Enteric bacteria cultured on Triple Sugar Iron agar slants. Gas production has lifted the agar in the tube on the left. Bacteria in the tube on the right are producing H₂S, visible as a black precipitate. Photo by Tracey Martinson.
# Table of Contents

Tentative lab schedule 5  
Lab rules 6  

**Exercises**  
1. Microscopy 7  
2. Aseptic technique 15  
3. Microbial and fungal ubiquity 19  
4. Staining techniques 21  
5. Tests used to identify unknown bacteria 27  
6. Pure culture techniques 35  
7. Bacterial population counts 39  
8. Microbial growth curves 47  
9. Chemical control of microbial growth 51  
10. Bacteria of the oral and nasal passages 55  
11. Bacteria of the gastrointestinal tract 61
# Lab Schedule Spring 2007

<table>
<thead>
<tr>
<th>Date</th>
<th>Topic</th>
<th>Lab exercise</th>
<th>Notes/Items due</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan. 23</td>
<td>Introduction</td>
<td></td>
<td>Quiz 1</td>
</tr>
<tr>
<td></td>
<td>Lab safety</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jan. 30</td>
<td>Aseptic technique</td>
<td>2</td>
<td>Quiz 2</td>
</tr>
<tr>
<td></td>
<td>Staining</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Feb. 6</td>
<td>Microbial ubiquity</td>
<td>4</td>
<td>No quiz!</td>
</tr>
<tr>
<td></td>
<td>Identification of bacteria</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Feb. 13</td>
<td>Pure culture techniques</td>
<td>6</td>
<td>Quiz 3</td>
</tr>
<tr>
<td></td>
<td>Bacterial population counts</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Feb. 20</td>
<td>Microbial growth curves</td>
<td>8</td>
<td>Quiz 4</td>
</tr>
<tr>
<td>Feb. 27</td>
<td>Bacterial transformation</td>
<td>Handout</td>
<td>Quiz 5</td>
</tr>
<tr>
<td>Mar. 6</td>
<td>Chemical control of microbial growth</td>
<td>9</td>
<td>Quiz 6</td>
</tr>
<tr>
<td></td>
<td>Turn in lab manual for grading</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mar. 20</td>
<td>Tobacco mosaic virus lab</td>
<td>Handout</td>
<td>No quiz!</td>
</tr>
<tr>
<td>Mar. 27</td>
<td>Flora of the oral and nasal tract</td>
<td>10</td>
<td>No quiz!</td>
</tr>
<tr>
<td>Apr. 3</td>
<td>Flora of the GI tract</td>
<td>11</td>
<td>Quiz 7</td>
</tr>
<tr>
<td>Apr. 10</td>
<td>Ice-nucleating bacteria</td>
<td>Handout</td>
<td>Quiz 8</td>
</tr>
<tr>
<td></td>
<td>Turn in lab manual for final grading</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apr. 17</td>
<td>Synthetic blood typing</td>
<td>Handout</td>
<td>Quiz 9</td>
</tr>
<tr>
<td>Apr. 24</td>
<td>Synthetic epidemic</td>
<td>Handout</td>
<td>Quiz 10</td>
</tr>
<tr>
<td></td>
<td>Disease tracking using ELISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>May 1</td>
<td>Lab practical</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Lab Rules

1. Attendance in lab is mandatory. Please be on time.
2. No one who appears to be under the influence of alcohol or drugs will be permitted in the lab.
3. No children, visitors, or students not enrolled in this course are permitted in the lab.
4. Shoes and appropriate dress must be worn at all times. Secure long hair and loose sleeves.
5. Wear a lab coat or apron—they are easier to sterilize than your clothing, should you spill a culture or staining reagents.
6. Leave outerwear and backpacks on the hooks outside the lab.
7. Be careful with Bunsen burners—keep them away from microscopes, out from under the top shelf of the lab bench, and watch your hair. Never leave the flame unattended. Wear safety glasses if heating glass slides in the flame, as the slides can sometimes shatter.
8. Place pipettes, thermometers, and test tubes so that they don’t roll of the lab bench.
9. Always place used pipettes, swabs, and other materials in the biohazard bags provided so that they can be autoclaved and disposed of properly. Do NOT throw trash in the autoclave bag.
10. No eating or drinking in lab.
11. Never lick your fingers, or put your fingers in your mouth. No mouth pipetting.
12. Treat every organism as a potential pathogen.
13. Consider all chemicals hazardous. Never taste any chemical or inhale any vapor.
14. Treat spilled cultures with disinfectant before cleaning them up. Cover the spill with a paper towel. Spray the paper towel with disinfectant until the towel is soaking wet. Let this sit for 10 minutes. Wearing gloves, pick up the paper towels and discard in the autoclave bag. Clean the spill area three times with disinfectant, discarding of contaminated towels in the autoclave bag. Ask the instructor or T.A. for help as soon as the spill occurs.
15. Carry microscopes with two hands. Remember to wipe the oil off the lenses before putting the microscope away.
16. No radios, tape players, or CD players in the lab.
17. Please bring any safety concerns to the attention of your instructor or T.A.
18. Notify the T.A. or instructor of any accident, no matter how minor.
19. At the beginning of each lab period, clean your bench with disinfectant. Clean it again at the end of lab.
20. WASH YOUR HANDS before leaving the lab, even if it’s only for a break. It is also a good idea to wash your hands before beginning the lab exercises.
EXERCISE 1
MICROSCOPY

Objectives:
1. To become familiar with the operation of a light microscope
2. To observe several microorganisms using light microscopy
3. To determine the size of several microorganisms

Introduction:
Microscopes are one of the most important tools available to a microbiologist. In a clinical setting, accurate diagnosis of a disease may require the determination of cell size and shape, arrangement of cells, and whether or not the cells are motile. In addition, there are many staining procedures that used to distinguish between different bacterial species. All of these determinations require the use of a microscope.

There are many kinds of microscopes, including bright-field, dark-field, phase contrast, fluorescence, and electron microscopes. In this course, we will be using bright-field microscopes, which can magnify objects up to about 1000X. For observing bacteria, it is generally necessary to use the highest magnification possible in order to see them clearly. This also means that the microscope must be adjusted properly so things won’t appear fuzzy, and operated correctly so the lenses don’t get out of alignment.

The usefulness of a light microscope is determined primarily by two factors: magnification and resolving power. **Magnification** is a function of the lens systems of a given microscope.

Modern light microscopes have two lens systems, an **objective lens system** and the **ocular lens system**. The light microscopes we have are equipped with a 10X ocular lens, and three objective lenses of 10X, 40X, and 100X. Some microscopes may have a fourth objective lens of 4X.

When you look at an object under a light microscope, the objective lens system magnifies the object to produce a **real image**, which is projected up into the focal plane of the ocular
lens system. This lens system then produces a virtual image that you see when you look through the microscope. The total magnification of an object is a function of both lens systems, and is determined by multiplying the magnification of the ocular lens and the magnification of the objective lens that is in place. For example, using an ocular lens of 10X together with an objective lens of 10X gives a total magnification of 100X.

The resolving power of a lens system is defined as the smallest distance at which two points can be seen separately, and is a function of the wavelength of light, the design and positioning of the condenser, and the numerical aperture of the objective lens. The condenser collects and focuses light onto the slide containing the organism being studied. The numerical aperture is a function of the wavelength of light coming from the condenser, the half angle of the lens aperture, and the refractive index of the medium between the slide and the front of the lens. In most light microscope systems, the wavelength of light and the half angle of the lens aperture are not readily changed. However, using immersion oil when using the highest-powered objective lens (100X) will improve the resolving power. This works because immersion oil has the same refractive index as glass, and so the amount of light reaching the objective lens is maximized (less light is lost). Using the highest magnification and immersion oil lenses, the best light microscopes can distinguish between objects that are about 0.2 µm apart.

**Care and handling of microscopes**

1. Always carry a microscope with two hands—one hand under the base of the microscope and the other hand holding onto the stand.
2. Keep the microscope at least 6 inches from the edge of the lab bench, and keep all excess electrical cord up on the bench and out of the way of burners.
3. Do not touch the surfaces of the glass lenses with your fingers. They scratch easily.
4. Always remove the oil from the surface of the oil immersion lens with a Q-tip or lens paper BEFORE putting the microscope away. This will ensure that the lens is clean and not encrusted with immersion oil the next time you hope to look through it.
5. Use only Q-tips or lens paper to clean the lenses. Never use Kimwipes, Kleenex, or paper towels—they will scratch the lenses.

6. Do not tamper with any parts of the microscope. If it doesn’t work properly, ask your T.A. for assistance.

7. Always start your observations using the lowest-powered objective (e.g. 4X or 10X), and always put the lowest-powered objective back into place when putting the microscope away.

8. Never use the coarse adjustment knob to focus while the 40X and 100X objective lenses are in place—use the fine adjustment knob. Use of the coarse adjustment knob will throw the lenses out of parfocal alignment, and make your life more difficult. Trust me.

9. Always unplug the microscope by pulling on the plug, not the cord.

10. Never look through the microscope while rapidly reducing the distance between the objective lens and the slide. Watch from the side so that you avoid damaging the lens by running it into the slide.

**Using the microscope**

Some of the more common problems encountered when using a microscope stem from a failure to focus in the right plane. With practice, you will become efficient at locating and focusing on microorganisms. To help reduce frustration, always follow the steps outlined below when using a microscope.

1. With the lowest power objective lens in place, put the slide on the microscope stage. The material to be studied should be facing up, and should be in the center of the light source.

2. With the coarse adjustment knob, move the stage as close to the lens as it will go (looking at the stage from the side, not through the lens).

3. Looking through the ocular lens, focus on the organism of interest by slowly turning the fine adjustment knob. To help find the right plane to focus in, it is helpful to gently move the slide back and forth on the stage while turning the fine adjustment knob.

4. Once you have focused on the organism of interest using the lowest powered objective, center it in your field of view.
5. Gently rotate the next highest objective (the high-dry objective) into place. Always look from the side to avoid running the objective into the slide.

6. Very small adjustments to the fine adjustment knob should be all that is necessary to bring the organism of interest into focus. DO NOT use the coarse adjustment knob with anything other than the low power objective.

