MODULE 7

Aseptic Transfer of Microbes

PREREQUISITE SKILL

None

MATERIALS

culture tube rack
empty culture tubes with closures (2)
sterile nutrient agar slants (3)*
sterile tube of nutrient broth*
slant culture of *Serratia marcescens*†
inoculating equipment: inoculating loop
Bunsen burner or Fisher burner
burner striker

*Prepared by the student if the instructor so indicates, otherwise, media from Module 2 "Preparing and Dispensing Media," can be used.
†*Serratia* has been associated with disease.

OVERALL OBJECTIVE

Master the aseptic transfer of bacteria from one tube to another.

Specific Objectives

1. Define the terms aseptic technique and pure cultures.
2. Explain why aseptic technique is important when transferring bacteria.
3. Practice the method used to transfer bacteria from one tube to another using empty culture tubes (dry runs).
4. Perform a dry run for your instructor.
5. Transfer living bacteria growing on a nutrient agar slant to three uninoculated slants using manual manipulations you learned in Specific Objective 3, and obtain pure cultures.
6. Identify any errors or omissions by another person whom you observe doing an aseptic transfer.
7. Demonstrate your ability to transfer bacteria aseptically from a slant culture to broth.

DISCUSSION

Microbes are present everywhere, so extreme care must be exercised not to introduce unwanted organisms into a pure culture and not to infect yourself. Unwanted bacteria are introduced by direct contact with contaminated surfaces or your hands, that is, by touching media or inner surfaces of the tube with any object that has not been sterilized. Bacteria are airborne, and can enter tubes via air currents. Using aseptic technique can keep outside microorganisms from contaminating the transfer culture. Using aseptic technique will protect you and those around you from contamination. Laboratory accidents do not happen frequently if you always practice good aseptic technique. If you handle bacteria correctly, you will have only the desired organisms growing in your transfer culture. If you transfer a pure, uncontaminated culture, only this particular bacterium will grow. A pure culture is one in which only a single species is found.

Module 7, "Aseptic Transfer of Microbes," shows step by step how to manipulate pure-culture transfers without contaminating the culture, yourself, or the lab. When transferring bacteria from one tube to another, you must be aware of airborne bacteria so they do not contaminate the material with which you are working.

ACTIVITIES

Activity 1: Aseptic Tube Transfer Dry Runs

The following manipulations are basic to most experiments performed in the laboratory portion of this course, and require observation and practice. Study the techniques, illustrated in Figures 7-1 through 7-15.

Imitate the actions taken in the figures in several dry runs with the two empty culture tubes in your tube rack. Do a step at a time, checking yourself.

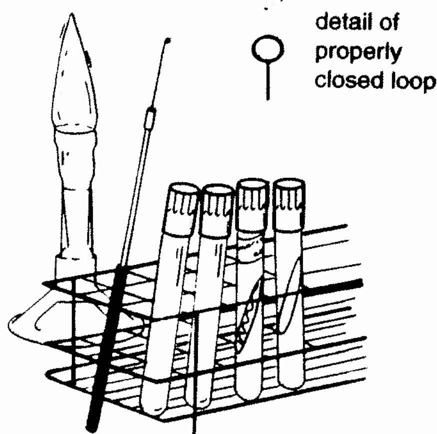


FIGURE 7-1
Equipment needed to transfer bacteria aseptically.

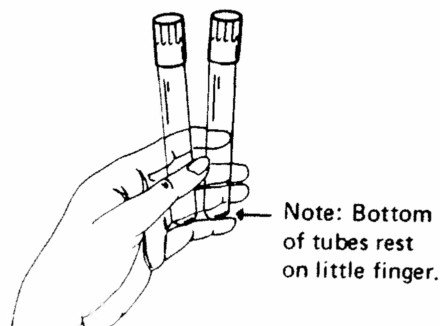


FIGURE 7-2
Hold both culture tubes in your left hand, or right hand if you are left-handed. Do *not* hold them vertically once you remove the closures.

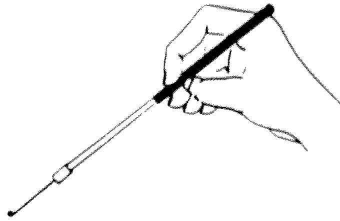


FIGURE 7-3
Hold the inoculating loop in your right hand, like a pencil, or left hand if you are left-handed.

