

Fundamentals of Microbiology
Atlanta Metropolitan State College

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Introduction to the Microscopic World

Beyond the naked eye, there exists a world that can only be seen through the aid of magnification. The human eye can only discern objects that are approximately 0.1mm in size. That is quite an amazing feat! Unfortunately, microscopic organisms are much smaller. For instance, most bacteria range from 0.5 to 10 micrometers in size. Because of this, we use special instruments called microscopes to magnify these tiny organisms so that we can readily observe them.

Organisms are divided into 3 domains of life: Archaea, Prokarya, and Eukarya. You may be most familiar with Eukarya, which include the double membraned organelled eukaryotes. Some of the members of this domain include Arthropods, Plants, Fungi and Animals. Members of Prokarya are also pretty well known. For example, all bacteria are prokaryotes and are defined by the lack of intracellular organelles. Members of Archaea are a bit more diverse, as they vary in size and cellular structure. Interestingly, Archaeans most closely resemble prokaryotes, but have a greater genetic association to eukaryotes. In this lab, we will study a few members of each domain and describe the similarities between them.

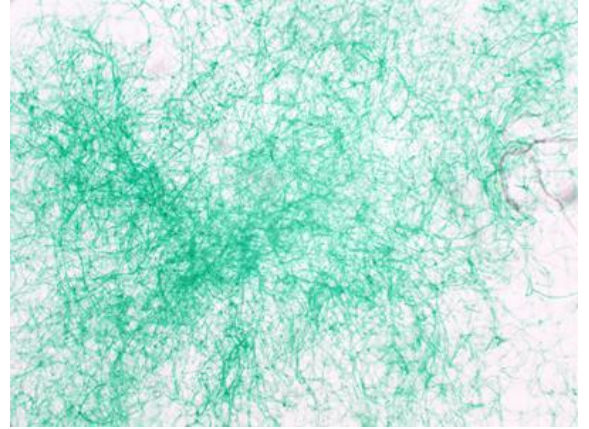


Figure 1.1: *Penicillium mycelium* conidiophores at 1000x magnification.

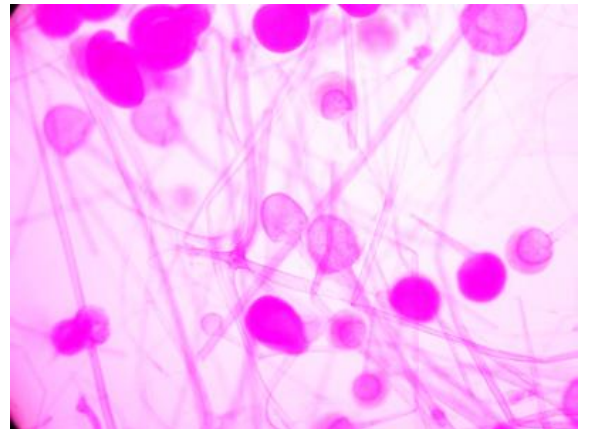


Figure 1.2: *Rhizopus nigricans* at 1000x magnification.

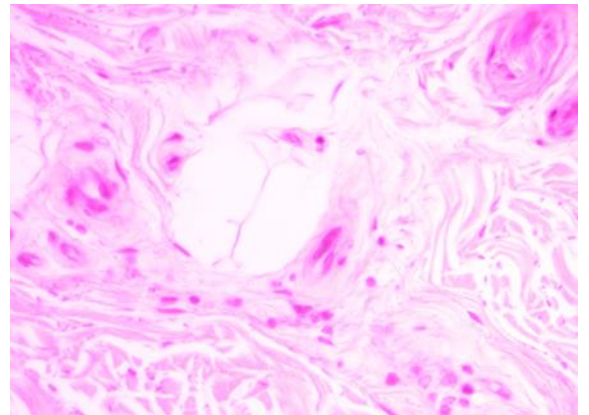


Figure 1.3: Human stratum corneum at 1000x magnification.

Learning Objectives

Students should be able to describe the structural differences between Archaeans, Eukaryotes and Prokaryotes.

Materials and Equipment

Saccharomyces cerevisiae slides
Aspergillus nigricans slides
Entamoeba histolytica slides
Staphylococcus aureus slides
Escherichia coli slides
Bacillus subtilis endospore slides
Halobacterium salinarium slides
Mixed Rotifers slides
Epithelial tissue slides
Mesenchymal tissue slides
Microscope

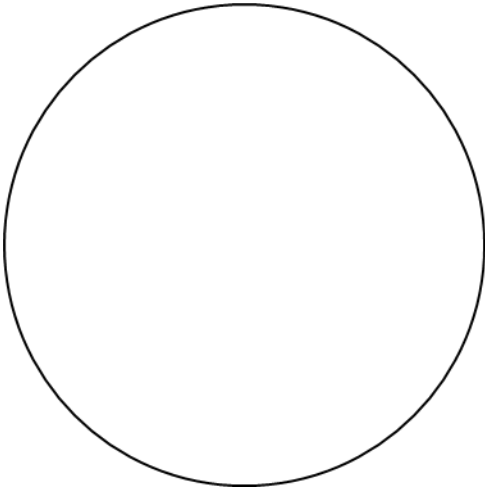
Procedure

Slide Analysis

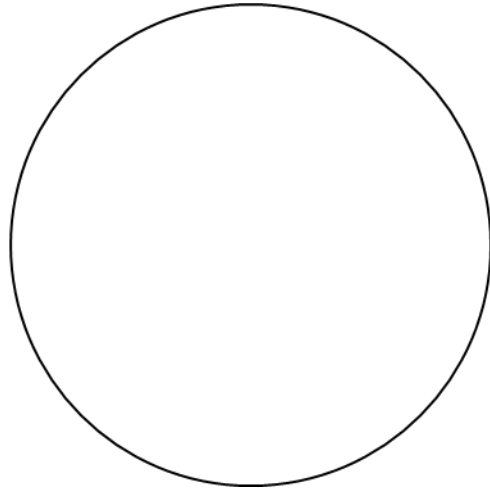
Prep Stage

- ☐ Obtain prepared slides from instructor and visualize at 10x, 20x, and 40x magnification
- ☐ Document your observations in the Results section.

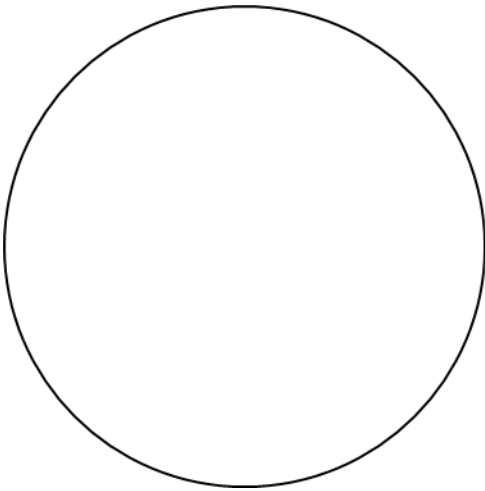
Results



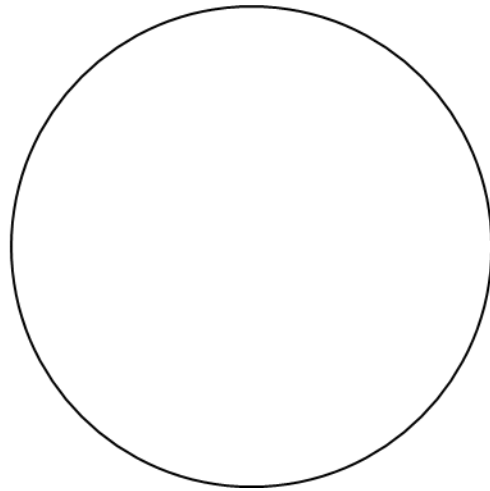
Slide: _____



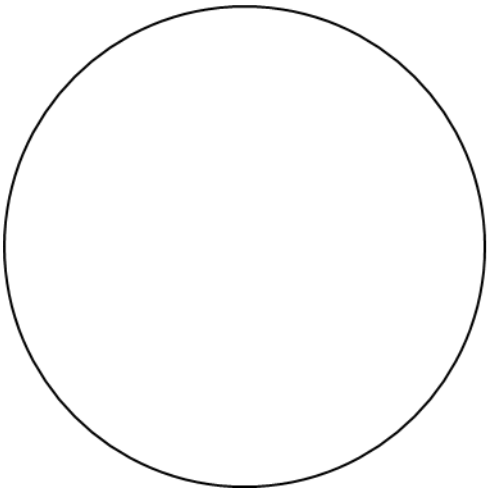
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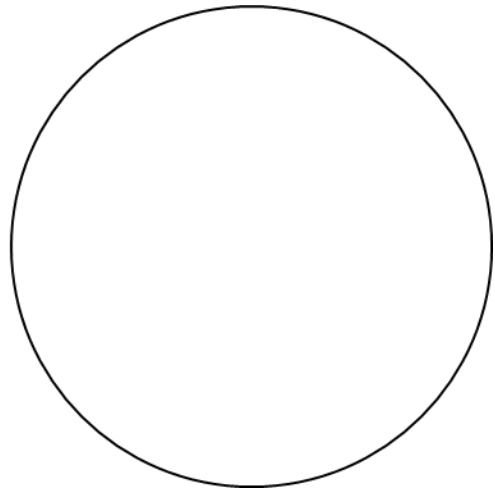
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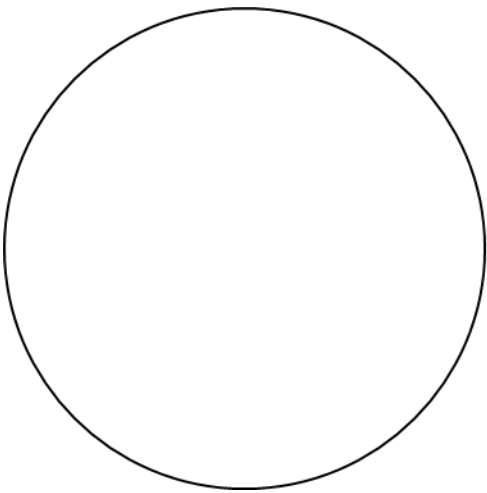
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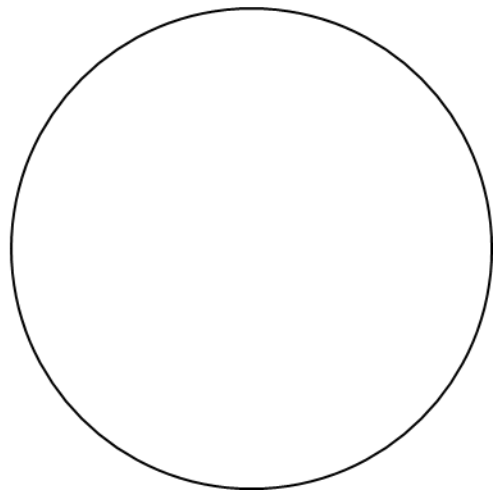
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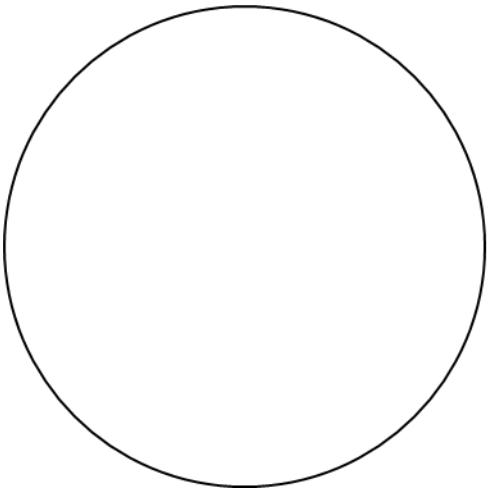
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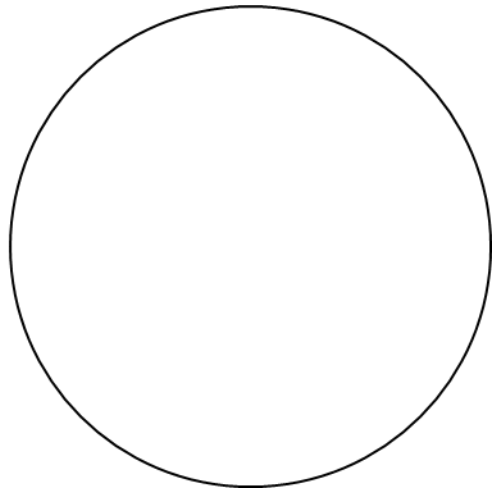
Slide: _____