7. Once you have focused on your organism using the high-dry objective, you may increase to the highest power (the 100X lens). Since our lenses are **oil immersion** lenses, you MUST use immersion oil with these lenses at all times (or risk scratching the lens on the glass slide). To do this, rotate the high-dry lens half-way, carefully put a small drop of immersion oil on the slide, then carefully rotate the oil immersion lens into place. Use the fine adjustment knob to focus on the organism.

8. Increase the light level if the background is too dim. Do this first by opening up the diaphragm. If that is still not enough, increase the voltage of the lamp (this prolongs the life of the lamps).

**Putting the microscope away**

When you are finished using the microscope, it is important that you take a few minutes to do the following. This will help ensure that the microscope is clean and in good working condition the next time you want to use it.

1. Remove the slide from the stage.
2. Use lens paper or a Q-tip to clean any immersion oil off the oil immersion objective lens (and the high-dry lens in the event that you had to rotate that lens into place after you had already put immersion oil on a slide).
3. Rotate the low power objective lens into place.
4. Turn off the light, unplug, and carefully wrap the electrical cord around the base.
5. Return the microscope to the cabinet where you got it.

**Materials:**

Microscopes
Microscope with ocular micrometer
Stage micrometer
Immersion oil
Procedure:
1. 4 pts. Calculate the total magnification for each of the objective lenses on your microscope.

<table>
<thead>
<tr>
<th>Objective lens power</th>
<th>X</th>
<th>Ocular lens power</th>
<th>= Total magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Look at a commercially prepared slide. Draw a picture of what you observe. Be sure to note the magnification at which you did the drawing (2 pts.).

3. Prepare and observe a wet mount of the material provided.

   Wet mounts are used to observe living organisms, or when more accurate estimates of cell size are desired. Wet mounts can be prepared from both liquid and solid cultures.

   To prepare a wet mount from a liquid culture (2 pts.):
1. Use a dropper or a sterile loop to put a drop of liquid on a clean slide.
2. Put a cover slip over the drop. If liquid oozes out the sides, your drop was too big.

To prepare a wet mount from solid fungal or bacterial growth (2 pts.):
1. Put a drop of water on a slide.
2. Pick up a small amount of material using a sterile loop and gently disperse it in the drop of water.
3. Put a cover slip over the drop.

4. Measure a microorganism.

   The size of a microorganism is typically expressed in units of micrometers (µm); 1 millimeter = 1000 µm. Size determinations are made using a microscope equipped with an ocular micrometer. The ocular micrometer has uniformly spaced lines etched onto its surface, like an unmarked ruler. The ocular micrometer must be calibrated against a stage micrometer, which has lines etched 0.01 mm (10 µm) apart. Note that the ocular micrometer only needs to be
calibrated once for a given microscope. However, it must be calibrated for EACH of the objective lenses.

To calibrate the ocular micrometer, first align its left edge with the left edge of the stage micrometer. Then find a place where a line on the stage micrometer aligns with one of the lines in the ocular micrometer. Count how many spaces of the stage micrometer are included within a given number of spaces on the ocular micrometer.

Using one of the microscopes set up for this, calibrate the 40X and 100X objectives on the microscope (4 pts.).

<table>
<thead>
<tr>
<th></th>
<th>40X</th>
<th>100X</th>
</tr>
</thead>
<tbody>
<tr>
<td># spaces on ocular micrometer</td>
<td></td>
<td></td>
</tr>
<tr>
<td># spaces on stage micrometer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To calibrate the ocular micrometer, use the relationship:

\((\#\ \text{stage spaces}) \times (0.01\ \text{mm}) = (\#\ \text{ocular spaces}) \times (\text{value of 1 ocular space, in mm})\)

You know the \# of stage spaces and the \# of ocular spaces; you need to solve for the value of 1 ocular space. Thus,

\[1\ \text{ocular space (mm)} = \frac{[(\#\ \text{stage spaces}) \times (0.01\ \text{mm})]}{\#\ \text{ocular spaces}}\]

For example, if 10 spaces on the ocular micrometer = 5 spaces on the stage micrometer, then:

\[1\ \text{ocular space (mm)} = \frac{[5\ \text{stage spaces} \times 0.01\ \text{mm}]}{10\ \text{ocular spaces}}\]

\[= \frac{0.05\ \text{mm}}{10}\]

\[= 0.005\ \text{mm} (= 5\ \mu\text{m})\]

Calculate (4 pts.):
High-dry power (40X):

1 ocular space (mm) = [_______ x 0.01 mm] ÷ [_______]

1 ocular space = _________ mm

= _________ µm

Oil immersion (100X):

1 ocular space (mm) = [_______ x 0.01 mm] ÷ [_______]

1 ocular space = _________ mm

= _________ µm

Next to the microscope used for calibration is another microscope with an ocular micrometer and a slide of prepared bacterial cells. Use your calculations above to estimate the size of one bacterial cell (2 pts.). Don’t forget to check the magnification!

Length of one bacterial cell = ______ ocular spaces at ______ X magnification

The length of the bacterial cell = _______ µm
EXERCISE 2
ASEPTIC TECHNIQUE

Objectives:
1. To learn aseptic techniques for working with bacterial cultures

Introduction:
Microorganisms include bacteria, fungi, and molds, and they are everywhere—in the air, in water, and on every kind of surface imaginable. Their widespread presence means that microbiologists have to take certain precautions when working with bacteria to avoid contamination. When studying bacteria, it is important that the bacterial culture be pure, meaning that it contains only cells of that bacterial species. Otherwise, the results of any tests or procedures are meaningless. From a clinical standpoint, it is imperative to avoid cross-contamination of samples, lest the wrong diagnosis be made.

In this lab we will learn methods for transferring and working with bacterial cultures aseptically, so that the cultures do not get contaminated. You will learn to transfer broth culture to broth, slant culture to broth, broth culture to slant, and slant culture to plate culture.

Materials (per student):
Test tube rack labeled with your name (share half with a partner)
1 5 mL nutrient broth culture of bacteria
1 nutrient agar slant culture of bacteria
2 5-mL tubes of sterile nutrient broth
1 nutrient agar plate
Inoculating loop
Period 1.

Procedure:

Part 1: Transfer of bacteria from broth to broth.

1. Using your Sharpie pen, label your plates and tubes with your first name (and last initial if necessary). When labeling plates, always label the BOTTOM of the plate rather than the lid.
2. Light your Bunsen burner.
3. Sterilize your loop by holding it in the flame of the Bunsen burner until it glows red. It works best to hold the loop at a slant so that 1 to 1 1/2” of the loop is in the flame. Avoid heating the handle of the loop.
4. Let the loop cool thoroughly. You may set the loop down on the bench, but make sure the sterilized end does not touch anything. Failure to let the loop cool thoroughly can kill the bacteria that you are trying to transfer with the loop. You know it’s too hot if the broth sizzles when you put the loop into it, or if the agar melts when you touch the loop to an agar plate.
5. With the loop in your dominant hand, pick up the broth culture in the other hand and shake it gently to make sure it’s mixed.
6. With the little finger of the hand holding the loop, grasp the cap of the tube and pull it off.
7. Immediately flame the mouth of the tube, but do not hold it in the flame too long or the tube may crack. Remove the tube from the flame.
8. Insert the loop into the culture, making sure you don’t go too deep and risk exposing the culture to an unflamed portion of your loop.
9. Remove the loop, flame the mouth of the culture, and replace the cap. Set the tube down in the rack.
10. Pick up the new tube of sterile broth, remove the cap as before, and flame the mouth of the tube.
11. Without flaming the loop, insert the loop containing some of the original culture into the new tube. Remove the loop and flame the mouth of the tube before replacing the cap.
12. Flame the loop to sterilize it.
Part 2. Transfer of bacteria from agar to broth.

1. With a loop sterilized and cooled as described in Part 1 in one hand, pick up your agar slant culture of bacteria in the other. Remove the cap as before, and flame the mouth of the tube.
2. Insert the loop and pick up a small amount of bacterial growth. Remove the loop, flame the mouth of the tube, and replace the cap. Put the culture back in your rack.
3. Pick up a fresh tube of nutrient broth, remove the cap, and flame the mouth of the tube.
4. Insert the loop containing the bacteria from the slant culture and stir gently to dislodge the bacteria into the broth.
5. Remove the loop, flame the mouth of the tube, replace the cap, and set it in your rack.
6. Sterilize your loop.

Part 3. Transfer of bacteria from broth to agar.

1. With a loop sterilized and cooled as described above, pick up your broth culture of bacteria. Remove the cap as before, and flame the mouth of the tube.
2. Insert the loop to get a drop of culture. Remove the loop, flame the mouth of the tube, and replace the cap. Put the culture back in your rack.
3. Carefully lift the lid of your agar plate with the hand that is not holding the loop. Do NOT set the lid down anywhere at anytime, and try to avoid breathing on the plate while the lid is off.
4. Carefully streak the loop back and forth over the surface of the plate so that the plate looks like this:

![Image of streaked agar plate]

5. Put the lid back down and flame your loop.
6. Note: this type of streak plate is designed for maintenance of cultures that are already PURE. A different method of streaking is used when trying to purify different species of bacteria from a common source (e.g. from a throat swab or from an environmental sample). We will learn about those methods in Lab exercise 3.

When you are finished with this part of the lab, give your labeled test tube rack and plates to your T.A. They will be incubated at 37 °C for 48 hours and then refrigerated until next lab period. Place your used cultures in the designated disposal area.

Period 2.
Observations and questions.

1. 1 pt. Did something grow in all of your transfers? If not, what could have been the problem?

2. 1 pt. Do any of your agar plates appear to be contaminated?

3. 1 pt. How would you know if your broth cultures were contaminated with a microbe other than the one you wanted to grow?
EXERCISE 3
MICROBIAL AND FUNGAL UBIQUITY

Introduction:
In this lab, we will look at the ubiquity of microorganisms in our environment. Tryptic soy agar is a standard complex medium, suitable for culturing many types of bacteria. Sabouraud’s agar is especially useful for growing molds, due to its low pH. This makes it a selective medium, as other types of microorganisms will not grow under those conditions.