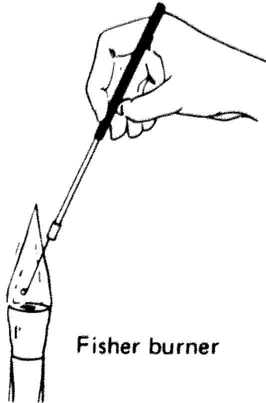


FIGURE 7-4
Flame the inoculating loop in the Bunsen burner, holding it upright so all the nichrome wire gets red-hot at once. Allow the loop to cool so you do not cremate the living bacterial cells you are about to transfer.

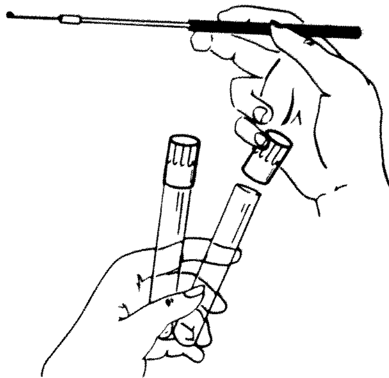


FIGURE 7-5
Remove the closures one at a time from both culture tubes by wrapping the little finger of your right hand around the closure of the tube nearest your right hand. Grasp only the uppermost portion of the closure so the open end does not touch the heel of your hand.

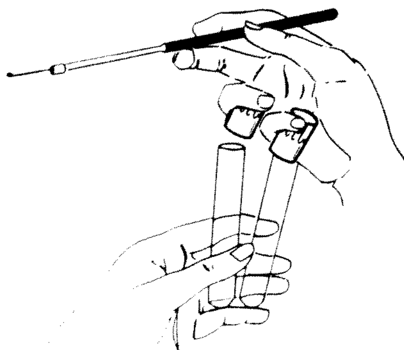


FIGURE 7-6
Remove the second closure with the finger next to your little finger in the same manner, that is, by wrapping the finger around the second closure. Approach this tube by reaching *between* the two tubes. *Do not* attempt to reach behind the tubes.

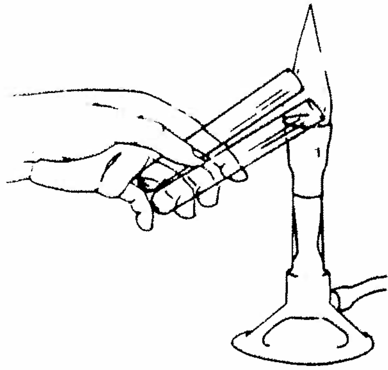


FIGURE 7-7
Flame the necks of the uncovered tubes by passing them back and forth through the flame twice. Hold the tubes in a nearly horizontal position.

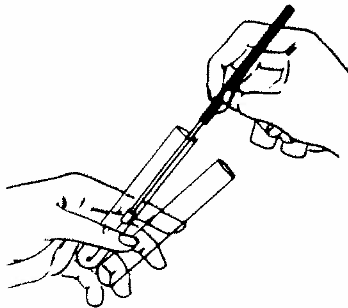


FIGURE 7-8
Insert the inoculating loop into the pure culture (or the substituting practice tube), and remove a small amount of bacteria. Note the position of the closures in the right hand.

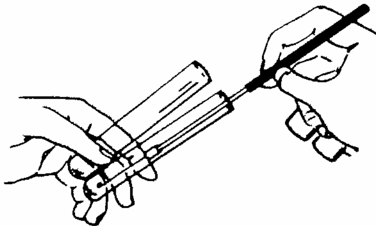


FIGURE 7-9
Transfer the inoculum (small amount of bacteria) to the surface of the uninoculated slant (or the substitute practice tube).

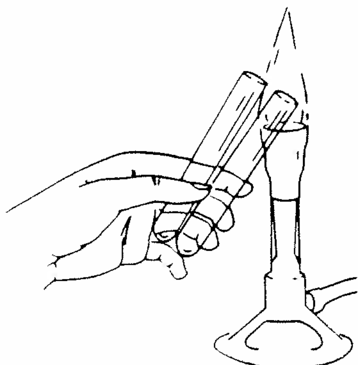


FIGURE 7-10
Reflame the necks of both tubes.