Slide: _____



Slide: _____



Slide: _____

Lab Notes

Smear Preparation

Although staining is a critical component in the identification of microbiological organisms, smear preparation ultimately determines the quality of any results. Typically, bacteria are applied to a clean microscope slide and heat fixed, but this varies depending upon the desired stain. For instance, heat fixation would melt the bacterial capsule and render any capsule staining results useless. As the semester goes on, you will perform smear preparation many times. In this lab, you will practice preparing a smear from both liquid and solid cultures.

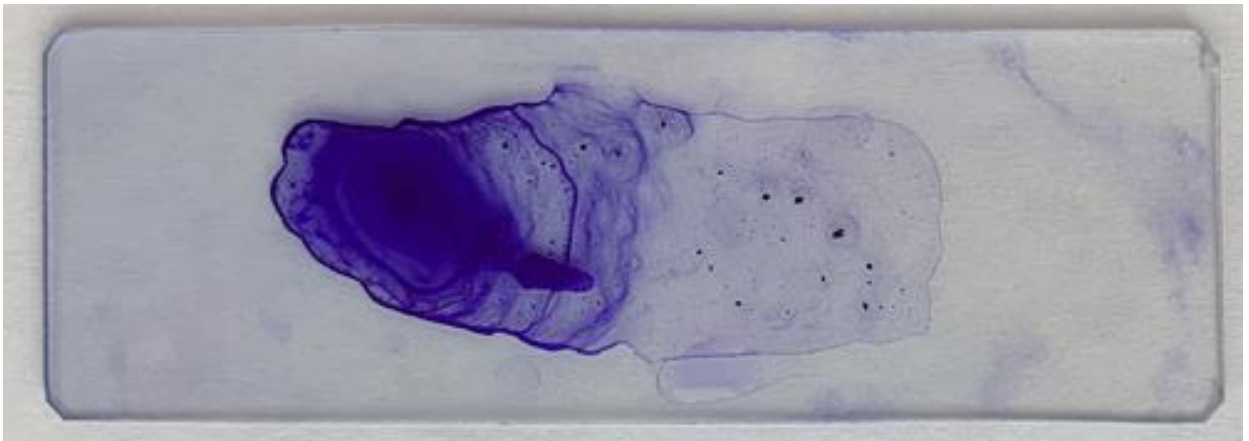


Figure 2.1: An example of a poorly prepared smear. Too many bacteria were applied to the slide (too thick) and resulted in accumulation of crystal violet and erroneous results.

Learning Objectives

After completing this exercise, students will be able to prepare bacterial smears from liquid and solid cultures.

Materials and Equipment

- Bacterial culture in broth
- Bacterial culture on agar plate
- Bunsen burner
- Inoculating loop
- Clean slides
- Microscope
- Water bottle
- Wax Pencil

Procedure

Part 1: Smear Preparation from a Liquid Culture

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve agar plate from the refrigerator, remove the parafilm wrapping and sit the plate on the benchtop with the agar side facing up.
- ☐ Using a wax pencil, label your slides as shown in Figure 2.2. *Draw one circle on the bottom/underside of the slide.*
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

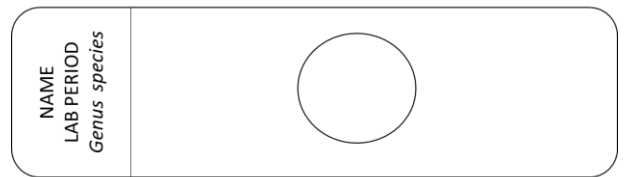


Figure 2.2: A properly labeled slide.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

Smear Preparation from a Liquid Culture

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Gently resuspend the bacteria into the medium by holding the culture tube between your palms and alternating moving them back and forth. The bacteria should no longer be settled on the bottom of the culture tube, as shown in Figure 2.3.
- ☐ Place culture tube back on test tube rack.

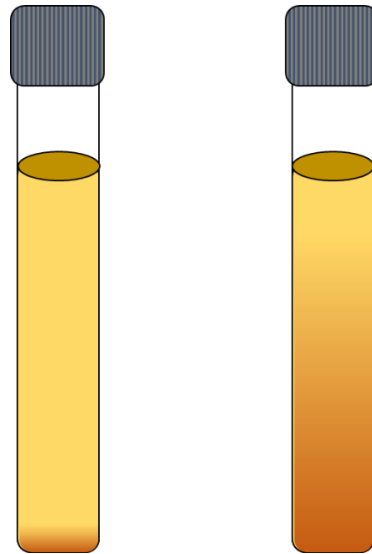


Figure 2.3: Bacteria in culture tubes. Left: Bacteria is settled on the bottom of the culture tube. Right: Bacteria has been resuspended into the medium and is ready for inoculation.

- ☐ Retrieve the inoculating loop and place in the inner cone of the flame, as shown in Figure 2.4. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.

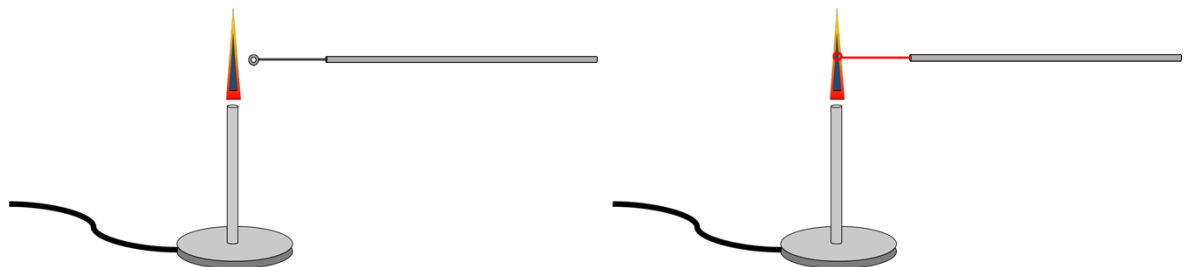


Figure 2.4: Sterilizing the inoculating loop. **Left:** Place the inoculating loop parallel to the benchtop. **Right:** Insert the inoculating loop into the inner blue cone of the flame. Slowly move the inoculating in and out of the flame until the entire wire is bright red.

- ☐ Retrieve the culture tube and hold with your left hand. Be sure to work close to the Bunsen burner for the remaining steps.
- ☐ Using the pinky finger of your right hand, remove the cap of the culture tube. *Do not set down.*

- Quickly pass the mouth of the culture tube through the flame of the Bunsen burner as shown in the left panel of Figure 2.5.
- While working close to the flame of the Bunsen burner, quickly insert the inoculating loop into the culture tube, as shown in the right panel of Figure 2.5. Be sure to not touch the inner walls of the culture tube. If the wire of the inoculating loops touches anything besides the medium, you must re-sterilize the loop. Quickly remove the loop and keep close to flame.

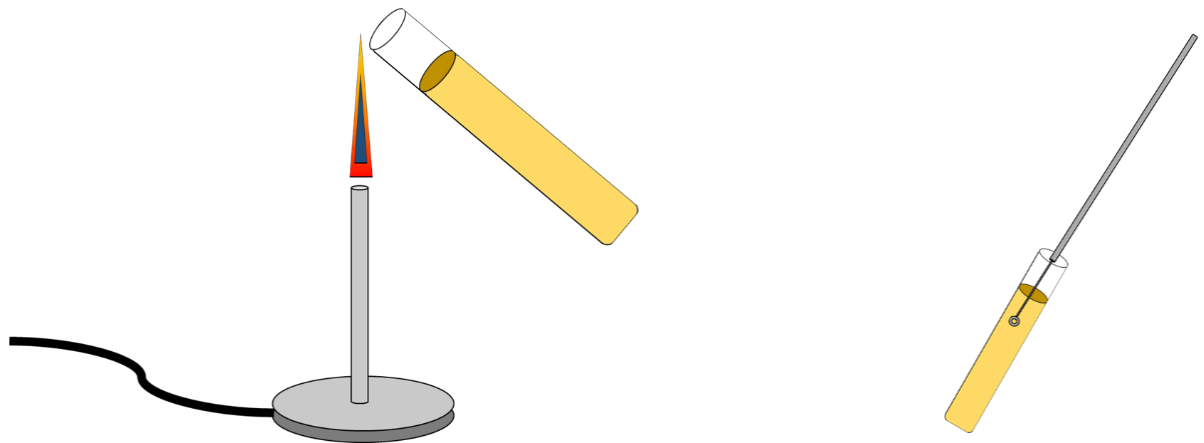


Figure 2.5: Left. Remove cap and slightly tilt culture tube before flaming the mouth of the tube. Right. Slightly tilt culture tube and insert inoculating loop. Be sure to not touch anything but the medium with the inoculating loop.