Materials (per student):
1 tryptic soy agar (TSA) plate
1 Sabouraud’s agar (SA) plate
Sterile cotton swabs
Sterile water

Procedure:
Part 1. Exposure of TSA plate.
1. Choose a method for exposing your TSA plate.
   a. Expose plate to the air for 30 minutes (can be in lab, in another room, or outside).
   b. Expose plate by pressing your lips to the agar briefly.
   c. Expose plate by gently pressing your fingertips to the agar.
   d. Expose plate by pressing several coins to the agar. Remove coins after a minute or so.
   e. Comb or brush your hair over the agar.
   f. Expose by some other creative means.

2. Label your plate (on the bottom) with your name and your method of exposure.
Part 2. Exposure of SA plate.

1. Choose a method for exposing your SA plate.
   a. Expose plate to the air for 30 minutes.
   b. Expose plate to the tray of detritus for 30 minutes.
   c. Use a sterile swab moistened with sterile water to sample for molds in the location of your choice. Gently streak the swab across your SA plate.

2. Label your plate (on the bottom) with your name and the source of the mold.

When you are finished, give your plates to your TA for incubation until next week.

Period 2.

Observations and Questions:

1. 4 pts. Describe your TSA and SA plates.
EXERCISE 4
STAINING TECHNIQUES

Objectives:
1. To learn different staining techniques for identifying and observing microorganisms
2. To use these stains to observe your unknown bacterium

Introduction:
The development of staining techniques was of great importance to microbiology. Since many bacteria do not have pigments, it can be difficult to see individual cells under a light (bright-field) microscope. Stains range from simple to complex. Simple stains involve only one reagent, and stain all bacteria similarly. More complex stains involve multiple reagents, and are often differential. This means that they stain different types of bacteria differently.

One of the most important differential stains in microbiology is the Gram stain. Bacteria are classified into two main groups based on how they stain with this procedure. Gram-positive bacteria stain purple, while Gram-negative bacteria stain pink/red. The basis for the different stain result lies in the structure of the cell walls of these two groups of bacteria. Gram-positive bacteria have a large peptidoglycan layer and lack a lipopolysaccharide (LPS) layer. Gram-negative bacteria have a much thinner layer of peptidoglycan, but have a complex LPS.

The first step in the Gram stain involves staining heat-fixed cells with crystal violet. Crystal violet enters all cells and stains them purple. The next step is to fix the crystal violet in the cell using Gram’s iodine. The third step involves washing the cells in ethanol, and this is where the difference arises. For Gram-positive cells, the thick peptidoglycan layer dehydrates in the presence of ethanol, effectively locking the crystal violet inside. For Gram-negative cells, the LPS layer and thin peptidoglycan layer allows the ethanol to permeate the cell and remove the crystal violet. This renders them colorless. In order to see them, safranin is used as a counterstain. This stains the Gram-
negative cells pink/red. Gram-positive cells also pick up the safranin, but since they still contain crystal violet, they will appear dark purple.

There are also specialized stains for detecting the presence of endospores, capsules, and flagella. The presence of these cellular features is an important identification tool, as only certain genera of bacteria produce endospores, and not all bacteria have capsules or flagella. In addition, the flagellar pattern is used to identify different bacterial species.

In this exercise, you will practice some of the more commonly used staining procedures, ranging from simple stains and negative stains, to the more complex Gram stain. You will also learn how to stain for endospores and capsules.

**Materials (for all):**
- Microscope slides
- Cover slips
- Inoculating loops
- Broth cultures of various bacteria
- Microscopes
- Various stains

**Procedure:**

**Part 1. Simple staining with methylene blue.**

1. Using aseptic technique, transfer 2-3 loopfuls of liquid culture to a clean slide.
2. Allow the smear to air dry.
3. Heat-fix the smear by passing the slide through the burner flame a few times or by setting it on the slide warmer (60 °C) for 10 minutes. Heat-fixing helps kill the bacteria so that they don’t autolyse during staining, and also helps ensure that the bacteria stick to the slide and don’t come off during staining and washing steps.
4. Use a clothespin to hold the slide (otherwise you will get blue fingers!) and cover the smear with methylene blue for 30-60 seconds.
5. Wash off excess stain with distilled water.
6. Gently blot smear with absorbent paper so that it is dry.
7. Examine your smear and record your observations below (1 pt.).

Organism: _______________________
________________________________
________________________________
________________________________

Part 2. Negative staining with nigrosin.
1. Place a small drop of nigrosin stain at the end of a clean slide.
2. Using aseptic technique, mix a drop of broth culture (use a different one than in Part 1) into the drop of nigrosin. Avoid spreading the drop or allowing it to dry out.
3. Hold the edge of another slide at an angle to the first slide and spread the drop out along the first slide so that a gradient of dye forms.
4. Let the dye smear air dry, but do NOT heat fix. This can distort the cells.
5. Observe your smear under the microscope and record your observations below (1 pt.).
Organism: _______________________
________________________________
________________________________
________________________________

1. Using a marker, make 2 circles on a clean microscope slide. Make them big enough to contain the smears, and far enough apart so they don’t get cross contaminated. Mark one with a “+” and one with a “—”. Turn the slide over so that the ink is on the bottom.
2. Using aseptic technique, transfer 2-3 loopfuls of an *E. coli* broth culture to the circle marked “—“. Transfer 2-3 loopfuls of a *Bacillus subtilis* broth culture to the circle marked “+”. Let the slide air dry completely.
3. Heat fix the slide.
4. Use a clothespin to hold the slide, and apply **crystal violet** to each of the smears for 60 seconds.
5. Pour off the crystal violet and apply **Gram’s Iodine** for 60 seconds.
6. Pour off iodine and add fresh iodine for 1 minute.
7. Wash the slide with distilled water.
8. Decolorize the smears with **95% ethanol** for 15-30 seconds. This step is CRITICAL. Failure to decolorize completely will lead to confusing results. The time required depends on the thickness of the smear. Practice makes perfect.
9. Gently wash slide with distilled water to remove alcohol.
10. Apply **safranin** for 30 seconds.
11. Wash with distilled water.
12. Air dry.
13. Observe your smears under the microscope. Gram-negative organisms, such as *E. coli* will appear pink/red. Gram-positive organisms, such as *Bacillus sp.*, will appear dark purple.
14. If your Gram-stain results are questionable, you might want to repeat the process. This is a very important stain in microbiology and it requires a bit of practice to become proficient at it. A key aspect is to avoid making your smears too thick. You only need a few cells to make observations, after all.

**Part 4. Endospore staining.**

1. Using aseptic technique, make smears of each of two *Bacillus* cultures: *B. cereus* (24-hour), and *B. cereus* (1 week). Air dry and heat fix.
2. Place small pieces of paper towels over each smear. The paper towel pieces should be smaller than the slide.
3. Flood the papers over the smears with **malachite green**, and carefully place the slides over a beaker of boiling water.
4. Steam slides for 5 minutes, adding more stain as needed to keep the papers wet.
5. Remove the papers and discard in the trash.
6. Wash the smears carefully with distilled water.
7. Counterstain with **safranin** for 30 seconds.
8. Wash the smears with distilled water and blot dry.
9. Examine the smears microscopically and record your observations below (2 pts.).
Part 5. Capsule stain.

1. Prepare smears of *Klebsiella pneumoniae*. Air-dry the slides, but do not heat-fix as this will distort the capsules.

2. Flood the slide with *crystal violet* for 4-7 minutes.

3. Rinse slide thoroughly with 20% *copper sulfate* solution.

4. Blot dry and examine under oil immersion.

5. Record your observations below (1 pt.).

Organism: __________________

____________________________

____________________________
EXERCISE 5
TESTS USED TO IDENTIFY UNKNOWN BACTERIA

Objectives:
1. To learn how to perform some standard tests used to identify unknown bacteria

Introduction:
The ability to identify bacteria isolated from a sample is important to the study of ecology, the management of infectious disease, the safeguarding of food and water supplies, etc. Proper identification is necessary in order to understand the hazards posed by the presence of that particular bacterium. There are many tests used to identify bacteria, many of which are based on particular metabolic traits. Basic information, such as Gram-stain reaction and morphology, coupled with the origin of the strain, can be used to determine which tests to perform.

In this exercise, you will be given pure cultures of two bacteria to test. The tests you will use include the fluid thioglycollate test (to determine oxygen class), phenol red glucose, phenol red lactose, and phenol red mannitol (to determine ability to ferment those sugars), nitrate test (to determine the ability to reduce nitrate to nitrite, N₂, or some other product), Voges-Proskauer test (to determine whether your bacteria produce neutral products from fermentation), citrate test (to determine whether your bacteria can utilize citrate as an energy source), oxidase test (to determine whether your bacteria have cytochrome c oxidase; helps distinguish between pseudomonad species (+) and enteric species (−)), and catalase test (to determine whether your bacteria have the enzyme catalase, which catalyzes the release of O₂ from H₂O₂). In the second lab period, you will use your test results to confirm the identity of your test bacteria.
Materials (per student):
2 plate cultures of different bacteria (18 hours)
2 tubes of each:
  Fluid thioglycollate medium
  Phenol red glucose w/Durham tube
  Phenol red lactose w/Durham tube
  Phenol red mannitol w/Durham tube
  Nitrate w/Durham tube
  Voges-Proskauer medium
  Simmons citrate slants
3% hydrogen peroxide for catalase test
oxidase test strips/cards
microscope slides
sterile plastic loops for oxidase test

Period 1.
Procedure:
Part 1. Metabolic tests.
1. Fluid thioglycollate test.
   a. Label your two fluid thioglycollate tubes with your name and the names of your test bacteria.
   b. Using a flamed and cooled loop, obtain a small amount of one of your test cultures. Flame the tube, replace the cap, and set in your rack.
   c. Aseptically transfer the bacteria to your fluid thioglycollate tube using the stab method: gently stab your loop straight down to the bottom of the tube and pull it back out. Do not stab twice and take care not to mix or stir the medium, which is very soft.
   d. Flame the mouth of the tube, replace the cap, and carefully set it in your rack. Don’t forget to flame your loop when you are finished.
   e. Repeat these steps to inoculate a tube with your second bacterium.
   f. Set tubes at one end of your rack.
g. The fluid thioglycollate tubes will be incubated at room temperature. If possible, you should look at these tubes in **24-48 hours**, as oxygen diffuses into the medium over time and can lead to ambiguous results. Record your results in Table 5.1.