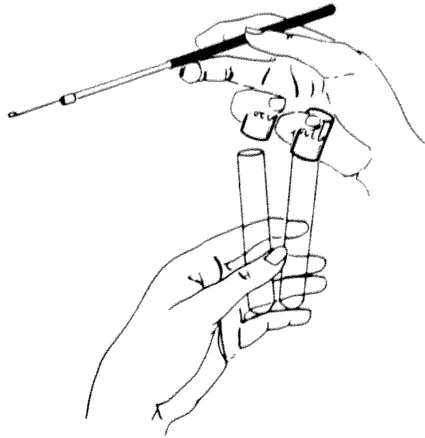


FIGURE 7-11
Recap the tubes, placing the closures on the same tubes from which they came. Return the first cap, that is, the closure held by your little finger to the tube you uncapped first.

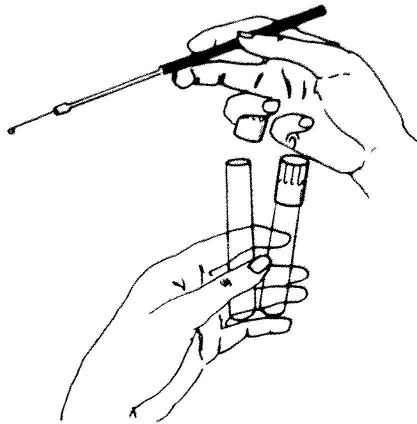


FIGURE 7-12
Recap the second tube.

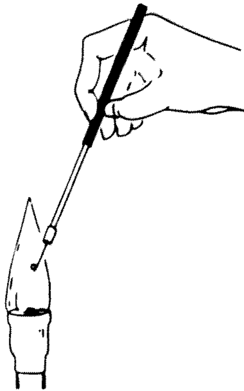


FIGURE 7-13
Reflame the loop, killing any remaining bacteria.

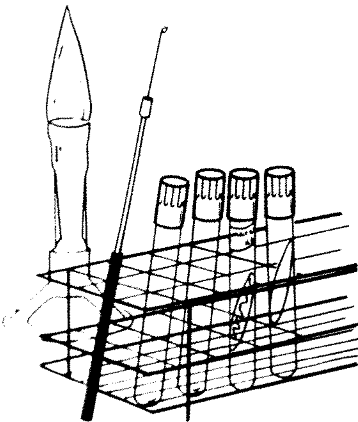


FIGURE 7-14
Return the tubes and the loop to the culture tube rack.

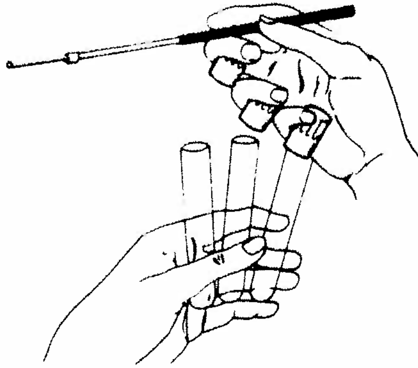


FIGURE 7-15
Once you've master this technique, you'll be able to manipulate three tubes and closures simultaneously. One inoculum can inoculate two or three tubes.

The following checklist summarizes the manipulations. Once you learn the correct handling of the equipment shown in the figures, you may need the checklist as a reminder only.

Checklist

1. Hold both tubes in your left hand and the inoculating loop in your right hand, as shown in Figures 7-2 and 7-3.*
2. Flame the inoculating loop until all the wire is hot. Allow the loop to cool for approximately 30 seconds to avoid cremating the bacteria you are about to transfer.
3. Remove the closures one at a time from both culture tubes. Remove the closure closest to your right hand first by wrapping the little finger of your right hand around it. Remove the second closure with the finger next to the little finger on your right hand with the same wrapping motion. Approach the second tube by reaching between the two tubes. Do not attempt to reach behind the tubes.
4. Flame the neck of the uncovered tubes by passing the tubes back and forth through the flame twice. Hold the tubes at less than a 45° angle when they are uncapped.
5. Insert the inoculating loop into the stock culture (or empty practice tube substituting for it) and remove a small amount of bacteria.
6. Transfer this inoculum, or small amount of bacteria, to the surface of the uninoculated slant (or the empty practice tube substituting for it).
7. Reflame the neck of both tubes by passing the tubes back and forth through the flame twice.
8. Recap the tubes, placing the closures on the same tubes from which they came. Recap the tube closest to your right hand first.
9. Flame the inoculating loop.
10. Return the tubes and loop to the culture tube rack.