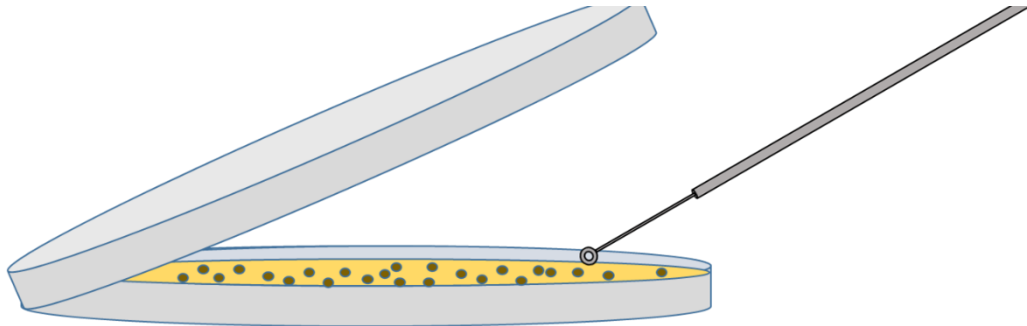
- Quickly re-flame the mouth of the culture tube and immediately replace the cap. Set the culture tube back on the test tube rack.
- Touch the inoculating loop to the region of the slide where you made a circle with the wax pencil. Move around in a tight circular motion to spread out the bacteria.
- Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- Allow to air dry for 10-30 minutes.
- The slide is now ready for staining or storage.

Smear Preparation from a Solid Culture

- Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.

- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to the region of the slide where you made a circle with the wax pencil.
- ☐ Retrieve the streaked nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the petri dish (no more than 45 degrees). As shown in Figure 2.6, carefully pick a small and isolated colony from the plate and close the petri dish. Set the petri dish on the benchtop.

Figure 2.6: Picking a colony from an agar plate. Be sure to tilt the lid no more than 45 degrees to prevent debris from falling inside of plate causing contamination.



- ☐ Add the colony to the region of the slide where you made a circle with the wax pencil. Move the inoculating loop around in a tight circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- ☐ Allow to air dry for 10-30 minutes.
- ☐ The slide is now ready for staining or storage.

Lab Notes

Simple Staining

The instructor will provide you with a sample that contains the unknown bacteria. The objective of this exercise is for students to be able to separate bacterial cultures into a pure isolate. To simplify this, you will be provided with a sample that contains bacteria that produce two different pigments. Upon proper streaking, students will have distinct colonies of bacteria upon which to gram stain for characterization.

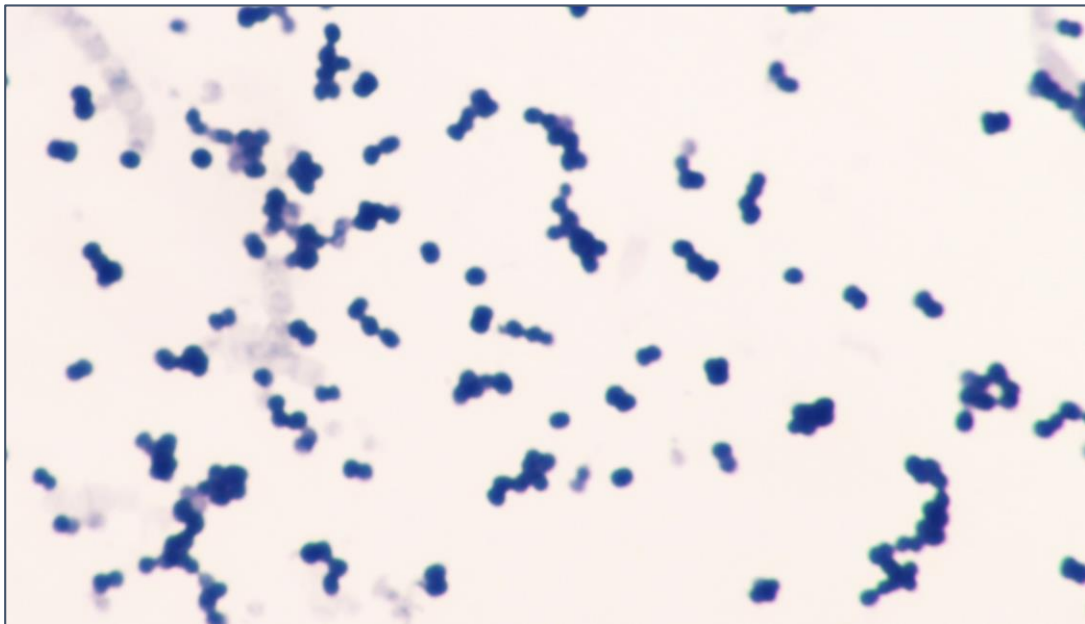


Figure 3.1: *Sarcina subflava* stained with methylene blue at 1000x magnification.

Learning Objectives

This exercise is meant to reinforce streaking and proper aseptic technique. Additionally, students will learn how to differentiate between different bacterial growth characteristics and gram staining results.

Materials and Equipment

Bacterial culture in broth
Bunsen burner
Inoculating loop
Clean slides
Microscope
Immersion Oil
Methylene Blue
Bibulous Paper
Water bottle
Wax pencil

Procedure

Smear Preparation from a Liquid Culture

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve agar plate from the refrigerator, remove the parafilm wrapping and sit the plate on the benchtop with the agar side facing up.
- ☐ Using a wax pencil. *Draw one circle on the bottom/underside of the slide.*
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

Smear Preparation from a Liquid Culture

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Gently resuspend the bacteria into the medium by holding the culture tube between your palms and alternating moving them back and forth. The bacteria should no longer be settled on the bottom of the culture tube.
- ☐ Place culture tube back on test tube rack.
- ☐ Retrieve the inoculating loop and place in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.

- ☐ Let loop cool for approximately 15 seconds.
- ☐ Retrieve the culture tube and hold with your left hand. Be sure to work close to the Bunsen burner for the remaining steps.
- ☐ Using the pinky finger of your right hand, remove the cap of the culture tube. *Do not set down.*
- ☐ Quickly pass the mouth of the culture tube through the flame of the Bunsen burner.
- ☐ While working close to the flame of the Bunsen burner, quickly insert the inoculating loop into the culture tube. Be sure to not touch the inner walls of the culture tube. If the wire of the inoculating loops touches anything besides the medium, you must re-sterilize the loop. Quickly remove the loop and keep close to flame.
- ☐ Quickly re-flame the mouth of the culture tube and immediately replace the cap. Set the culture tube back on the test tube rack.
- ☐ Touch the inoculating loop to the region of the slide where you made a circle with the wax pencil. Move around in a tight circular motion to spread out the bacteria.
- ☐ Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- ☐ Allow to air dry for 10-30 minutes.
- ☐ The slide is now ready for staining.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

Simple Staining

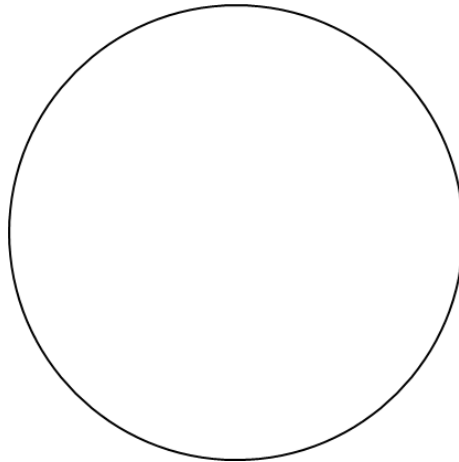
Methylene Blue Procedure

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Secure the slide with a clothespin and quickly pass the slide through the flame 3 to 4 times to heat fix the bacteria to the slide.
- ☐ Set the slide on the staining tray rack.
- ☐ Turn off the gas supply to the Bunsen burner.
- ☐ Flood the slide with Methylene Blue for 1 minute. Pour off excess stain into staining tray.
- ☐ Tilt the slide and carefully rise the slide with water until the water runs clear. *Be careful to not apply too much water pressure and remove the sample from the slide.*
- ☐ Using bibulous paper, carefully dry the front and back of slide. Carefully blot the area for the analysis.
- ☐ Set the slide back on the staining tray rack.
- ☐ The slide is now ready for analysis.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE PROCEEDING. IF NECESSARY, SLIDES CAN BE STORED AND STAINED AT A LATER TIME.

- ☐ Set the microscope on the benchtop and retrieve the immersion oil.
- ☐ Set the objective to 10x and focus the smear into your field of view.
- ☐ Be mindful to select a field of view that clearly shows the bacteria in a monolayer. Crowded areas of bacteria give erroneous results.
- ☐ Sequentially move up in objective magnification until you are focused on the target area at 40x magnification.
- ☐ Swing the objective halfway to 100x and add a drop of immersion oil to the area of analysis.
- ☐ Focus the specimen at 100x.
- ☐ Document your observations in the Results section.

Results



Bacterium: _____

Shape: _____

Arrangement: _____

Questions

1. Why was it necessary to only use one stain?
2. Can methylene blue stain any bacterial cell?
3. What are the chemical properties of methylene blue?

[illegible]

Gram Staining

Bacterial cell walls are not created equal. Some bacteria have a thick layer of peptidoglycan fortifying their cell walls, whereas other bacteria have a much thinner layer but are enveloped by an additional outer membrane. To accurately classify bacterial cells, one must be able to determine its Gram status. In, 1884 Christian Gram developed a differential staining method that allows us to quickly visualize the difference in bacterial cell wall composition. In the method, one treats heat fixed cells with Crystal violet, Iodine, Alcohol and Safranin.