2. **Catalase test.**
   a. Place a drop of 3% hydrogen peroxide on a clean slide.
   b. Using a sterile loop, pick a **small** amount of one of your test bacteria from your plate and emulsify in the drop of H$_2$O$_2$.
   c. Look carefully to see if bubbles form, indicating the presence of the enzyme catalase. Record your results in Table 5.1.
   d. Repeat the procedure for your other bacterium and record the results.

3. **Oxidase test.**
   a. Obtain 2 oxidase test strips or one of the test cards.
   b. Using a sterile PLASTIC loop, pick a **small** amount of one of your test bacteria from your plate and smear it on the test area of the strip or card.
   c. Color change should occur rapidly—yellow/buff is a negative result, purple is a positive result.
   d. Record your result in Table 5.1.
   e. Repeat this test with your other test bacterium and record the result.

4. **Carbohydrate fermentation tests.**
   a. Obtain 2 tubes of each: phenol red glucose/dextrose, phenol red lactose, and phenol red mannitol. Label one of each type with the names of your test bacteria.
   b. Using aseptic technique, inoculate 1 set of tubes with one of your test bacteria. It’s ok to shake your loop gently to dislodge the bacteria if necessary.
   c. Using aseptic technique, inoculate the second set of tubes with your other bacterium.
   d. Place all tubes in your rack for incubation.
5. **Nitrate tests.**
   a. Obtain 2 nitrate tubes and label appropriately.
   b. Using aseptic technique, inoculate one tube with one of your test bacteria using a STAB technique and do NOT stir the media. The media is made so that part of the tube is anaerobic, and it is important to maintain this state.
   c. Repeat the process for your other test bacterium.
   d. Carefully set your tubes in your rack to incubate for a week at room temperature.

6. **Voges Proskauer tests.**
   a. Obtain 2 tubes of Voges-Proskauer medium and label them appropriately.
   b. Using aseptic technique, inoculate one tube with one of your test bacteria. Repeat the process for your other test bacterium.
   c. Incubate tubes in your rack at room temperature for at least 2 days before performing the Voges-Proskauer test (see Period 2, below).

7. **Citrate test.**
   a. Obtain 2 Simmons citrate agar slants, and label appropriately.
   b. Using aseptic technique, inoculate one tube with one of your test bacteria, using the “streak-stab” method. First, streak the slant then stab your loop into the middle of the slant. It is helpful to use an inoculating needle, rather than a loop.
   c. Repeat the process to inoculate a slant with your other test bacterium.
   d. Incubate tubes in your rack at room temperature.

**Part 2. Staining and morphology**

**Procedure:**
1. Prepare Gram-stains of both of your test bacteria. Record the results in Table 5.1.
2. Observe the morphology of both bacteria and record this information in Table 5.1.
Period 2. Test results:

Fluid thioglycollate test.
1. Examine your fluid thioglycollate tubes within 24-48 hours and note where bacterial growth occurs.
2. Determine the oxygen class of your test bacteria and record your results in Table 5.1.
   a. **Strict aerobes**: grow only at or slightly below the surface of the medium
   b. **Facultative**: will grow throughout the tube, but with heavier growth near surface
   c. **Microaerophiles**: grow just below the surface of the medium, but not right on the surface
   d. **Strict anaerobes**: grow only at bottom of the tube
   e. **Aerotolerant anaerobes**: grow equally well throughout tube as they neither require O\textsubscript{2} for growth nor are harmed by it

Carbohydrate fermentation.
1. Examine your phenol red tubes. The culture medium will be yellow if your bacteria have fermented the sugar in the tube. If the bacteria are not capable of fermenting the sugar in the tube, the tube will be red. Look for gas bubbles in the Durham tubes.
2. Record your results in Table 5.1.

Nitrate reduction test.
1. Examine the Durham tubes in each of your tubes for bubbles. If there is gas in the Durham tube, and NOT in the phenol red glucose tube, then you can be certain that the gas produced in the nitrate test is N\textsubscript{2}. Your bacteria are DENITRIFIERS, and have converted the nitrate in the medium to N\textsubscript{2}.
2. If there is no gas in the tube or you think the gas may be from fermentation, add a few drops of Nitrate Reagent A (sulfanilamide) to the tube. Then add a few drops of Reagent B (N-1-Naphthylethylene diamine dihydrochloride). A positive test will turn magenta in color, while a negative test will not change color.
3. A positive test indicates that nitrite is present, and your organism has reduced nitrate to nitrite.
4. A negative test means one of two things: either your bacteria didn’t reduce nitrate at all, or reduced it to something beyond nitrite. To distinguish between these two possibilities, add a small amount of powdered zinc to the tube that has tested negative so far. If there is any nitrate left in the tube, the zinc will reduce it to nitrite, which will then react with Reagents A and B to produce a magenta color. If this happens, then your bacterium is a NON-REDUCER of nitrate. If the medium does not change color, then your bacterium has reduced the nitrate to something beyond nitrite.

5. Record your results in Table 5.1.

**Voges-Proskauer test.**

1. Take a 2.5 mL sample of each of your cultures and put each into a clean tube.
2. Add Voges-Proskauer reagents A and B as per instructions on the kit.
3. Shake tubes well, but don’t spill!
4. If acetoin or 2,3-butanediol were formed by the culture, the test will be a pink-red color (= positive test).
5. If no pink-red color develops, the test is negative. Allow up to 20 minutes for color development before determining that the test is negative.
6. Record your results in Table 5.1.

**Citrate utilization test.**

1. Examine your Simmons citrate agar slants.
2. Your bacterium is positive for citrate utilization if the slant has turned blue. A negative result will look much like an uninoculated tube.
3. Record your results in Table 5.1.
11 pts. Table 5.1. Results of identification tests

<table>
<thead>
<tr>
<th>Test/procedure</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>Oxidase</td>
<td></td>
</tr>
<tr>
<td>Fermentation: glucose</td>
<td></td>
</tr>
<tr>
<td>Fermentation: lactose</td>
<td></td>
</tr>
<tr>
<td>Fermentation: mannitol</td>
<td></td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td></td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td></td>
</tr>
<tr>
<td>Citrate utilization</td>
<td></td>
</tr>
<tr>
<td>Oxygen class</td>
<td></td>
</tr>
</tbody>
</table>

Compare your results with the dichotomous key provided, and with other students in the lab who worked with the same bacterium. Do your results agree? If not, where could problems have occurred?
### Key for identifying unknown bacteria

<table>
<thead>
<tr>
<th></th>
<th>Gram</th>
<th>Morph</th>
<th>Cat</th>
<th>Ox</th>
<th>Glu</th>
<th>Lac</th>
<th>Mann</th>
<th>Nit</th>
<th>VP</th>
<th>Cit</th>
<th>O2</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>+</td>
<td>Rod</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aero.</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>Rod</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Fac. maybe</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>+</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aero.</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>+</td>
<td>Rod</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aero.</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>+</td>
<td>Coccus</td>
<td>+</td>
<td></td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Aero. Yellow</td>
</tr>
<tr>
<td><em>Micrococcus roseus</em></td>
<td>+</td>
<td>Coccus</td>
<td>+</td>
<td></td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Aero. Red/pink</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td>Coccus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac. Yellow</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>+</td>
<td>Coccus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac. White</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>+</td>
<td>Coccus</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aero. Pyocyanin (green/blue) produced</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Fac. Red @ 30°C</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Yersinia enterolytica</em></td>
<td>—</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fac.</td>
</tr>
<tr>
<td><em>Neisseria subflava</em></td>
<td>—</td>
<td>Coccus</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aero. or Fac.</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>—</td>
<td>Coccus</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Strict Aero.</td>
</tr>
</tbody>
</table>
EXERCISE 6
PURE CULTURE TECHNIQUES

Objectives:
1. To understand the principles behind pure culture techniques
2. To learn how to obtain a pure culture

Introduction:
When you streak a swab sample taken from the environment onto a nutrient agar plate, you can expect to see several types of colonies on the plate after it has been incubated. The colonies may differ in size, color, and shape, and each different type of colony represents a different species of bacteria. A look at the results of the streaks you did last week will reveal the great diversity of bacteria in natural populations. Many microbiologists wish to study individual species of bacteria, however, and in order to do that it is necessary to work with a pure culture.

There are two basic methods for obtaining a pure bacterial culture, the Streak Plate method, and the Pour Plate method. Both techniques are based on the assumption that a single bacterium on the agar surface will give rise to one colony. All bacterial cells within a colony are clones of the original bacterium that landed there when the plate was streaked or poured.

In the Streak Plate method, a sample of bacteria is streaked onto an agar plate using a loop and the loop is sterilized. Another set of streaks is made by crossing the first set of streaks a few times to pick up a few bacteria and then streaking back and forth to spread these out. The loop is flamed again, and the process repeated two more times. In the last set of streaks, the bacteria will be spread thinly enough that individual colonies will be well isolated from one another. The investigator can then pick individual colonies, which may represent pure cultures. Typically, additional streak plates are necessary before a culture can be termed “pure”, and this must be confirmed with microscopic observations and physiological tests.
The **Pour Plate** method involves taking a loopful of a mixed bacterial culture and diluting it into molten agar (50 °C), and mixing. A loopful of this is then transferred to a second tube of molten agar and mixed. Finally, a loopful of the second tube is transferred to a third tube of molten agar and mixed. The three tubes of molten agar are then poured into Petri plates, allowed to set, and incubated. The first dilution will almost certainly have colonies that touch each other and/or are not distinct. The second or third dilution, however, will probably have distinct, well-isolated colonies that can be picked for further purification. The main disadvantages of the Pour Plate method are that the bacteria must be able to withstand 50 °C agar for several minutes, you must work fast to ensure that the agar does not solidify before you can pour the plates, and it requires facilities for keeping the agar molten. For work in field situations, the Streak Plate method is more suitable.

In this lab exercise, you will use the streak plate method to separate different bacterial species from a mixed culture.