Activity 2: Demonstration Given to a Classmate (optional)

Using the same empty culture tubes, ask another student to observe you as you perform aseptic technique. Use the step-by-step procedure, referring to the figures and instructions accompanying them. Stop after each step so your classmate can refer to the checklist to determine whether your technique is correct. Repeat the instructions in the checklist until you can follow the procedure in flowing succession.

*Reverse if you are left-handed.

Activity 3: Dry Run for Instructor

Practice enough dry runs so that the manipulations of aseptic tube transfer become natural. Demonstrate the technique to your laboratory instructor and don't proceed to Activity 4 until he or she approves it.

Activity 4: Aseptic Transfer of Living Bacteria

You are now ready to work with living microbes. Most bacteria are not pathogenic, but those that do cause disease can be terribly harmful. Protect yourself and your classmates by handling living bacteria correctly.

Every time you prepare an inoculation, label it with waterproof ink. Include your name, date of inoculation, organism, source if appropriate (such as a throat swab), type of medium, temperature of incubation, and module and/or activity number. See Figure 7-16 for an example.

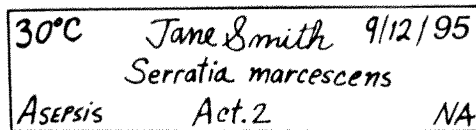


FIGURE 7-16
Proper label.

1. In your culture tube rack, place a slant of *Serratia marcescens*, a red pigment-producing bacterium, and three uninoculated slants of nutrient agar.
2. Label the nutrient agar slants.
3. Using the technique you used in your dry runs, transfer a small amount of living bacteria (inoculum) to the nutrient agar slants.
   - Do not cut or gouge the agar surface. Allow the loop to glide over the agar surface from the bottom of the tube to the top of the slanted surface with a slight side-to-side motion.
4. Incubate the slants at 30°C for 48 hours.

Next lab period, examine the transfer slants for colony morphology and pigment production. If your aseptic technique was performed correctly, the growth will appear smooth and confluent and only red-pigmented bacteria will be present. If one bacterial species only is growing on the transfer slants, you practiced aseptic technique successfully. Sketch the growth on the NA slants on the worksheet.

Activity 5: Aseptic Transfer from a Slant Culture to Broth

1. Place a tube of sterile nutrient broth in the culture tube rack alongside the slant of red pigment-producing bacteria.
2. Label the tube as detailed in Activity 4.
3. Transfer a small amount of living bacteria to the tube of sterile broth.
   - Immerse the loop, with the inoculum, in the broth and shake it vigorously two or three times to deposit a few cells in the sterile broth.
4. Place the newly inoculated broth tube in a coffee can or basket in the 30°C incubator for 48 hours.

During your next lab period examine the broth tube for turbidity (cloudiness) and red pigment. Write your conclusions on aseptic transfer on the worksheet.

Phonetic Pronunciation

Serratia marcescens = sir-race´-ya mar-cess´-unz

Take the post test. If you do not answer all the questions correctly, review the module.

MODULE 22

Simple Stain

PREREQUISITE SKILL

Completion and mastery of Module 21, "Preparing a Bacterial Smear;" and Module 4, "Compound Microscope for the Study of Microbes."

MATERIALS

staining equipment:
- stainless steel pan or other receptacle
- staining rack
- wash bottle
- bibulous paper pad

6 smears from Module 21

methylene blue stain*

* Prepared by the student if the instructor so indicates. Formula at the end of the module.

OVERALL OBJECTIVE

Perform a simple bacterial stain and describe the chemical reaction involved.