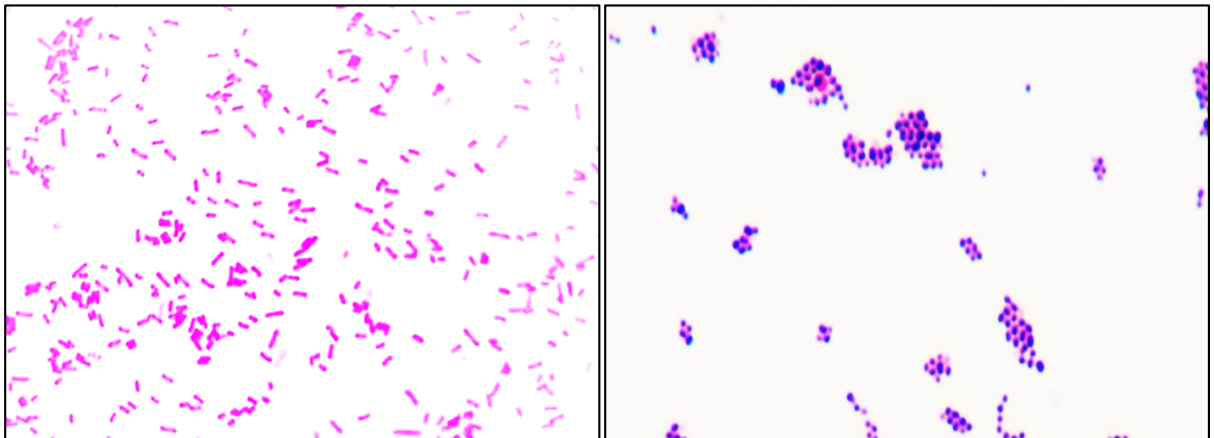
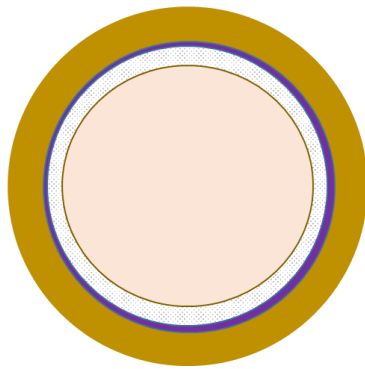


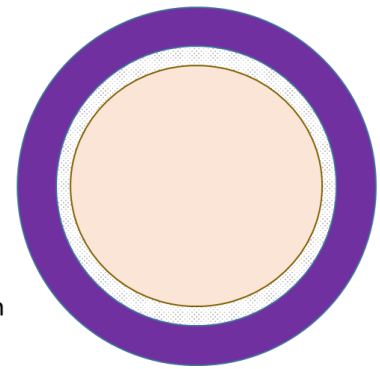
Figure 4.1: Gram stained bacterial cells. **Left panel:** Gram negative *E. coli* cells. **Right panel:** Gram positive *S. aureus* cells.

In this technique, crystal violet is added to a bacterial smear as the primary stain. Both gram positive and gram negative cells with stain purple. Mechanistically, the chloride ion of crystal violet disassociates in solution and crystal violet is now charged and free to traverse the cell wall. When iodine is added, it binds the charged crystal violet molecule. Next, alcohol is added as a decolorizer to remove any unbound crystal violet. The crystal violet-iodine complex is too large to pass through the peptidoglycan crosslinks and is retained in gram positive cells. However, the complex is simply washed away in gram negative cells because of the lack of a prominent peptidoglycan layer. Last, the smears are counterstained with safranin which stains cells pink.

Gram negative cell wall



Gram positive cell wall



Key





	Cytoplasm
	Cell membrane
	Periplasmic space
	Layer of peptidoglycan

Figure 4.2: Left panel: Gram negative cell wall. Right panel: Gram positive cell wall.

Learning Objective

After completing this exercise, you should be able to determine the gram status of bacterial cells through gram staining.

Materials and Equipment

Escherichia coli agar plate
Staphylococcus aureus agar plate
Bunsen burner
Inoculating loop
Nutrient agar plate
Clean slides
Gram staining kit
Staining Tray
Microscope
Water bottle
Bibulous paper
Immersion oil
Wax pencil

Procedure

Gram Staining from an Agar Plate

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve agar plate from the refrigerator, remove the parafilm wrapping and sit the plate on the benchtop with the agar side facing up.
- ☐ Take a picture of the plate to document in your lab notebook. Document the different pigments and growth characteristics of the bacterial colonies in the results section of this lab exercise.
- ☐ Retrieve the Gram staining kit, staining tray, clean slides, water bottle, inoculating loop, bibulous paper, microscope and immersion oil.
- ☐ Using a wax pencil, label your slides as shown in Figure 4.3. *Draw one circle on the bottom/underside of the slide.*, about 15mm apart from your staining zones. They must be close together, but not touching, in order to accurately stain and decolorize. Be sure to write in your lab notebook which zone, A or B, corresponds to the which smear. For example, Zone A: thin smear, Zone B: moderate smear.
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

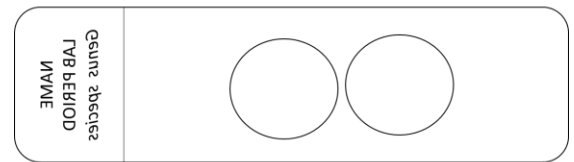


Figure 4.3: A properly labeled slide.

CHECKPOINT: BE SURE THAT YOU HAVE ALL GRAM STAINING REAGENTS AND EQUIPMENT BEFORE BEGINNING.

Gram Staining

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to Zone A.
- ☐ Retrieve the streaked nutrient agar plate and hold with your left hand.

- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the petri dish (no more than 45 degrees).

NOTE: Be sure to transfer a very small amount bacteria to the slide. Using too much bacteria will result in a crowded slide with poor staining. If you can easily see the bacteria on the loop, you are transferring too much.

- ☐ Carefully pick a small and isolated colony from the plate and close the petri dish. Set the petri dish on the benchtop.
- ☐ Add the colony to Zone A and move around in a circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to Zone B.
- ☐ Retrieve the streaked nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the petri dish (no more than 45 degrees).
- ☐ Carefully pick a small and isolated colony from the plate and close the petri dish. Set the petri dish on the benchtop.
- ☐ Add the colony to Zone B and move around in a circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Allow to air dry for 10-30 minutes.

CHECKPOINT: TURN OFF GAS SUPPLY TO THE BUNSEN BURNER TO MINIMIZE RISK OF FIRE. BE SURE TO HAVE THE GRAM STAINING KIT ON BENCHTOP AND WITHIN REACH.

- ☐ Light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Secure the slide with a clothespin and quickly pass the slide through the flame 3 to 4 times to heat fix the bacteria to the slide.
- ☐ Set the slide on the staining tray rack.
- ☐ Flood the slide with Crystal Violet for 1 minute. Pour off excess crystal violet into staining tray.

- ☐ As shown in Figure 4.4, tilt the slide and carefully rise the slide with water until the water runs clear. *Be careful to not apply too much water pressure and remove the sample from the slide.*

- ☐ Set the slide back on the staining tray rack. Add Gram's iodine to the slide and let sit for 1 minute.

- ☐ Pour off excess iodine into staining tray.

- ☐ Tilt and carefully rise the slide. Set the slide on the staining tray rack.

- ☐ Rinse the slide with alcohol for 3 seconds to decolorize unbound crystal violet.

Quickly tilt slide and rinse

with water. Set the slide on the staining tray rack. *Be careful to not leave alcohol on slide too long as it may decolorize Gram positive cells also.*

- ☐ Add safranin to the slide to counterstain the bacterial cells. Allow to stain for 30 seconds.
- ☐ Quickly pour off excess safranin into staining tray.
- ☐ Tilt and carefully rinse the slide for no longer than 3 to 5 seconds.
- ☐ Carefully blot the slide dry with bibulous paper. The slide is now ready for visualization under the microscope.
- ☐ Return all reagents and equipment to their appropriate stations.

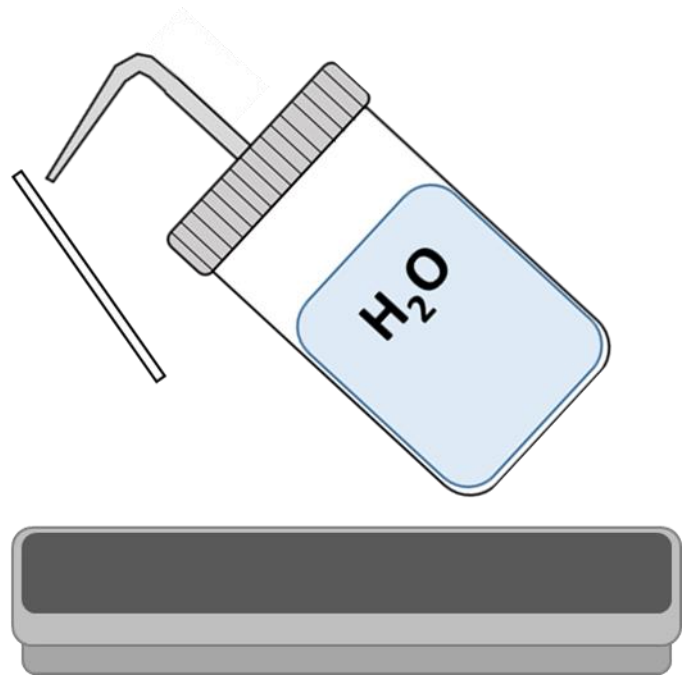
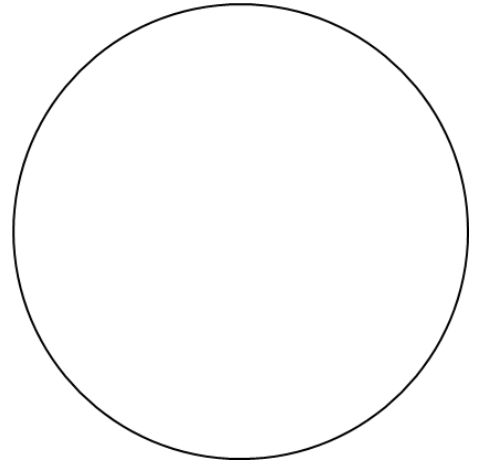
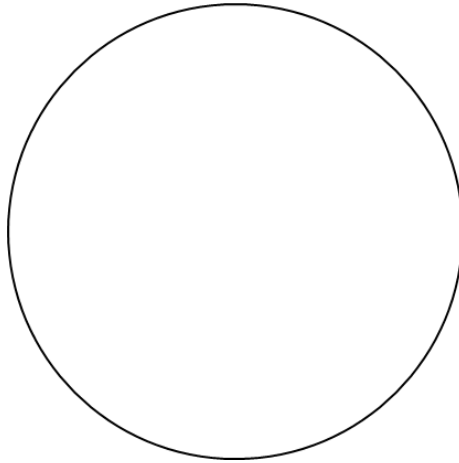


Figure 4.4: Tilt the slide and carefully rinse the slide with water in a wash bottle. The liquid should run into the staining tray.

Results

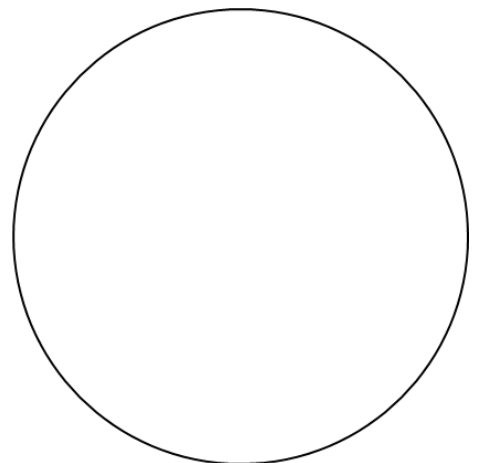
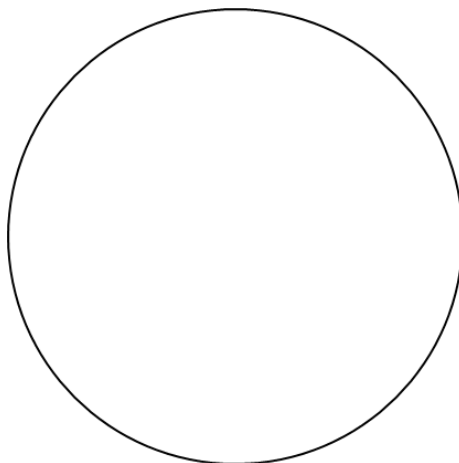
Use this section to document your results. Use a pen or pencil to draw the what you observe under the microscope.