**Materials (per student):**
1. Tryptic soy agar (TSA) plate
2. Mixed culture of bacteria
3. Loop

**Period 1.**

**Procedure:**

1. Label one of your plates with your name and the word “Mixed”.
2. Using aseptic technique (sterile loop, flamed tube, etc.), obtain a loopful of the mixed bacterial culture.
3. After flaming the lip of the tube and replacing the cap, set the tube down in your rack.
4. Carefully lift the lid of your TSA plate (but remember, do NOT take the lid all the way off, expose it to the air, or set it down), and make several streaks back and forth along one edge of the plate as shown (#1).
5. Put the lid back down, and flame your loop. Cool the loop thoroughly. This is critical because a hot loop will kill any bacteria that it comes into contact with.
6. Lift the lid and with the newly sterilized and cooled loop make a second set of streaks as shown (#2). Put the lid back down, flame your loop, and cool thoroughly.

7. Repeat this process as shown two more times, making streaks as shown (#3 and #4). DO NOT run the last set of streaks into the first set.

When you are finished, give your plate to your T.A. and put the mixed culture in the disposal area.

**Period 2.**

**Observations and questions.** (complete next week)

Examine your streak plates.

1. 2 pts. Did you get good colony separation from your streak of the mixed bacterial culture? Describe the types of colonies you observe.
EXERCISE 7
BACTERIAL POPULATION COUNTS

Objectives:
1. To understand the difference between direct and indirect measurements of bacterial growth
2. To learn how to do a dilution series
3. To learn how to do a viable plate count
4. To learn how to correlate an indirect measurement of bacterial numbers (optical density) with a direct measurement (viable plate count)

Introduction:
It is often useful to know the number of bacteria present in a culture or in a particular sample. Such techniques are used not only by microbiologists studying some aspect of microbial growth, but are also important for determining the amount of bacteria present in food, water, and milk.

There are many techniques for measuring microbial growth or population size, but they can be divided into two main groups, based on whether the population size is determined directly or indirectly. Direct counts include counting cells under the microscope (with or without special stains), using electronic particle counters, or counting colonies on spread plates (also called a viable plate count). Indirect methods provide an estimate of cell numbers and can be done by measuring dry weight, the optical density of a culture, or by measurements of total protein. Indirect methods have the advantage of being more rapid than direct methods, but in order to be meaningful, an indirect method must first be correlated to a direct method.

In this exercise, we will use viable plate counts (a direct method) and optical density measurements (an indirect method) to estimate the number of bacteria in a broth culture. Keep in mind that viable plate counts will tend to underestimate the number of bacteria in a culture because only bacteria that are alive and capable of growing under the incubation
Period 1.

Materials (per 4 students):
10 mL tryptic soy broth (TSB) culture of *Micrococcus luteus*, *Bacillus cereus*, OR *Staphylococcus aureus*
2 sterile 99 mL dilution blanks (r.o. water)
5 sterile 9 mL dilution tubes (r.o. water)
1 mL pipettes
5 tryptic soy agar (TSA) plates
glass hockey stick and ethanol for flaming

Procedure:
1. Label all plates with the name of someone in your group, and the name of your bacterium (**important!**).
2. Label 5 TSA plates as follows:
   \[10^{-5} \quad 10^{6} \quad 10^{-7} \quad 10^{8} \quad 10^{-9}\]
3. Label 2 99 mL dilution blanks as follows:
   \[10^{-2} \quad 10^{4}\]
4. Label 5 9 mL dilution blanks as follows:
   \[10^{-5} \quad 10^{6} \quad 10^{-7} \quad 10^{8} \quad 10^{-9}\]
5. NOTE: The following steps require ASEPTIC transfers. Don’t forget to flame the mouths of all bottles and tubes, both before and after removing or adding samples. The pipettes are already sterile, so it is not necessary to flame them. However, you must take care to avoid cross-contamination of your dilutions by using a fresh pipette when directed to do so. Avoid touching the pipette tip to anything, even after you have used it (it is covered with bacteria). Dispose of all pipettes in the autoclave bag provided.
6. **Student 1**: Aseptically transfer 1 mL of culture into the \(10^{-2}\) dilution blank, and shake for 1 minute. Dispose of the pipette.
7. **Student 2:** Using a NEW pipette, aseptically transfer 1 mL from the $10^{-2}$ bottle to the $10^{-4}$ dilution blank. Shake for one minute. Discard pipette in the appropriate place.

8. **Student 3:** Using a NEW pipette, aseptically transfer 1 mL of the $10^{-4}$ bottle to the $10^{-5}$ dilution blank.

9. Using the SAME pipette, transfer 0.5 mL of the $10^{-5}$ dilution to the center of the plate marked “$10^{-5}$”. Discard the pipette.

10. **All members read:** Using an alcohol-flamed hockey stick that has been cooled thoroughly, evenly disperse the liquid across the surface of the plate. It is important to get it as even as you can, and avoid having lots of liquid accumulate at the edges of the plate. Let the plates sit so the liquid can absorb into the agar. Moving the plates around too soon can result in all the bacteria being at the periphery of the plate (and you won’t be able to count them).

11. TIP: alcohol-flame the hockey stick after all spreading procedures so that it can cool while you are doing the next dilution.

12. **Student 4:** Using a NEW pipette, transfer 1 mL of the $10^{-5}$ dilution to the $10^{-6}$ dilution blank and shake for 1 minute.

13. Using the SAME pipette, transfer 0.5 mL of the $10^{-6}$ dilution to the TSA plate marked “$10^{-6}$”, and spread evenly using the hockey stick. Discard the pipette.

14. **Student 1:** Using a NEW pipette, transfer 1 mL of the $10^{-6}$ dilution to the $10^{-7}$ dilution blank and shake for 1 minute.

15. Using the SAME pipette, transfer 0.5 mL of the $10^{-7}$ dilution to the TSA plate marked “$10^{-7}$”, and spread as before. Discard the pipette.

16. **Student 2:** Using a NEW pipette, transfer 1 mL of the $10^{-7}$ dilution to the $10^{-8}$ dilution blank, and shake for 1 minute.

17. Using the SAME pipette, transfer 0.5 mL of the $10^{-8}$ dilution to the TSA plate marked “$10^{-8}$”, and spread as before. Discard the pipette.

18. **Student 3:** Using a NEW pipette, transfer 1 mL of the $10^{-8}$ dilution to the $10^{-9}$ dilution blank, and shake for 1 minute.

19. Using the SAME pipette, transfer 0.5 mL of the $10^{-9}$ dilution to the TSA plate marked “$10^{-9}$” and spread as before. Discard the pipette.

20. **All:** breathe. You have just completed your first dilution series!
21. Let plates sit for at least 10 minutes before inverting and stacking. Place your stack of plates in the 37 °C incubator.

22. Plates will be incubated for 48 hours and then refrigerated until the next lab period.

**Part 2. Measuring the optical density of dilutions of a bacterial culture.**

**Materials (per 4 students):**

- Bacterial culture used in Part 1
- 4 5-mL tubes of TSB
- 5 mL sterile pipettes
- spectrophotometer set to read absorption at 686 nm

**Procedure:**

1. Label 3 of the tubes “1/2”, “1/4”, and “1/8”. These values represent the dilutions that you will be making. Label the 4th tube “Blank”.

2. Pipette 5 mL of your bacterial culture into the “1/2” tube. Mix by gently flicking the bottom of the tube with one finger, or by rolling the tube in your hand. Be careful not to invert the tube or it will leak.

3. Pipette 5 mL from the “1/2” tube to the tube marked “1/4” and mix. Put the “1/2” tube in your rack—you will need it in a minute.

4. Pipette 5 mL from the “1/4” tube to the tube marked “1/8” and mix. Put both tubes in your rack.

5. Fill a clean cuvette with broth from the “Blank” tube and insert into the spectrophotometer. Zero the spectrophotometer.

6. Remove the cuvette and pour the broth into the waste container provided. Do not rinse cuvette.

7. Fill the cuvette with sample from the “1/8” tube. Place in the spectrophotometer and record the absorption value in the table below.

8. Important: DO NOT zero the spectrophotometer at this point, or you will have to start all over (think about what you would be doing here).

9. Remove the cuvette and pour the broth into the water container provided. Rinse with distilled water.

10. Repeat steps 7 and 8 for the “1/4” and “1/2” tubes.
Table 7.1. Optical density of diluted bacterial cultures (3 pts.).

<table>
<thead>
<tr>
<th>Species:</th>
<th>1/8 dilution (0.125)</th>
<th>1/4 dilution (0.25)</th>
<th>1/2 dilution (0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If you did your dilutions accurately, the 1/4 dilution should have approximately half the absorption of the 1/2 dilution, and about twice the absorption of the 1/8 dilution.

In general, the linear relationship between absorption and cell numbers holds up until absorption reaches about 1.0. Beyond this point, the cell numbers are so high that some of the light initially scattered by one cell may be scattered forward to the detector by another cell, thus causing the amount of light hitting the detector to increase. This is why dilutions are made.

**Period 2 (finish next week).**

**Part 3. Linking viable plate counts with turbidity measurements: generating a conversion factor.**

Using viable plate count data and optical density data obtained from the same culture, it is possible to calculate a conversion factor. The conversion factor relates optical density (which is unit-less) to the number of viable cells in a culture. The conversion factor can be used in later experiments (using the same bacterial species) to convert optical density readings to estimates of the numbers of viable cells in a culture, thus avoiding the need for time-consuming viable cell counts.

To calculate the conversion factor, you first need to determine the number of viable cells per mL of your original culture. As a rule, plates are counted that have from 30-300 colonies on them. If there are more than 300, the plate will be impossible to count. If there are fewer than 30, it is statistically unreliable.

Look at your dilution plates and find the dilution that has from 30 to 300 colonies on it. You probably only have one plate that is useable, and this makes mathematical sense.
For example, if you have 20 colonies on the $10^{-7}$ dilution, by definition, the $10^{-6}$ plate will have 200, and the $10^{-5}$ plate will have 2000.

