medium that is used for the detection of sulfur reduction, indole production, and motility. It is recommended for the differentiation for gram negative enteric bacilli (coliforms). We will be looking for the results of sulfur reduction and indole production only.

1. Sulfur Reduction

Coliforms degrade the protein casein that is in the SIM medium into amino acids. One particular amino acid is cysteine. SIM medium also contains an iron- containing compound and sulfur in the form of sodium thiosulfate. Sulfur reduction produces hydrogen sulfide gas in the anaerobic process. There are two different courses of action. These can be achieved depending on enzymes produced. The enzyme **cysteine desulfurase** breaks down cysteine to produce pyruvic acid, ammonia, and **hydrogen sulfide** gas. The other enzyme that serves in sulfur reduction is thiosulfate reductase. Sodium thiosulfate is a terminal electron acceptor in anaerobic respiration, and **thiosulfate reductase** catalyzes the reaction of reducing thiosulfate (sulfur) to the hydrogen sulfide gas product. The iron- containing compounding the SIM medium will react with the hydrogen sulfide gas to produce ferrous sulfide, showing a black precipitate to indicate a positive result. (Figure 16.10).

- Observe color of medium.

2. Indole test

SIM medium is also used to detect for the indole production. Bacteria partially breakdown the amino acid tryptophan to produce pyruvic acid, ammonia, and **indole** by using the enzyme **tryptophanase**. (Figure 16.11)

- Add ¼ to ½ of a dropper full of Kovac's reagent to the tube in order to see a layer on top of the semisolid medium.
- Observe color of reagent.

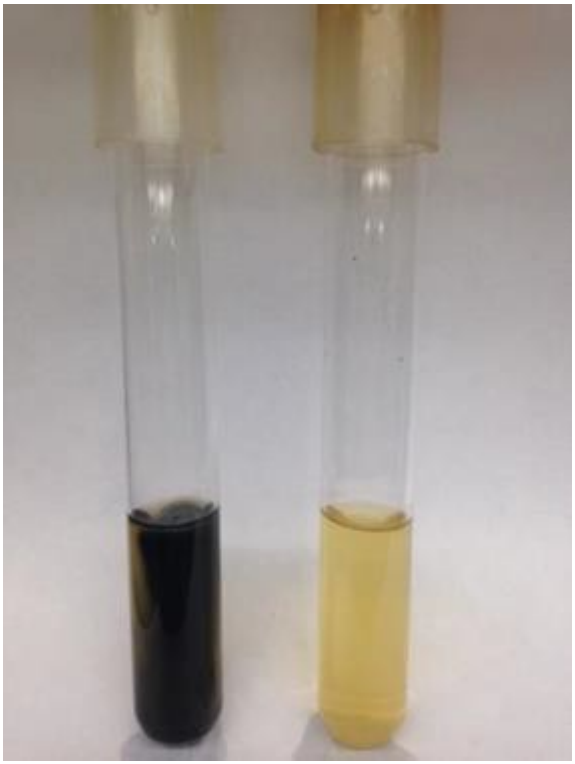


Figure 16.10: Hydrogen Sulfide Production: The tube on the left is positive (black precipitate) while the tube on the right is negative (no color change).

Figure 16.11: Indole Productio
is positive (red color) while th
negative

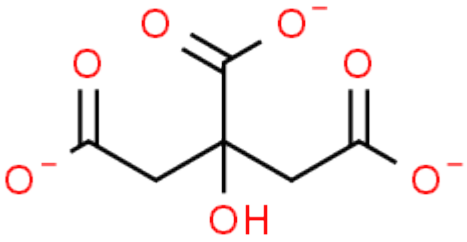
(no color change/ yellow).

i. Citrate Utilization

Citrate utilization uses the medium Simmon's Citrate agar that contains sodium citrate as **a sole carbon source**. It also

contains inorganic ammonium salts as a sole nitrogen source. Citrate is metabolized in the citric acid cycle (or TCA) by the enzyme citrate permease. For uptake from the medium, citrate transporters must be able to handle the three negative charges (by symport or antiport) which will typically increase pH. The agar contains a pH indicator called bromthymol blue. In alkaline conditions, the green agar will turn to a deep blue color. (Figure 16.12).

- Observe color of medium.



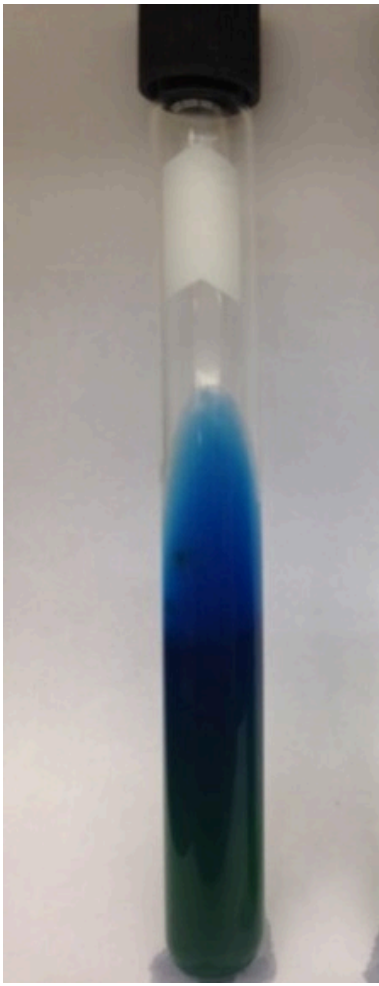


Figure 16.12: Citrate Utilization: The tube on the left is positive (deep blue color) while the tube on the right is negative (no color change/ green). Just a blue tinge at the top is also a negative.

j. IMViC tests

Four of the metabolic tests can be grouped together naming them as the IMViC tests (I: indole test; M: methyl red test; V: Voges-Proskauer test; and C: citrate utilization test). These tests are used to differentiate bacteria that belong to the family Enterobacteriaceae for water analysis looking for fecal contamination, differentiating *Escherichia coli* and *Klebsiella aerogenes*. *E. coli* is found in human and animal intestines whereas *K. aerogenes* is a soil bacteria. These bacteria are morphologically and culturally similar. However, on these four tests, they physiologically differ. The two bacteria give exactly the opposite reactions.

	Indole	**Methyl Red**	**Voges-Proskauer**	**Citrate**
E. coli	+	+	–	–
K. aerogenes	–	–	+	+

Table 16.1: IMViC tests: Comparing the physiological difference between
E. coli and K. aerogenes.

Important:

After you have collected all your data, interpret the results and use the "Unknown Booklet of Organisms" to identify your unknown. Fill out your Descriptive Lab Chart, and discuss with your instructor your unknown identification. **Do not discard any of your test tubes, plates, or oxy-swab** until the unknown identification has been verified correctly. If you have your unknown identification incorrect, your instructor can analyze your tests to see if everything has been interpreted correctly.

Sometimes bacteria do not produce an enzyme that it typically should or vice versa. This may alter your deduction of the identification. Do not worry about a few mismatches, except for Gram-stain and cell shape. The metabolic tests can get you to a correct result even if a few of them don't work correctly.

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