Smear Thickness: _____

Arrangement: _____

Gram Status (+/-): _____



Smear Thickness: _____

Arrangement: _____

Gram Status (+/-): _____

Questions

1. Which smear gave the best results?
2. Does the thicker smear stain evenly? Why or why not?
3. How does crystal violet bind to the bacterial cell wall?

Lab Notes

Acid Fast Staining

Members of the Genus *Mycobacterium* have cell walls unlike that of other bacteria. Similar to gram positive bacteria, Mycobacteria also have very thick cell walls. However, they have a cell wall that is comprised of much less peptidoglycan. Instead, Mycobacteria have an abundance of mycolic acids. These acids give the Mycobacteria cell walls a high lipid content. As a result, its waxy nature repels traditional stains such like crystal violet. By heating the smear in combination with the addition of phenol based carbolfuscin, the cell wall becomes more permeable to the dye. As a result, the acid fast bacteria retain the pink dye, carbol fuschin, whereas non-acid fast bacteria are stained with methylene blue.

Learning Objective

After completing this exercise, students will be able to differentiate between acid-fast and non acid-fast bacterial cells.

Materials and Equipment

Bacillus subtilis agar plate
Mycobacterium smegmatis agar plate
Bunsen burner
Inoculating loop
Nutrient agar plate
Clean slides
Carbol Fuscin
Methylene Blue
Staining Tray
Microscope
Water bottle
Bibulous paper
Immersion oil
Wax pencil

Procedure

Gram Staining from an Agar Plate

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve agar plate from the refrigerator, remove the parafilm wrapping and sit the plate on the benchtop with the agar side facing up.
- ☐ Take a picture of the plate to document in your lab notebook. Document the different pigments and growth characteristics of the bacterial colonies in the results section of this lab exercise.
- ☐ Retrieve the Acid Fast staining kit, staining tray, clean slides, water bottle, inoculating loop, bibulous paper, microscope and immersion oil.
- ☐ Using a wax pencil, label your slides as shown in Figure 5.1. *Draw one circle on the bottom/underside of the slide.*
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

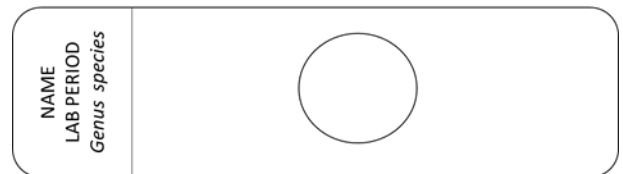


Figure 5.1: A properly labeled slide.

CHECKPOINT: BE SURE THAT YOU HAVE ALL GRAM STAINING REAGENTS AND EQUIPMENT BEFORE BEGINNING.

Acid Fast Staining

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to the target circle on the slide.
- ☐ Retrieve the streaked *Mycobacterium smegmatis* nutrient agar plate and hold with your left hand.

- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the petri dish (no more than 45 degrees).

NOTE: Be sure to transfer a very small amount bacteria to the slide. Using too much bacteria will result in a crowded slide with poor staining. If you can easily see the bacteria on the loop, you are transferring too much.

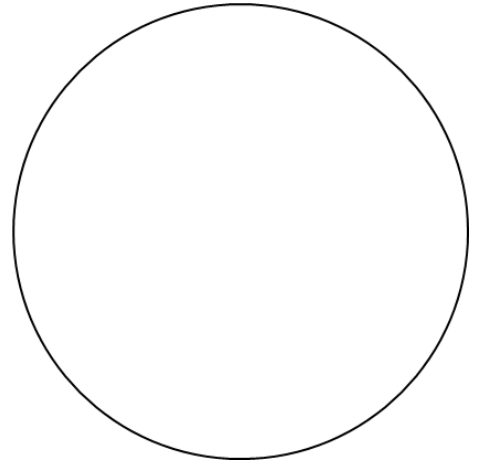
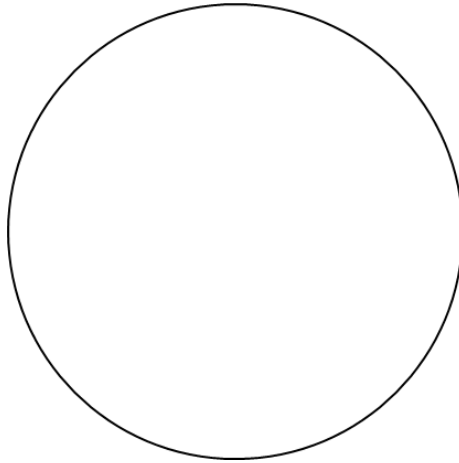
- ☐ Carefully pick a small and isolated colony from the plate and close the petri dish. Set the petri dish on the benchtop.
- ☐ Add the colony to the target circle and move around in a circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Repeat this protocol for the streaked *Bacillus subtilis* nutrient agar plate.
- ☐ Allow to air dry for 10-30 minutes.

CHECKPOINT: TURN OFF GAS SUPPLY TO THE BUNSEN BURNER TO MINIMIZE RISK OF FIRE. BE SURE TO HAVE THE ACID FAST STAINING KIT ON BENCHTOP AND WITHIN REACH.

- ☐ Light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Secure the slide with a clothespin and quickly pass the slide through the flame 3 to 4 times to heat fix the bacteria to the slide.
- ☐ Set the slide on the staining tray rack.
- ☐ Flood the slide with Carbol Fuschin for 5 minutes.
- ☐ Pour off excess Carbol Fuschin into staining tray.
- ☐ Tilt the slide and carefully rise the slide with water. *Be careful to not apply too much water pressure and remove the sample from the slide.*
- ☐ Set the slide back on the staining tray rack. Decolorize with acid alcohol for 1 minute. At this point, there should be no more stain running off the slide.
- ☐ Rinse briefly with water to stop decolorization reaction.
- ☐ Add methylene blue to the slide to counterstain the bacterial cells. Allow to stain for 30 seconds.
- ☐ Quickly pour of excess methylene blue into staining tray.
- ☐ Tilt and carefully rinse the slide for no longer than 3 to 5 seconds.
- ☐ Carefully blot the slide dry with bibulous paper. The slide is now ready for visualization under the microscope.
- ☐ Return all reagents and equipment to their appropriate stations.

Results

Use this section to document your results. Use a pen or pencil to draw the what you observe under the microscope.



Bacterium: _____

Arrangement: _____

Acid Fast (+/-): _____

Questions

4. How did the results of the two different bacterial samples differ?

5. If the *Bacillus* was in a sporulated state, do you think this would influence its acid fast status?

6. How do mycolic acids differ from teichoic acids?

Lab Notes

Endospore Staining

Typically, disinfectants and sterilants are quite capable of killing bacteria. However, certain bacteria have the ability to induce a dormant stage where they will remain until favorable conditions are presented. For instance, members of the Genus *Bacillus* rapidly undergo this sporulation process. As a spore, bacteria are highly resistant to heat and chemicals. For these reasons, it is often necessary to test for the presence of spores. In this experiment, vegetative cells will stain pink and endospores will stain a vibrant green.

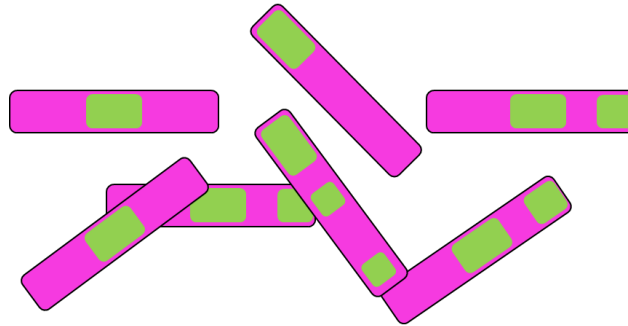


Figure 6.1. An endospore stain of bacilli. Endospores are stained green.

Learning Objectives

After completing this exercise, students will be able to identify and differentiate endospores from actively dividing bacterial cells.

Materials and Equipment

Bacillus cereus culture on nutrient agar slant (72-96hr culture)

Bunsen burner

Inoculating loop

Clean slides

Microscope

Immersion Oil

Malachite Green

Safranin

Bibulous Paper

Water bottle

Wax pencil

Hot Plate

Beaker

Mesh Plate

Procedure

Smear Preparation from a Liquid Culture

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve agar slant from the preparatory stain.
- ☐ Fill a 500mL beaker with water and set on hot plate. Place a metal mesh over the beaker. The water should begin a slow boil, releasing steam.
- ☐ Using a wax pencil. *Draw one circle on the bottom/underside of the slide.*
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

Smear Preparation from an Agar Slant

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to the region of the slide where you made a circle with the wax pencil.
- ☐ Retrieve the culture tube and hold with your left hand. Be sure to work close to the Bunsen burner for the remaining steps.
- ☐ Using the pinky finger of your right hand, remove the cap of the culture tube. *Do not set down.*
- ☐ Quickly pass the mouth of the culture tube through the flame of the Bunsen burner.
- ☐ While working close to the flame of the Bunsen burner, quickly insert the inoculating loop into the culture tube. Be sure to not touch the inner walls of the culture tube. If the wire of the inoculating loops touches anything besides the agar, you must re-sterilize the loop. Quickly remove the loop and keep close to flame.
- ☐ Quickly re-flame the mouth of the culture tube and immediately replace the cap. Set the culture tube back on the test tube rack.

- ☐ Touch the inoculating loop to the region of the slide where you made a circle with the wax pencil. Move the loop of the inoculating loop around in a circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- ☐ Allow to air dry for 10-30 minutes.
- ☐ The slide is now ready for staining.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES BEFORE BEGINNING. CHECK THE HOTPLATE- MAKE SURE WATER IS BOILING AND RELEASING STEAM.

Endospore Staining

- ☐ Place a small piece of bibulous paper over the smear.
- ☐ Saturate the bibulous paper with Malachite Green.
- ☐ Secure the slide with a clothespin or forceps and sit the slide on the mesh atop the steaming beaker on the hotplate for 5 minutes. If the bibulous paper begins to dry out, add more Malachite Green.
- ☐ After 5 minutes, remove slide from the heat source.