To determine the concentration of cells in the original culture, simply take the number of colonies on the plate, multiply by the inverse of the dilution, and divide by the volume that you plated. For example, if you counted 250 colonies on the $10^{-6}$ plate, and you plated 0.5 mL of the original culture, then the concentration of bacteria in the original culture will be (2 pts.):

$$\frac{250 \times 10^6 \text{ cells}}{0.5 \text{ mL}} = 500 \times 10^6 \text{ cells/mL}$$

# colonies: ___________  Concentration in the original culture: ________________ cells/mL
Dilution factor: ___________  ___________________  
Volume plated: ___________

Now that you know the concentration of cells in the original culture, you can relate that to your optical density readings taken from the same culture. Let’s say your original culture had $2 \times 10^7$ cells/mL. The “1/2” dilution would have a cell density of $1 \times 10^7$ cells/mL. Let’s say the “1/2” dilution had an absorption value of 0.7. You can use these values to set up a ratio:

$$\frac{1 \times 10^7 \text{ cells/mL}}{0.7 \text{ O.D.}} = \frac{x \text{ cells/mL}}{1.0 \text{ O.D.}}$$

Solving for $x$, we find that 1.0 O.D. reading is equal to $1.43 \times 10^7$ cells/mL, which is your conversion factor.

Set up a ratio to determine your conversion factor (2 pts.):

$$\frac{\text{__________ cells/mL}}{\text{O.D.}} = \frac{x \text{ cells/mL}}{1.0 \text{ O.D.}}$$

Conversion factor: 1 O.D. = ________________ cells/mL
In later experiments using the same bacterial species, you can just multiply your O.D. reading by the conversion factor to obtain the concentration of cells in the culture you are working with in those experiments. For example, if your O.D. reading is 0.25, then the cell density in that culture is $3.57 \times 10^6$ cells/mL.

Using your conversion factor, estimate the cell density of a culture with the following optical density values (3 pts.):

0.152

0.645

0.892
EXERCISE 8
MICROBIAL GROWTH CURVES

Objectives:
1. To investigate some factors contributing to microbial growth
2. To construct microbial growth curves

Introduction:
There are many factors controlling microbial growth, including temperature, oxygen, pH, and water availability. All bacteria have a minimum, maximum, and optimum temperature for growth. At low temperatures, enzymatic activity is slowed and growth is minimal. At high temperatures, enzymatic activity is high, and growth is rapid. Some bacteria are strict aerobes, and will not grow well under conditions where O\textsubscript{2} concentration is low. Others are facultative anaerobes, which grow best with lots of O\textsubscript{2}, but can switch to anaerobic respiration if O\textsubscript{2} levels are reduced. Some microbes are halophiles (have an absolute requirement for moderate to high concentrations of salt) or halotolerant (can tolerate some salt in their environment, but do not require it for growth). Microbes that cannot tolerate high salt become dehydrated and cannot grow.

In this lab, we will investigate the effects of either temperature or oxygen on the growth of three different species of bacteria.

Materials (per group of 4 students):
2 200 mL TSB cultures of Micrococcus luteus, Bacillus cereus, OR Staphylococcus aureus (NOTE: use the same organism that you used for Exercise 7)
Stir plates
Spectrophotometer and cuvettes
10 mL tube of TSB to use as blank
Period 1.

Procedure:
1. Obtain two 200 mL cultures of your bacterium (the same one used in Exercise 7).
2. Decide which factor you want to manipulate (temperature or aeration rate), and how you are going to vary them (i.e. what two stir plate settings or what two temperatures). Label the flasks with the experimental condition.
3. Use sterile TSB to zero the spectrophotometer at 686 nm (reading absorption). Record the absorption of each of your two cultures in Table 8.1. Use a separate Pasteur pipette for each culture. Discard the samples in the waste beaker after each measurement.
4. Set up your cultures and note the time in Table 8.1. This is time “0”.
5. Determine O.D. readings every 20-30 minutes for both of your cultures. Record the data in Table 8.1 (5 pts.).
6. Use your conversion factor from Exercise 7 to determine the concentration of cells at each time point for both cultures, and record the results in Table 8.1 (5 pts.).

Table 8.1. Effects of _____________________ on growth of ____________________.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Absorption reading</th>
<th>Calculated cell density</th>
<th>$\log_{10}$ cell #</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7. Construct growth curves of \textbf{cells/mL vs. time} for both of your experimental conditions and attach to page 42. Label the phases of growth seen in each curve (5 pts).

8. Determine the \(\log_{10}\) of each cell concentration where the cultures were in exponential phase, and record in Table 8.1 (5 pts.).

9. Construct growth curves of \(\log_{10} \text{cells/mL vs. time}\) for both of your experimental cultures and attach to page 42. Only include those time points where cultures were in exponential phase (e.g. the plot should be linear) (5 pts.).

10. Fit a regression line to each curve, and include the equations on the graph (3 pts.).

11. From the slope (m) of each line, determine \(g\) (remember that \(g = 0.301/m\)) (2 pts.).
   
   a. \underline{__________________________} \quad g = \underline{\quad} \text{min.}

   b. \underline{__________________________} \quad g = \underline{\quad} \text{min.}

Questions:

1. 5 pts. Discuss the variation in \(g\) between your different experimental conditions. Compare your results with those observed by others working with the same bacterial species.
Microbial growth curves
EXERCISE 9
CHEMICAL CONTROL OF MICROBIAL GROWTH

Objectives:
1. To distinguish between a disinfectant and an antiseptic
2. To distinguish between bacteriocides and bacteriostatics
3. To evaluate the effectiveness of various disinfectants and antiseptics

Introduction:
Inhibition of microbial growth is of great importance to everyone—in lab settings, in the food industry, and in clinical settings. It is important to distinguish between the complete elimination of all microbes (sterilization) and the mere reduction in their numbers. Depending on the situation, sterilization may not be possible (or even desirable) but a reduction in microbial load can be achieved in virtually all situations. For example, in a research or testing laboratory, glassware and culture media can be completely sterilized using autoclaves or by filter sterilization of non-autoclavable media. In the food industry, many foods cannot withstand the high temperatures involved in autoclaving, and filter sterilization may not be practical. Methods have been developed, however, for reducing the microbial load of our foods. One of the most familiar of these is pasteurization, which has greatly improved the shelf life of milk and other food products and reduced the incidence of food poisoning. In a clinical setting, it is imperative that microbes be eliminated or reduced as much as possible in order to avoid cross-contamination and infection of patients. Disinfectants are used to reduce the numbers of microbes on non-living surfaces, while antiseptics are used to reduce the microbial population on living tissue.

Antibacterial chemicals can be classified according to their effect on bacteria. Chemicals that kill bacteria are called bactericidal, while those that both kill the cells and cause them to lyse are called bacteriolytic. Chemicals that only inhibit the growth of bacteria are said to be bacteriostatic. In this case, growth of the bacteria will resume once the chemical is removed.

In this exercise, you will look at how various disinfectants affect microbial growth. You will first investigate the effects of disinfectants on growth as a function of
exposure time. Finally you will attempt to determine if the disinfectant is bacteriostatic, bacteriolytic, or bacteriocidal in its function.

Period 1.
Evaluating the effects of disinfectants on microbial growth over time.

Materials (per 4 students):
2x 200-mL culture of Micrococcus luteus, Bacillus cereus, or Staphylococcus aureus (NOTE: use the same species used in Ex. 7 and 8)
disinfectant
2 stir plates
1 10 mL tube of TSB
spectrophotometer and cuvettes
2 100 mL flasks of TSB
2 TSB plates

Procedure:
1. Obtain two 200 mL cultures of your bacterial species for which you have conversion factor data.
2. Label one culture “Control”, and label the other with the name of the disinfectant chemical that you are going to test.
3. Using TSB as a blank, obtain initial absorption readings for your two cultures and record in Table 9.1 (1 pt.).
4. Add disinfectant to your experimental flask as instructed. Place both flasks on the stir plates, using the same stir setting for both.
5. Note the time in Table 9.1 (this is time “0”).
6. Using a fresh Pasteur pipette each time, obtain absorption readings for both of your cultures every 15 minutes or so for at least 1 hour, and record in Table 9.1 (4 pts.).
7. Calculate the cell density for both cultures at each time point and record in Table 9.1 (5 pts.).
8. At the end of the experiment, pipette 0.1 mL of each of your cultures into 100 mL flasks of TSB (use a separate tube for each culture!). Mix well. This represents a
1000-fold dilution, which will help reduce the # of colonies on your plates (below) and also dilute the disinfectant in the experimental sample.

9. Pipette 0.1 mL of your diluted cultures onto separate TSB agar plates and spread with an alcohol-flamed and cooled glass hockey stick. Let sit for a few minutes and give the plates to your T.A. for incubation.

Conversion factor (from Ex. 7): _________________________________

Table 9.1. Effects of ________________ on growth of ________________.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th></th>
<th>Experimental</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorption</td>
<td>Cell density</td>
<td>Absorption</td>
<td>Cell density</td>
</tr>
<tr>
<td>Initial (t=0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Period 2. Results from plate incubations:

1. 4 pts. Using your final cell concentration in Table 9.1 and the dilutions (0.1 mL in 100 mL, then 0.1 mL plated), how many colonies would you expect to see on each of your plates (show your work)?

2. 2 pts. What conclusions can you draw about your disinfectant (e.g. is it bacteriostatic, bacteriolytic, or bacteriocidal)?
EXERCISE 10
BACTERIA OF THE ORAL AND NASAL PASSAGES

Objectives:
1. To isolate and study some of the bacteria that are present as normal flora of the mouth and nose
2. To learn how selective and differential media can be used to isolate and distinguish specific species of bacteria

Introduction:
The mouth and nose harbor many species of microorganisms. Some species of the respiratory tract are pathogenic. In healthy individuals, they are held at bay by immune defenses. However, when injury to the respiratory mucosa occurs, the pathogens are capable of multiplying and causing disease. In the mouth, there are a number of species that are essentially parasites. Their growth alters the conditions of the mouth such that the individual is more susceptible to tooth decay and ulcerations.

Some of the normal flora of the mouth and nose that are opportunistic pathogens include:

- *Streptococcus mitis*: causes ulcerations on the root of an injured or diseased tooth
- *Streptococcus pyogenes*: causes strep throat
- *Candida albicans*: a yeast that causes “thrush” in small children, elderly people, or immunocompromised individuals
- *Streptococcus pneumoniae*: can cause pneumonia following a respiratory infection or if tissue resistance is low

In this exercise, you will look at some of the microflora that are present in your mouth and nose. We will use several kinds of media.