Specific Objectives

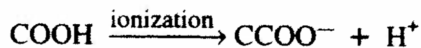
1. Explain how a basic stain, such as methylene blue, colors the surface of a bacterium.
2. Describe the steps involved in performing a simple stain.
3. Name an acid dye and a basic dye for staining bacteria.
4. Name the part of a cell stained by an acidic dye and by a basic dye.
5. Determine the correct amount of cells needed to make a good smear for microscopic observation.

DISCUSSION

The chemical compounds used to stain bacteria are called dyes. Unstained cells are practically transparent, so we stain bacteria to make them more visible. Stained bacteria are almost always used for microscopic observations.

Dyes can be acidic or basic. Acidic dyes such as acid fuchsin and eosin have a

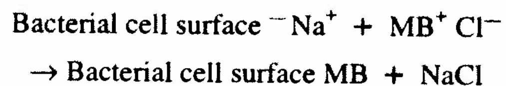
strong affinity for basic portions of the cell, that is, for the cytoplasmic components that are alkaline. Basic dyes such as crystal violet, methylene blue, and safranin have a strong affinity for acid portions of the cell. The surface of a bacterial cell is acidic because of a large number of carboxyl groups located on the surface. The carboxyl groups (COOH) are the acid portions of amino acid molecules, and many amino acids are combined in the cell wall. Therefore, when ionization of the carboxyl groups takes place, the surface of the cell has negative charges. For example,



$$COOH \xrightarrow{\text{ionization}} CCOO^- + H^+$$

In nature, however, the hydrogen ion is replaced by another positive ion such as Na^+ or K^+ and the H^+ bonds with oxygen to form water. The surface of a bacterial cell could be represented as in Figure 22-1.

Basic dyes are commercially prepared as salts. For example, when we purchase methylene blue, it is really methylene blue chloride. When rehydrated, methylene blue chloride ionizes to have a positive charge on the colored part of the molecule, that is, MB^+. It is this cation that allows us to say that methylene blue is basic, because in electrolysis, MB^+ will move to the negative electrode. A law of chemistry is that unlike charges attract, so the MB^+ molecules ionically bond to negative charges on the surface of the bacterium. When the positive and negative charges bond, the cell is stained. Following is an equation that demonstrates staining.



$$\text{Bacterial cell surface } {}^-Na^+ + MB^+ Cl^-$$
$$\rightarrow \text{Bacterial cell surface MB} + NaCl$$

Staining bacteria is, therefore, an exchange of positive and negative charges between molecules to form an ionic bond. If a single dye is used, the stain is called a simple stain. In Module 23, "Gram Stain," you will be doing a differential stain that employs two dyes of different colors.

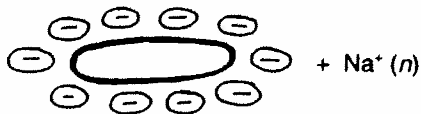


FIGURE 22-1
Negative charges predominate on a bacterial cell surface.

ACTIVITY

Caution: Many dyes are potential carcinogens. Handle all stains carefully to avoid spilling them on your skin. Use slide forceps and/or surgical gloves when working with stain-covered slides.

Staining Bacterial Smears with a Simple Stain

1. Place Slide 1, saved from Module 21, on a staining rack.
2. Flood the slide with methylene blue. Allow the methylene blue to react with the smear for 1 minute.
3. Using a slide forceps, tilt the slide so the stain runs off into the staining pan or staining sink as shown in Figure 22-2.
4. Using water in a squeeze bottle, wash off the excess stain while the slide is tilted over the staining receptacle.
5. Blot the smear gently with bibulous paper to remove the water.
   - Do not rub because it will remove the stain.

Repeat this staining procedure with the remaining five smears. If you stain more than one smear at a time, do not allow the stain to dry on the smear because the drying stain will crystallize and obscure the bacteria. Prevent crystallizing of the stain by using an excess of stain to flood the slide and by rinsing the stain immediately with water, that is, while the stain drains off the smear.