CHECKPOINT: SET SLIDE ON STAINING RACK AND TURN OFF HOT PLATE TO REDUCE THE RISK OF BURN/FIRE.

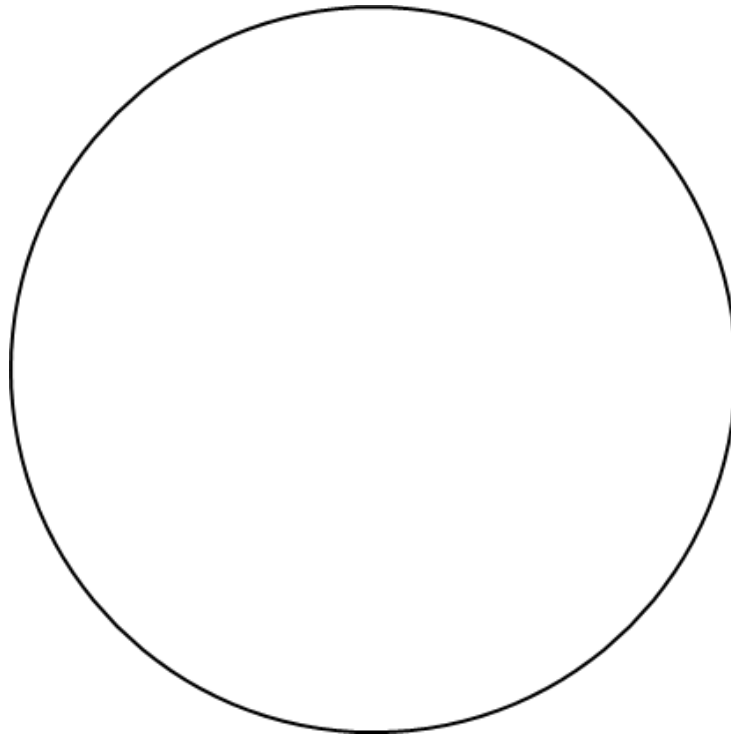
- ☐ Discard the bibulous paper in the appropriate waste disposal container.
- ☐ Tilt the slide and carefully rise the slide with water. Be careful to not apply too much water pressure and remove the sample from the slide.
- ☐ Counterstain slide with Safranin for 2 minutes.
- ☐ Tilt the slide and carefully rise the slide with water until the water runs clear. Be careful to not apply too much water pressure and remove the sample from the slide.
- ☐ Using bibulous paper, carefully dry the front and back of slide. Carefully blot the area of the analysis.
- ☐ Set the slide back on the staining tray rack.
- ☐ The slide is now ready for analysis.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE PROCEEDING. IF NECESSARY, SLIDES CAN BE STORED AND STAINED AT A LATER TIME.

- ☐ Set the microscope on the benchtop and retrieve the immersion oil.
- ☐ Set the objective to 10x and focus the smear into your field of view.

- ☐ Be mindful to select a field of view that clearly shows the bacteria in a monolayer. Crowded areas of bacteria give erroneous results.
- ☐ Sequentially move up in objective magnification until you are focused on the target area at 40x magnification.
- ☐ Swing the objective halfway to 100x and add a drop of immersion oil to the area of analysis.
- ☐ Focus the specimen at 100x.
- ☐ Document your observations in the Results section.

Results



Bacterium: _____

Stain: _____

Percentage sporulated: _____

Questions

1. For what reason do endospores form?
2. How long can endospores survive?
3. Why is it necessary to heat the bacterial smear?

Lab Notes

Environmental Effects on Bacterial Growth

Bacteria are the one of the most abundant organisms on the earth, inhabiting a variety of sub ecosystems. In the hydrothermal vents at the bottom of the ocean, one could expect to find heat tolerant microorganisms, known as thermophiles. In oceans, one may also find bacteria with an unusually high tolerance for salt. These bacteria are known as halophiles. Also, in ecosystems devoid of oxygen, you would find anaerobic bacteria. These anaerobes do not use oxygen as the terminal acceptor in the electron chain, but rely on other molecules such as nitrate and sulfate. In this lab experiment, students will test the dependencies of bacteria on temperature, salt and oxygen.

Learning Objective

Students will be able to explain the effects of salt concentration, oxygen and temperature on bacterial growth.

Materials and Equipment

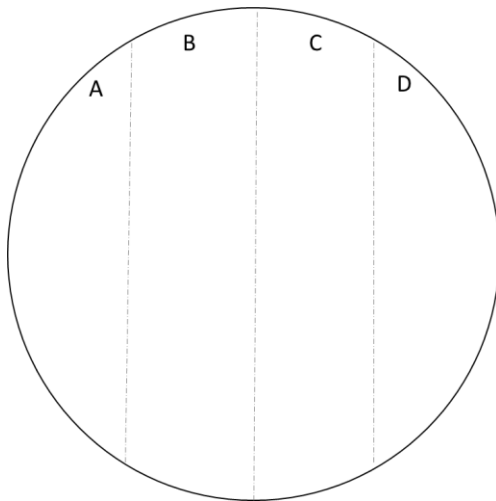
E. coli
S. aureus
S. epidermidis
Halobacterium salinarium
B. megaterium
Clostridium sporogenes
Serratia marcescens
Chromobacter violaceum
Bacillus stearothermophilus
Bunsen burner
Inoculating loop
MSA plates
Nutrient agar plates: 1 each per student: 0%NaCl, 5% NaCl and 10% NaCl
Fluid Thioglycollate Medium Broth: 3 per student
Nutrient agar plates: 4 per student
Culture tube labels
Wax pencil

Procedure

Part 1: Streaking Nutrient Agar for Temperature Dependency

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve culture broths from instructor's stock bacteria rack.
- ☐ Using a fine tip marker, mark the bottom of a sterile nutrient agar plate as shown in Figure 7.1. The petri dish should be divided into four zones and labeled as Zone A, B, C and D.
- ☐ Four nutrient agar plates should be labeled as above, but with each labeled as 25C, 30C, 37C, and 50C, respectively.
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.



The zones should be labeled in your notebook as the following:

Zone A: *Serratia marcescens*

Zone B: *Chromobacter Violaceum*

Zone C: *Bacillus megaterium*

Zone D: *Bacillus stearothermophilus*

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

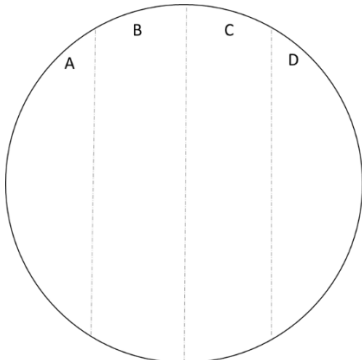
Streaking from a Liquid Culture

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Gently resuspend the bacteria into the medium by holding the *Serratia marcescens* culture tube between your palms and alternating moving them back and forth. The bacteria should no longer be settled on the bottom of the culture tube.

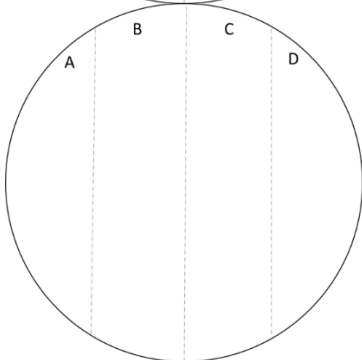
- ☐ Place culture tube back on test tube rack.
- ☐ Retrieve the inoculating loop and place in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Retrieve the culture tube and hold with your left hand. Be sure to work close to the Bunsen burner for the remaining steps.
- ☐ Using the pinky finger of your right hand, remove the cap of the culture tube. *Do not set down.*
- ☐ Quickly pass the mouth of the culture tube through the flame of the Bunsen burner.
- ☐ While working close to the flame of the Bunsen burner, quickly insert the inoculating loop into the culture tube. Be sure to not touch the inner walls of the culture tube. If the wire of the inoculating loops touches anything besides the medium, you must re-sterilize the loop. Quickly remove the loop and keep close to flame.
- ☐ Quickly re-flame the mouth of the culture tube and immediately replace the cap. Set the culture tube back on the test tube rack.
- ☐ Retrieve labeled nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the nutrient agar plate (no more than 45 degrees).
- ☐ Place the inoculating loop parallel to the agar and gently streak the plate in the appropriate marked Zone. The streak should be made in a straight line.
- ☐ Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- ☐ Repeat this process for Zones B-D using the appropriate bacteria.
- ☐ Make four of plates in total, labeled 25C, 30C, 37C and 50C.
- ☐ Incubate the plates in the respective incubators for 24-48h.

Results

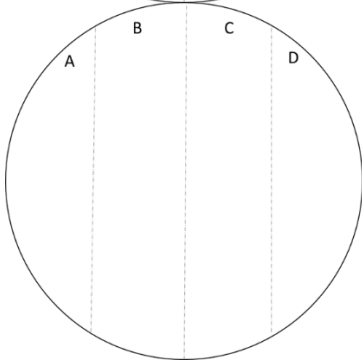
Use this section to document your results. Use a pencil or pen to indicate the growth in each zone for each plate incubated at 25C, 30C, 37C or 50C, respectively. Document the variation in amount of growth for each zone at each temperature. Appropriate descriptions are “no growth” “moderate growth” “heavy growth.” Also, indicate the optimal growth temperatures for each bacterium.



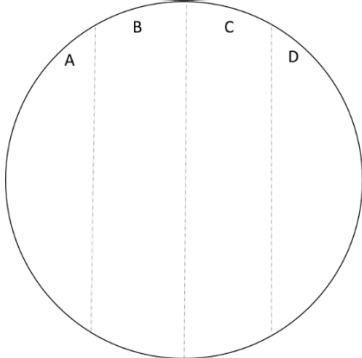
	Bacterium	Temp	Growth	Optimum?
Zone A				
Zone B				
Zone C				
Zone D				



	Bacterium	Temp	Growth	Optimum?
Zone A				
Zone B				
Zone C				
Zone D				



	Bacterium	Temp	Growth	Optimum?
Zone A				
Zone B				
Zone C				
Zone D				



	Bacterium	Temp	Growth	Optimum?
Zone A				
Zone B				
Zone C				
Zone D				

Questions

1. What causes the bacteria to grow at different temperatures?
2. Did you notice a difference in pigment production when growing bacteria at different temperatures? How can you account for this?
3. Where would one most likely find *Bacillus stearothermophilus* growing in the environment?