**Mannitol salt agar**: This media is both differential and selective. The media contains 7.5% NaCl, which permits only the growth of *Staphylococcus sp.* This makes it a selective medium because it selects for *Staphylococcus*. The media also contains mannitol and phenol red, which is a pH indicator.
*Staphylococcus aureus* can ferment mannitol, thus lowering the pH of the medium below 6.4. At this pH, the phenol red will turn yellow. *Staphylococcus epidermidis*, however, does not ferment mannitol. So, while it will grow on the mannitol salt agar plate, the plate remains red in color. This red/yellow color change allows us to distinguish between *S. aureus* and *S. epidermidis*, and thus makes the mannitol salt agar a **differential** medium. *Staphylococcus* species that ferment mannitol are pathogenic, while non-pathogenic species cannot ferment mannitol.

**Mitis-salivarius agar:** Mitis-salivarius agar is **selective** in that it enhances the growth of *Streptococcus* species, while inhibiting the growth of other bacterial species. It is also **differential** because different species of *Streptococcus* can be distinguished on the basis of colony morphology. *Streptococcus salivarius* forms large blue gum drop-like colonies due to the synthesis of levan and dextran. *Streptococcus mitis* and *Streptococcus pyogenes* form small blue colonies. *Streptococcus mutans*, implicated in dental caries, forms blue colonies that resemble burnt sugar or etched glass.

**BiGGY agar:** BiGGY agar contains bismuth sulfite, glycine, and dextrose. It is **selective** for *Candida* sp., while inhibiting the growth of other bacteria. It is also **differential**. *Candida albicans* will form black colonies without a metallic sheen and without color diffusion. Other species of *Candida* form black colonies with a metallic sheen and color diffusion. The *Candida* ferment the dextrose in the agar, using bismuth sulfite as an electron acceptor. This gives the colonies a black color.

**Blood agar:** Blood agar contains red blood cells. Bacteria that produce **hemolysins** can lyse red blood cells. There are two main types of hemolysis: α and β, both visible by a zone of red blood cell destruction around a colony. In α-hemolysis, potassium and other ions leak out of the red blood cells creating a green/brown colored zone around the colony. In β-hemolysis, red blood cells are completely lysed, resulting in a clear zone around the colony. Bacteria that do not lyse red blood cells are said to be γ-hemolytic (as in “gamma” not “γ”).
Hemolysis patterns are used to differentiate between several species of *Streptococcus* and *Staphylococcus*.

We will also examine our susceptibility to dental caries using **Snyder test agar**. This test is based on the assumption that an acidic environment aids in the decalcification of tooth enamel, thus making the tooth more prone to caries. Decalcification begins at pH 5.5, and becomes more rapid as the pH drops. Snyder test agar contains 2% dextrose and the pH indicator bromcresol green. The high concentration of dextrose (sugar) encourages the growth of cavity-causing bacteria (e.g. *Streptococcus mutans*), which produce lactic acid and lower the pH of the medium. Bromcresol green is green at pH > 4.8, but turns yellow at pH < 4.4. The rate at which the medium turns yellow is used to estimate one’s susceptibility to dental caries. The more lactic acid-producing bacteria in your mouth, the faster the pH will drop. One look at these results, and you may never eat sugar again…

**Period 1.**

**Materials (per student):**

- 1 Mannitol salt agar plate
- 1 Mitis salivarius agar plate
- 1 BiGGY agar plate
- 1 blood agar plate
- 1 tube of Snyder Test agar at 50 °C (do not take until you are ready)
- 1 sterile test tube for collecting saliva
- 1 sterile swab
- sterile saline for moistening swab
- 1 sterile 1-mL pipette
Procedure:

Period 1.

Part 1. Inoculation of plates.

1. Label your plates with your name. Divide the mannitol salt agar and blood agar plates in half, and label with “mouth” and “nose”. Label the Mitis-salivarius agar and the BiGGY agar plates with “mouth”.

2. Collect some of your saliva in a sterile test tube. Collect at least 1-2 mL. Vigorously mix your tube to disperse the bacteria.

3. Using PURE CULTURE TECHNIQUE, inoculate the “mouth” half of your **mannitol salt agar plate** with your saliva. You will probably have room for only 3 sets of streaks.

4. Using PURE CULTURE TECHNIQUE, inoculate the “mouth” half of your **blood agar plate** with a loopful of your saliva. You will probably have room for only 3 sets of streaks.

5. Use a flamed and cooled loop to inoculate your **BiGGY agar plate** with a loopful of saliva. Streak the loop back and forth over the entire plate.

6. Use a flamed and cooled loop to inoculate your **Mitis-salivarius agar plate** with a loopful of your saliva.

7. Use a little sterile saline to moisten your sterile swab. Carefully swab inside your nose, then use this to inoculate a small area of the “nose” half of your **mannitol salt agar plate**.

8. Using the same swab, swab a small area of the “nose” half of your **blood agar plate**.

9. Discard the swab in the autoclave bag.

10. Use your sterilized, cooled loop and PURE CULTURE TECHNIQUE to continue streaking the “nose” part of your **blood agar plate**.

11. Sterilize the loop and use it to continue streaking the “nose” part of the **mannitol salt agar plate** using PURE CULTURE TECHNIQUE.

12. Give your plates to your T.A. for incubation.
Part 2. Inoculation of Snyder Test Agar.
1. Obtain a sterile 1 mL pipette and 1 tube of Snyder Test Agar at 50 °C.
2. Pipette 0.2 mL of your saliva into the tube, being careful not to touch the side of the tube or the agar.
3. Immediately mix the tube by rolling it between your hands.
4. Label the tube with your name, and let it solidify.
5. Give the tube to your T.A. for incubation.
6. The tube will be incubated at 37 °C for 24-72 hours, and should be checked every 24 hours for color change (instructor and/or T.A.s will do this for you).
7. Place your pipette in the autoclave bag and your test tube in the disposal area.

Period 2.

Mannitol salt agar plate:
1. 2 pts. Describe the appearance of your plate. What does a yellow color mean?

2. 1 pt. What species of *Staphylococcus* are present in your nose?

3. 1 pt. What species of *Staphylococcus* are present in your mouth?

Mitis-salivarius agar plate:
1. 1 pt. Describe the types of colonies present on your Mitis-salivarius agar plate. Use a dissecting microscope if you’d like.

2. 1 pt. What species of *Streptococcus* are present in your mouth?

Blood agar plate:
1. 2 pts. What type(s) of hemolysis are apparent on each section of your blood agar plate?
   a. Nose: _______________________
   b. Mouth: ______________________

**BiGGY agar plate:**

1. 1 pt. Describe your BiGGY agar plate.

**Snyder Test Agar results:**

1. Tubes were checked every 24 hours and moved to the appropriate rack if they had turned yellow.
2. Find your tube in the labeled racks at the front of the room. Note the time written on the rack. This is the time at which your tube was noted to be yellow.
3. Circle your susceptibility rating in the table below (1 pt.).

**Table 10.1. Susceptibility to dental caries based on Snyder Test Agar results.**

<table>
<thead>
<tr>
<th>Time</th>
<th>Color</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>Green</td>
<td>(Check again at 48 hours)</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>Very susceptible</td>
</tr>
<tr>
<td>48 hours</td>
<td>Green</td>
<td>(Check again at 72 hours)</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>72 hours</td>
<td>Green</td>
<td>Not susceptible</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>Slightly susceptible</td>
</tr>
</tbody>
</table>
EXERCISE 11
BACTERIA OF THE GASTROINTESTINAL TRACT

Objective:
1. To characterize bacteria normally found in the gastrointestinal tract
2. To determine the antibiotic sensitivity of these bacteria

Introduction:
The large intestine is an important microbial habitat, supporting populations of over $10^{11}$ cells/g of feces. These resident bacteria are important to our biology in that they synthesize vitamins (e.g. Vitamin K and folic acid) and prevent the colonization and growth of pathogenic bacteria. They are also a source of opportunistic infections, and can cause disease in other parts of the body. For example Pseudomonas species are normal residents of the gastrointestinal tract, but can cause serious infections elsewhere in the body. Some of these infections can be particularly serious because of the existence of strains that are highly resistant to antibiotics.

The microbial population of the gastrointestinal tract consists primarily of anaerobes (Bacteroides, Bifidobacterium, Enterococcus, and Lactobacillus), and facultative anaerobes (Escherichia, Proteus, Citrobacter, and Enterobacter). Some of these enteric species can ferment lactose and are referred to as coliforms. Coliforms are generally not pathogenic. In contrast, non-lactose fermenting enterics tend to be pathogenic, and include such species as Shigella and Salmonella.

In this exercise, you will use various tests to identify an unknown enteric bacterium. We will use MacConkey agar and Eosin-Methylene blue agar to differentiate between coliforms and non-lactose fermenters. Coliforms form red colonies on MacConkey agar, and form blue/black/green colonies with a metallic sheen on EMB agar. Non-lactose fermenters form colorless colonies on MacConkey agar and colorless/amber/light purple colonies on EMB agar. You will characterize your unknown using a number of commonly used tests: Triple Sugar Iron (TSI) agar, Motility indole ornithine (MIO) agar, urease activity, and mannitol fermentation. Finally, you will examine the effects of various antibiotics on your unknown. Each of these tests will be
explained in detail below. You will use the results from your tests to try and identify your unknown.

**Explanation of tests.**

**TSI agar** contains three kinds of sugar (glucose, lactose, and sucrose), ferrous sulfate, and a pH indicator (phenol red), all in a base of nutrient agar. It is used to differentiate species of intestinal bacteria based on their metabolic patterns.

The concentration of glucose is much lower than that of lactose and sucrose (0.1% vs. 1.0%), and helps distinguish between species that can ferment only glucose vs. those that can also ferment lactose and/or sucrose. The agar is made as a slant, and inoculated by first streaking the surface of the slant (zig-zag) and then stabbing the loop to the bottom of the tube. This creates both an anaerobic environment and an aerobic environment in the same tube. For an organism that can only ferment glucose, the release of acid will cause the phenol red to turn yellow initially. However, because the concentration of glucose is so small, the bacteria quickly run out of it and turn to oxidizing amino acids for energy. This aerobic process causes the pH to increase so that the phenol red turns back to red in the slant portion of the tube. The butt of the tube (anaerobic) remains yellow. Organisms that can ferment lactose and/or sucrose revert to those energy sources when the glucose supply is exhausted. In this case, the entire slant will be yellow for several days. The production of gas can also be ascertained, as the gas will lift the agar in the tube and small bubbles may be visible in the agar.