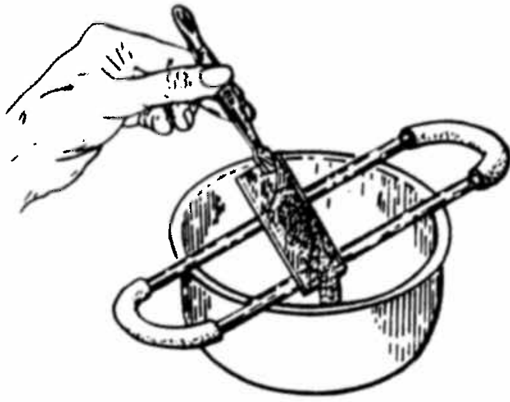


FIGURE 22-2
Draining a bacterial smear before rinsing.

After you stain all six smears and allow them to dry, examine them under an oil-immersion objective. Using the table on the worksheet, determine the quantity of cells you need to make a good smear.

Remember the number of loops from broth cultures and the amount of growth from a slant culture that yielded a good smear. You will be making smears throughout the course.

After you have completed the table on the worksheet, take the post test.

FORMULA FOR REAGENT

0.5% METHYLENE BLUE

methylene blue chloride	0.5 g
distilled water	100.0 ml

Dissolve the methylene blue in the distilled water.

MODULE 23

Gram Stain

PREREQUISITE SKILL

Mastery of Module 4, "Compound Microscope for the Study of Microbes;" Module 7, "Aseptic Transfer of Microbes;" Module 21, "Preparing a Bacterial Smear;" and Module 22, "Simple Stain."

MATERIALS

clean microscope slides (5 to 15)
inoculating equipment:
- Bunsen burner
- burner striker
- inoculating loop

staining equipment:
- stainless steel pan or other receptacle for staining process
- staining rack
- slide forceps
- wash bottle
- bibulous paper pad

Gram stain reagent set consisting of:*
- crystal violet
- Gram's iodine
- Acetone alcohol
- Gram's safranin

Slant cultures of:
- *Bacillus subtilis*
- *Escherichia coli*
- *Staphylococcus aureus*
- Candida albicans)
- unknown organisms – premade slides)

* Prepared in advance by the student if the instructor so indicates. Formulas for Gram reagents at the end of the module.

OVERALL OBJECTIVE

Become adept at Gram staining bacterial smears, and understand theoretical explanations for differing Gram reactions.

Specific Objectives

1. Discuss probable reasons for Gram reactions.
2. List five factors that can cause the Gram reaction of an organism to vary.
3. Gram-stain positive and negative organisms and obtain their characteristic, classic reactions.
4. Gram-stain unknown organisms and obtain the accepted Gram reaction for each.
5. Define the terms Gram reaction, mordant, Gram-positive, and Gram-negative.
6. State three physiological or cytologic characteristics that correlate with the Gram reaction of a bacterium.
7. Describe the Gram reaction, shape, and arrangement of five or six different organisms.

DISCUSSION

Microbiologists find the Gram stain a most useful aid in identifying bacteria. The Gram stain is a differential stain that requires both a primary stain and a counterstain. The primary stain is crystal violet, which is followed by an iodine solution. The iodine is called the mordant (a specialized term used in dyeing), which is a substance, often a metallic compound, that combines with a dye to form an insoluble colored compound. The insoluble precipitate is called the crystal violet–iodine complex. Gram-positive organisms do not retain the primary dye (after decolorization) if the iodine mordant is omitted. After decolorizing, usually with 95% ethanol or acetone alcohol, a safranin counterstain is applied to the smear. If the acetone alcohol decolorizer step is omitted from the procedure, all bacteria will appear Gram-positive.

Organisms that resist decolorizing and retain the crystal violet–iodine complex appear purple or deep blue in the microscope and are called Gram-positive. Conversely, cells that decolorize, or give up the crystal violet-iodine complex more rapidly will accept the safranin counterstain and appear red. These are Gram-negative organisms.

In 1884, Christian Gram accidentally discovered what eventually was called the Gram-staining reaction. He was studying the etiology of respiratory diseases at the Municipal Hospital in Berlin. As he attempted to stain biopsy specimens to differentiate microorganisms from the surrounding tissues, he applied crystal violet and then Lugol's iodine as a mordant. Both solutions were standard reagents at that time. The precipitate formed was so thick that Gram had to use 95% ethanol as a clearing agent. He found the tissue cells decolorized much more rapidly than the bacteria in them. Gram originally thought he had developed a differential stain for all bacteria in tissue, but he soon observed that some bacteria did not retain the primary stain but were decolorized with the tissue cells and accepted the counterstain. Hence there was no differentiation between these bacterial cells and tissue cells. This presents no problem, however, when bacterial cells are stained alone, that is, not in tissue cells.