Lab Notes

Procedure

Part 2: Oxygen Requirements

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve culture broths from instructor's stock bacteria rack.
- ☐ Retrieve 3 culture tube labels. Using a pencil, mark the label with the following information: Name, Class Period, Bacterium and FTM.
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

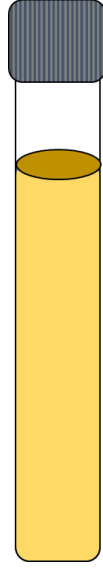
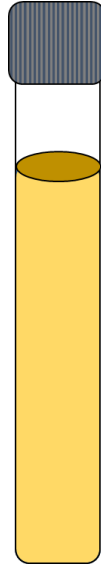
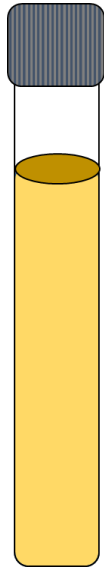
Broth to Broth Transfer

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Gently resuspend the bacteria into the medium by holding the *Staphylococcus aureus* culture tube between your palms and alternating moving them back and forth. The bacteria should no longer be settled on the bottom of the culture tube.
- ☐ Place the culture tube back on test tube rack.
- ☐ Retrieve the inoculating loop and place in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Retrieve the culture tube and hold with your left hand. Be sure to work close to the Bunsen burner for the remaining steps.
- ☐ Using the pinky finger of your right hand, remove the cap of the culture tube. *Do not set down.*
- ☐ Quickly pass the mouth of the culture tube through the flame of the Bunsen burner.
- ☐ While working close to the flame of the Bunsen burner, quickly insert the inoculating loop into the culture tube. Be sure to not touch the inner walls of the culture tube. If the wire of the inoculating loops touches anything besides the medium, you must re-sterilize the loop. Quickly remove the loop and keep close to flame.

- ☐ Quickly re-flame the mouth of the culture tube and immediately replace the cap. Set the culture tube back on the test tube rack.
- ☐ Retrieve labeled FTM culture tube and hold with your left hand.
- ☐ Using the pinky finger of your right hand, remove the cap of the FTM tube. *Do not set down.*
- ☐ Place the inoculating loop directly into the culture tube, being mindful to not touch neither the outside nor inside of the tube.
- ☐ Quickly pass the mouth of the culture tube through the flame of the Bunsen burner and immediately replace the cap. Set the inoculated FTM tube on the test tube rack.
- ☐ Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- ☐ Repeat this process for *E. coli* and *C. sporogenes*.
- ☐ Incubate the inoculated FTM broths at 37C for 24-48 hours.

Results

Use this section to document your results. Use a pencil or pen to sketch the growth characteristics of each bacterium in each of the FTM tubes, respectively. Using the space provided below, explain the differences in growth characteristics between each bacterium and the reason why each bacterium exhibits these characteristics.



Questions

4. What causes the bacteria to grow at different oxygen concentrations?
5. Why did the bacterial cultures grow in different regions of the FTM?
6. In what environment would you expect to find salt tolerant bacteria?
7. Would you expect to find anaerobic bacteria within the human body?

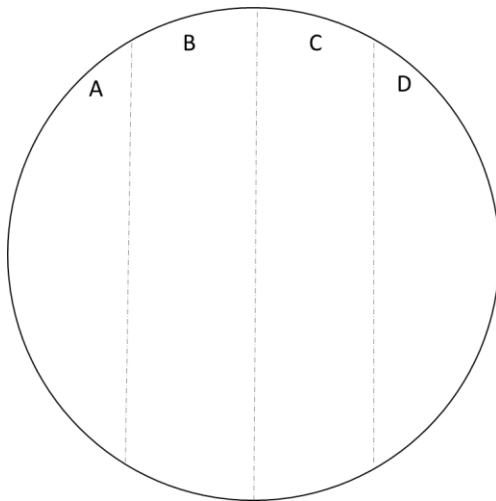
Lab Notes

Procedure

Part 3: Salt Tolerance

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve culture broths from instructor's stock bacteria rack.
- ☐ Using a fine tip marker, mark the bottom of a sterile nutrient agar plate as shown in Figure 7.2. The petri dish should be divided into four zones and labeled as Zone A, B, C and D.
- ☐ There should be 3 plates labeled total per student, one for each 0% NaCl, 5% 10% NaCl, and 15% NaCl.
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.



The zones should be labeled in your notebook as the following:

Zone A: *E. coli*

Zone B: *S. aureus*

Zone C: *S. epidermidis*

Zone D: *H. salinarium*

CHECKPOINT: BE SURE THAT YOU HAVE ALL SUPPLIES AND EQUIPMENT BEFORE BEGINNING.

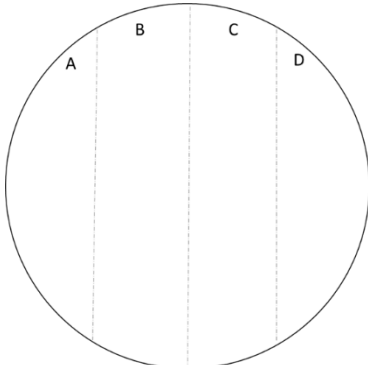
Streaking from a Liquid Culture

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Gently resuspend the bacteria into the medium by holding the *E. coli* culture tube between your palms and alternating moving them back and forth. The bacteria should no longer be settled on the bottom of the culture tube.
- ☐ Place culture tube back on test tube rack.

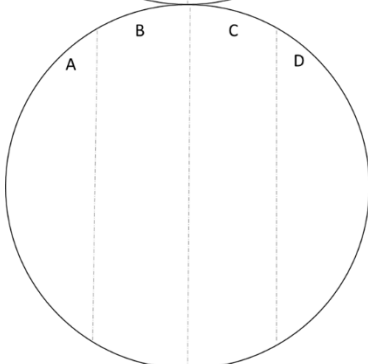
- ☐ Retrieve the inoculating loop and place in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Retrieve the culture tube and hold with your left hand. Be sure to work close to the Bunsen burner for the remaining steps.
- ☐ Using the pinky finger of your right hand, remove the cap of the culture tube. *Do not set down.*
- ☐ Quickly pass the mouth of the culture tube through the flame of the Bunsen burner.
- ☐ While working close to the flame of the Bunsen burner, quickly insert the inoculating loop into the culture tube. Be sure to not touch the inner walls of the culture tube. If the wire of the inoculating loops touches anything besides the medium, you must re-sterilize the loop. Quickly remove the loop and keep close to flame.
- ☐ Quickly re-flame the mouth of the culture tube and immediately replace the cap. Set the culture tube back on the test tube rack.
- ☐ Retrieve labeled nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the nutrient agar plate (no more than 45 degrees).
- ☐ Place the inoculating loop parallel to the agar and gently streak the plate in the appropriate marked Zone. The streak should be made in a straight line.
- ☐ Sterilize the wire of the inoculating loop in the flame of the Bunsen burner.
- ☐ Repeat this process for Zones B-D using the appropriate bacteria.
- ☐ Four nutrient agar plates in total should be streaked with the following concentrations: 0% NaCl, 5% NaCl, 10% NaCl, and 15% NaCL.
- ☐ Incubate the plates at 37C for 24-48h.

Results

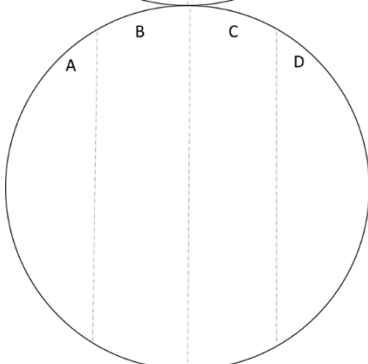
Use this section to document your results. Use a pencil or pen to indicate the growth in each zone for each plate incubated at the varying salt concentrations. Document the variation in amount of growth for each zone at each temperature in the table. Appropriate descriptions are “no growth” “moderate growth” “heavy growth.”



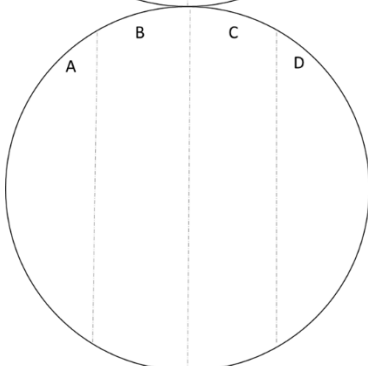
	Bacterium	[NaCl]	Growth
Zone A			
Zone B			
Zone C			
Zone D			



	Bacterium	[NaCl]	Growth
Zone A			
Zone B			
Zone C			
Zone D			



	Bacterium	[NaCl]	Growth
Zone A			
Zone B			
Zone C			
Zone D			



	Bacterium	[NaCl]	Growth
Zone A			
Zone B			
Zone C			
Zone D			

Questions

8. What causes the bacteria to grow at different salt concentrations?

9. What is the effect of salt on the water content in the cell? What effect does this have on the cell membrane?

10. In what environment would you expect to find salt tolerant bacteria?

11. How can you explain the presence of salt tolerant bacteria on human skin?

[illegible]

Bacterial Isolation from a Mixed Culture

The instructor will provide you with a sample that contains the unknown bacteria. The objective of this exercise is for students to be able to separate bacterial cultures into a pure isolate. To simplify this, you will be provided with a sample that contains bacteria that produce two different pigments. Upon proper streaking, students will have distinct colonies of bacteria upon which to gram stain for characterization.

Learning Objective

This exercise is meant to reinforce streaking and proper aseptic technique. Additionally, students will learn how to differentiate between different bacterial growth characteristics and gram staining results.

Materials and Equipment

- Unknown sample
- Bunsen burner
- Inoculating loop
- Nutrient agar plate
- Clean slides
- Gram staining kit
- Staining Tray
- Microscope
- Water bottle
- Bibulous paper
- Immersion oil
- Wax pencil

Procedure

Day 1: Streaking the Mixed Culture

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% ethanol and be sure that the area is free of clutter.
- ☐ Obtain 1 nutrient agar plate and label the bottom of the plate with your name, the date, class period and “Unknown.”
- ☐ Obtain 1 Unknown sample and an inoculating loop. Set on benchtop.
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

CHECKPOINT: TO REDUCE THE RISK OF A FIRE, BE SURE THAT BENCHTOP IS CLEAR OF CLUTTER AND THAT ALL NECESSARY MATERIALS ARE WITHIN REACH.

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Working closely to the flame, put the unknown sample in your left hand and the inoculating loop in your right hand.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ With the pinky finger of your right hand, unscrew the cap of the culture tube that contains your unknown sample. Keep the cap tucked between your pinky finger and closed palm of your hand.
- ☐ Quickly move the mouth of the culture tube over the flame, 2 or 3 times.
- ☐ Without touching the culture tube, “dip” the inoculating loop into the sample and twist. Withdraw the loop.
- ☐ Move the mouth of the culture tube over the flame and replace the cap.
- ☐ Set the culture tube back on culture rack.