The ability of some organisms to produce hydrogen sulfide (H$_2$S) by utilizing sulfur-containing amino acids can also be determined using TSI agar slants. The H$_2$S reacts with the ferrous sulfate in the agar to produce a black precipitate.

**Motility indole ornithine agar** is used to determine motility, indole production, and ornithine decarboxylase activity. The tubes are inoculated using a stab technique, and bacteria are motile if growth occurs away from the stab line. Indole is produced by bacteria that metabolize the amino acid tryptophan, and accumulates in the medium. Indole is detected by adding **Kovac's reagent**, which reacts with the indole to form a bright red color at the surface of the culture. Some bacteria can utilize ornithine via the enzyme ornithine decarboxylase. The medium contains an indicator dye, bromcresol purple, which is purple at pH >5.2, and yellow at acid pH. If the bacteria ferment
glucose, the fermentation products will reduce the pH, turning the tube yellow. The
acidic conditions activate decarboxylase enzymes that remove —COOH groups from
amino acids. In this case, bacteria that can decarboxylate ornithine will do so, producing
putrescine, an alkaline byproduct. The pH will increase, turning the tube back to purple.

Some bacteria (including enterics) can utilize **urea** as an energy source, forming
CO₂, ammonia, and water as end products. The ammonia builds up in the medium,
causing the pH to increase. A pH indicator in the **urea broth medium** will turn from
orange-red to purplish red or deep pink if the bacteria are metabolizing urea to produce
ammonia.

Some bacteria can ferment **mannitol**, and the accompanying acid production will
cause the pH of the medium to drop. Phenol red is an indicator dye that is red at pH >
6.5, but turns yellow at pH <6.4. The small Durham tube in the culture tube will trap gas
that is produced during fermentation, if any.

**Period 1.**

**Materials (per student):**

1 MacConkey broth culture of an enteric bacterium
1 MacConkey agar plate
1 Eosin-Methylene blue (EMB) agar plate
1 Triple sugar iron (TSI) agar slant
1 Motility indole ornithine (MIO) agar tube
1 5-mL tube of urea broth
1 5-mL tube of phenol red mannitol w/Durham tube
1 Mueller-Hinton agar plate
sterile swab
various antibiotic disks
1 test tube rack (can be shared with a partner)
Procedure:
1. Obtain a MacConkey agar plate and an EMB agar plate and label both with your name.
2. Inoculate a small area of the MacConkey agar plate, and a small area of the EMB agar plate.
3. Use a sterile plastic disposable loop and PURE CULTURE TECHNIQUE to inoculate the rest of the MacConkey agar plate.
4. Repeat step 4 with your EMB plate.
5. Give the plates to your T.A. for incubation at 37 °C.
6. Obtain your tubes of media and label them with your name and the number of your unknown. You will use your broth culture to inoculate the test media.
7. Using aseptic technique, inoculate the TSI agar slant by first streaking the surface of the slant (zig-zag) then stabbing to the bottom of the tube ONCE.
8. Place your tube in your rack. Flame and cool your loop.
9. Using aseptic technique, inoculate your MIO tube with your unknown by stabbing to the bottom of the tube once. Flame and cool your loop.
10. Using aseptic technique, inoculate your urea broth tube with your unknown. Flame and cool your loop.
11. Using aseptic technique, inoculate your phenol red mannitol tube with your unknown. Flame and cool your loop.
12. Use a sterile swab to inoculate your Mueller-Hinton agar plate as follows.
   a. Using aseptic technique, dip the sterile swab into the culture so that it is soaked.
   b. Swab the plate so that it is completely covered in bacteria. A good way to do this is to swab the plate as completely as possible in one direction. Then, using the same swab, streak the surface of the plate as completely as possible in a direction that is perpendicular to the first set of streaks. Finally, use the same swab to cover the plate at a 45° angle to the last set of streaks.
   c. Let the plate stand for 5 minutes.
13. Dispense 5 different antimicrobial disks onto your plate, being careful to avoid placing them too close together. Use a sterile loop to gently press on the disks so they
don’t move or come loose. Record the disk code and name of the antimicrobial in Table 11.1.

14. Give your inoculated tubes and plate to your T.A. for incubating at 37 °C.

15. Perform a gram stain of your unknown. Record both the Gram-stain result and the morphology of your unknown in Table 11.1.

**Period 2. Results of tests.**

**Materials (for all):**

- Kovac’s reagent
- Rulers

**Procedure:**

**MacConkey and EMB agar plates:**

1. **1 pt.** Examine your MacConkey agar plate from last week. Note the presence of colorless (non-lactose fermenting) or red (lactose fermenting) colonies. Describe the appearance of the colonies on your plate below.

2. **1 pt.** Examine your EMB agar plate from last week. Note the presence of metallic green, blue, black, or brown colonies (lactose fermenters) or colorless or light purple colonies (non-fermenters). Describe the appearance of the colonies on your plate below.

3. Record whether your isolate is a lactose fermenter or a non-fermenter in Table 11.1.

**TSI agar slants:**

1. Examine your TSI agar slant and record the results in Table 11.1. Be sure to record gas production, H₂S production, butt color, and slant color.
MIO agar tubes:
1. Examine your MIO tube for signs of motility. If growth is confined to the stab line, your bacterium is not motile. Record your result in Table 11.1.
2. Examine the color of your tube. If the tube is yellow, your organism is negative for ornithine decarboxylase. If the tube is purple, your organism is positive for the enzyme. Record this result in Table 11.1.
3. Wearing gloves, add 10 drops of Kovac’s reagent to your MIO tube. Shake the tube gently and look for a red color at the surface of the culture. A deep red color will develop if the test is positive. Record this result in Table 11.1.

Urease activity:
1. Examine your urea broth tube. A yellow color indicates a negative test. A magenta/dark pink color is a positive test. A tube with a thin layer of pink at the surface after more than 48 hours represents a delayed positive reaction (= weakly positive). Record your result in Table 11.1.

Mannitol fermentation:
1. Examine your phenol red mannitol tube. Remember, a red color means that mannitol was NOT fermented (no fermentation = no acid = no drop in pH = no color change). A yellow color indicates that fermentation has occurred. Check the Durham tube for gas production. Record your results in Table 11.1.

Antimicrobial sensitivity:
1. Observe your Mueller-Hinton agar plate.
2. Measure the zone of inhibition around each of your 5 disks. Record this information in Table 11.1, and note whether your isolate is resistant, intermediate, or susceptible, using the tables provided.
Table 11.1. Results of metabolic tests on enteric unknown (9 pts.).

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose fermentation</td>
<td></td>
</tr>
<tr>
<td>Gram-stain</td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial #1:</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial #2:</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial #3:</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial #4:</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial #5:</td>
<td></td>
</tr>
<tr>
<td>TSI agar: slant color</td>
<td></td>
</tr>
<tr>
<td>TSI agar: butt color</td>
<td></td>
</tr>
<tr>
<td>TSI agar: H₂S production</td>
<td></td>
</tr>
<tr>
<td>TSI agar: gas production</td>
<td></td>
</tr>
<tr>
<td>Motility (MIO tube)</td>
<td></td>
</tr>
<tr>
<td>Indole production (MIO tube)</td>
<td></td>
</tr>
<tr>
<td>Ornithine decarboxylase (MIO tube)</td>
<td></td>
</tr>
<tr>
<td>Urease activity</td>
<td></td>
</tr>
<tr>
<td>Mannitol fermentation, gas production</td>
<td></td>
</tr>
</tbody>
</table>

**Identification of your unknown.**

Use the results in Table 11.1 and the following key to identify your bacterium. Circle your identification (1 pt.). If you are unable to identify your bacterium from this key, talk to the T.A. or the instructor.
**Key to Gram-negative enterics.**

I. Lactose fermentation: positive
   A. Gas produced in TSI
      i. \( \text{H}_2\text{S} \) produced
         TSI: yellow butt, red or yellow slant
         Ornithine decarboxylase positive
         \( = \textit{Citrobacter} \)
      ii. \( \text{H}_2\text{S} \) not produced, TSI yellow butt and yellow slant
         a. Indole produced
            Urease negative
            \( = \textit{Escherichia} \)
         b. Indole not produced
            I. Motile (MIO)
               Ornithine decarboxylase positive
               \( = \textit{Enterobacter} \)
            II. Non-motile (MIO)
               Urease positive
               Ornithine decarboxylase negative
               \( = \textit{Klebsiella} \)
   B. Gas produced in TSI
      i. \( \text{H}_2\text{S} \) produced (TSI), motile (MOI)
         a. Urease negative; TSI: yellow butt, red slant
            I. Mannitol fermented
               Indole not produced
               \( = \textit{Salmonella} \)
            II. Mannitol not fermented
               Indole produced
               \( = \textit{Edwardsiella} \)
         b. Urease positive; TSI: yellow butt, red or yellow slant
            Ornithine decarboxylase negative
            Indole
               Produced: \( = \textit{Proteus vulgaris} \)
               Not produced: \( = \textit{Proteus mirabilis} \)
      ii. \( \text{H}_2\text{S} \) not produced (TSI)
         a. Pigmented at 25°C
            TSI: yellow butt, red or yellow slant
            Motile
            Ornithine decarboxylase positive
            \( = \textit{Serratia} \)
         b. Not pigmented at 25°C
            I. Ornithine decarboxylase positive
               TSI: yellow butt, red slant
               \( = \textit{Morganella} \)
            II. Ornithine decarboxylase negative
               Urease negative
               Indole produced
               Motile
               \( = \textit{Providencia} \)
II. Lactose fermentation: negative
   A. No gas or \( \text{H}_2\text{S} \) produced in TSI
      i. Pigmented at 25°C
         TSI: yellow butt, yellow or red slant
         Motile (MIO)
         Ornithine decarboxylase positive
         \( = \textit{Serratia} \)
      ii. Not pigmented at 25°C
         TSI: yellow butt, red slant
         Not motile (MIO)
         Urease negative
         Indole positive or negative
         \( = \textit{Shigella} \)