Streaking

- ☐ Retrieve labeled nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the nutrient agar plate (no more than 45 degrees).
- ☐ Place the inoculating loop parallel to the agar and gently streak the plate. In the first quadrant, the streak should be made in a tight zig-zag motion 8 to ten times.
- ☐ To streak the second quadrant, rotate the agar plate 90 degrees using your left hand.
- ☐ Streak the second quadrant by gently dragging the inoculating loop from the first quadrant into the second quadrant multiple times.
- ☐ To streak the third quadrant, rotate the agar plate 90 degrees using your left hand.
- ☐ Streak the third quadrant by gently dragging the inoculating loop from the second quadrant into the third multiple times.
- ☐ To streak the fourth quadrant, rotate the agar plate 90 degrees using your left hand.
- ☐ Streak the fourth quadrant by gently dragging the inoculating loop from the third quadrant into the fourth quadrant two times.
- ☐ Be sure to not change the angle of the loop, as this may damage the agar and compromise the streak.
- ☐ Replace petri dish lid and set the petri dish face up on the benchtop for a few minutes. This will allow the liquid to absorb into the media.

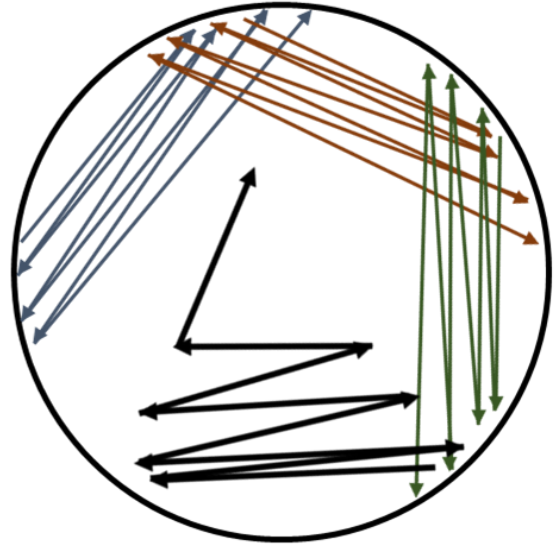


Figure 8.1: An illustration of a quadrant streak.

CHECKPOINT: TO REDUCE THE RISK OF A FIRE, BE SURE TO TURN OFF THE BUNSEN BURNER AFTER STREAKING YOUR PLATE. DISASSEMBLE AND PUT AWAY.

- ☐ After the liquid has been absorbed into the media, place in the incubator set to 25° Celsius. The plate should be set in the incubator with the agar side up.
- ☐ Allow the plate to incubate for 24-48 hours.

Day 2: Gram Staining from an Agar Plate

Prep Stage

- ☐ Put on personal protective equipment including gloves, safety glasses and labcoat.
- ☐ Wipe down benchtop with 70% alcohol and be sure the area is free of clutter.
- ☐ Retrieve agar plate from the refrigerator, remove the parafilm wrapping and sit the plate on the benchtop with the agar side facing up.
- ☐ Take a picture of the plate to document in your lab notebook. Document the different pigments and growth characteristics of the bacterial colonies in the results section of this lab exercise.
- ☐ Retrieve the Gram staining kit, staining tray, clean slides, water bottle, inoculating loop, bibulous paper, microscope and immersion oil.
- ☐ Using a wax pencil, label your slide as shown in Figure 8.2. *Draw two circles on the bottom of the slide, about 15mm apart from your staining zones. They must be close together, but not touching, in order to accurately stain and decolorize. Be sure to write in your lab notebook which zone, A or B, corresponds to the which bacterial colony. For example, Zone A: purple colony, Zone B: yellow colony.*
- ☐ Obtain a Bunsen burner and make sure the benchtop is free of clutter. Inspect the gas tubing for cracks and replace if necessary.

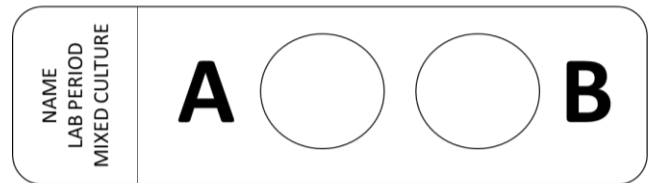


Figure 8.2: A properly labeled slide.

CHECKPOINT: BE SURE THAT YOU HAVE ALL GRAM STAINING REAGENTS AND EQUIPMENT BEFORE BEGINNING.

Gram Staining

- ☐ Connect the gas tubing and light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to Zone A.
- ☐ Retrieve the streaked nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the petri dish (no more than 45 degrees).

NOTE: Be sure to transfer a very small amount bacteria to the slide. Using too much bacteria will result in a crowded slide with poor staining. If you can easily see the bacteria on the loop, you are transferring too much.

- ☐ Carefully pick a small and isolated colony from the plate and close the petri dish. Set the petri dish on the benchtop.
- ☐ Add the colony to Zone A and move around in a circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Place the inoculating loop in the inner cone of the flame. Heat the loop and wire for 15 seconds, ensuring that that inoculating loop turns bright red.
- ☐ Let loop cool for approximately 15 seconds.
- ☐ Using an inoculating loop, carefully add 2 loopfuls of water to Zone B.
- ☐ Retrieve the streaked nutrient agar plate and hold with your left hand.
- ☐ Using the thumb and index finger of your left hand, slightly lift the lid of the petri dish (no more than 45 degrees).
- ☐ Carefully pick a small and isolated colony from the plate and close the petri dish. Set the petri dish on the benchtop.
- ☐ Add the colony to Zone B and move around in a circular motion to put the bacteria in solution. There should be no remaining clumps of bacteria left.
- ☐ Allow to air dry for 10-30 minutes.

CHECKPOINT: TURN OFF GAS SUPPLY TO THE BUNSEN BURNER TO MINIMIZE RISK OF FIRE. BE SURE TO HAVE THE GRAM STAINING KIT ON BENCHTOP AND WITHIN REACH.

- ☐ Light the Bunsen burner. Be sure to adjust the gas to achieve a coned shaped inner flame.
- ☐ Secure the slide with a clothespin and quickly pass the slide through the flame 3 to 4 times to heat fix the bacteria to the slide.
- ☐ Set the slide on the staining tray rack.
- ☐ Flood the slide with Crystal Violet for 1 minute. Pour off excess crystal violet into staining tray.

- ☐ As shown in Figure 8.3, tilt the slide and carefully rise the slide with water until the water runs clear. *Be careful to not apply too much water pressure and remove the sample from the slide.*

- ☐ Set the slide back on the staining tray rack. Add Gram's iodine to the slide and let sit for 1 minute.

- ☐ Pour off excess iodine into staining tray.

- ☐ Tilt and carefully rise the slide. Set the slide on the staining tray rack.

- ☐ Rinse the slide with alcohol for 3 seconds to decolorize unbound crystal violet.

Quickly tilt slide and rinse

with water. Set the slide on the staining tray rack. *Be careful to not leave alcohol on slide too long as it may decolorize Gram positive cells also.*

- ☐ Add safranin to the slide to counterstain the bacterial cells. Allow to stain for 30 seconds.
- ☐ Quickly pour off excess safranin into staining tray.
- ☐ Tilt and carefully rinse the slide for no longer than 3 to 5 seconds.
- ☐ Carefully blot the slide dry with bibulous paper. The slide is now ready for visualization under the microscope.
- ☐ Return all reagents and equipment to their appropriate stations.

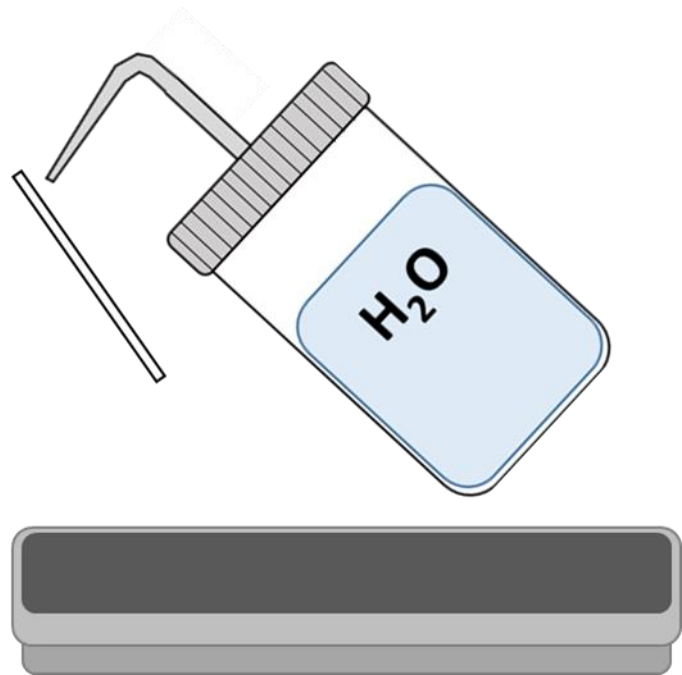
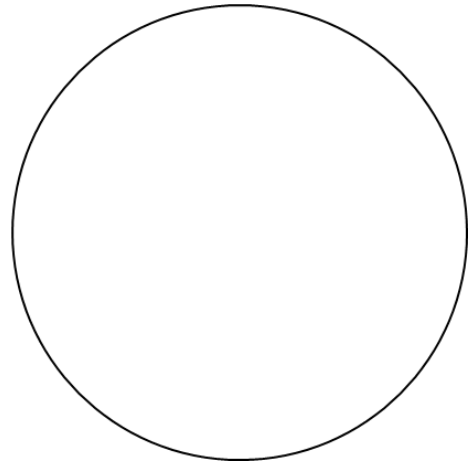
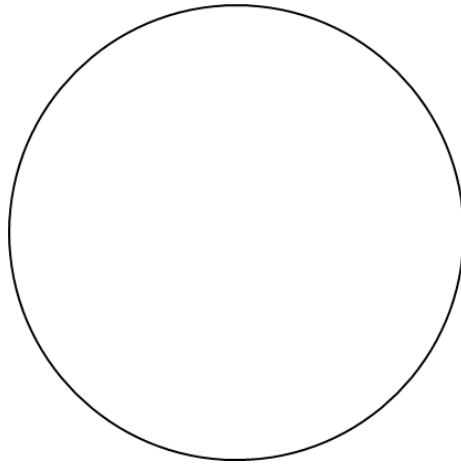


Figure 8.3: Tilt the slide and carefully rinse the slide with water in a wash bottle. The liquid should run into the staining tray.

Results

Use this section to document your results. Use a pen or pencil to draw the what you observe under the microscope.



Shape: _____

Arrangement: _____

Pigmentation: _____

Gram Status (+/-) _____

Questions

4. What would happen if you forgot to flame the inoculating loop between picking the different bacterial colonies?

5. What would happen if you rinse the slide too long when attempting to remove excess safranin?

6. How does crystal violet bind to the bacterial cell wall?

Lab Notes