Most living cells, including animal tissues, are Gram-negative. It is the Gram-positive characteristic that is distinctive. Some bacteria, yeasts, and a few molds are Gram-positive.

There is no universally accepted explanation for the differences in Gram reactions of certain cells. There are many theories to explain the differences, but none is completely satisfactory. The most widely accepted theory relates the Gram reaction to a difference in permeability of the cell wall based on structural differences. Gram-negative cells have a greater lipid content in their cell walls than Gram-positive cells. Lipids are soluble in alcohol and acetone, which are used as decolorizers in Gram staining. Removal of the lipid by the decolorizer is thought to increase the pore size of the cell wall, which accounts for the more rapid decolorization of Gram-negative cells.

Another theory suggests that a crystal violet-iodine-ribonucleate complex forms in Gram-positive cells but not in Gram-negative cells. This theory implies that the ribonucleic acids of the Gram-

positive cell cytoplasm must be different and bind more firmly with the crystal violet–iodine complex. The chemical bond formed with the Gram-positive ribonucleate is not readily broken by the decolorizer.

Remember, the differentiation of the Gram reaction is not an absolute, all-or-none phenomenon. It is based on the rate at which cells release the crystal violet–iodine complex to the decolorizer. Even Gram-positive organisms can show a Gram-negative reaction if decolorized too much. A number of other factors can result in variable Gram reactions, such as the following:

1. Improper heat fixing of the smear. If a smear is heated too much, the cell walls can rupture, causing Gram-positive cells to release the primary stain and accept the counterstain. This supports the theory that the Gram reaction depends on cell wall structure.
2. Cell density of the smear. An extremely thick smear may not decolorize as rapidly as one of ordinary density.
3. Concentration and freshness of the Gram-staining reagents.
4. Length and thoroughness of washing after crystal violet, and the amount of water remaining on the smear when iodine is added.
5. Nature, concentration, and amount of decolorizer applied.
6. Age of bacterial culture. Gram reactions are reliable only for cultures up to 24 hours old. Variability of Gram reaction in old cultures is also related to cell wall integrity and permeability.

Keep these variables constant to ensure reliable and consistent Gram differentiations. Practice the Gram-staining procedure repeatedly until you obtain consistent reactions. Taking time to practice will be time well spent because you will be using this differential stain constantly when studying microbes.

The Gram stain is indispensable in identifying unknown bacteria. This simple procedure will allow you to place any bacterium into one of five broad areas and, at the same time, eliminate the remaining four areas. That is, the organism will be either a Gram-positive rod, a Gram-negative rod, a Gram-positive coccus, a Gram-negative coccus, or Gram-nonreactive (including some Gram-negative spirilla). Gram-nonreactives are microorganisms that do not stain or stain poorly. The genus *Mycobacterium* and various spirochetes fall into this group. Acid-fast organisms are only weakly Gram-positive and are better studied with other staining procedures.

Gram-variable bacteria can appear both Gram-positive and Gram-negative on the same smear. Some organisms lose their Gram positivity in older cultures, possibly causing this Gram variability. Gram reactions stated in the course and in most reference works are for 24 hour cultures.

Determining the Gram reaction of the causative organism facilitates the diagnosis and treatment of bacterial disease. Gram-positive bacteria include the causative organisms of anthrax, rheumatic fever, diphtheria, botulism, septic sore throat, and boils. Representatives of the Gram-negative group include the organisms causing cholera, typhoid, dysentery, whooping cough, some food poisoning, bubonic plague, and numerous other diseases.

In later modules (Parts 5 and 6), you will demonstrate the correlation of the Gram reaction with the following physiological and cytologic traits:

1. Gram-positive organisms are more susceptible to penicillin, disinfectants, and dye bacteriostasis (inhibition of bacterial growth) than Gram-negative ones.
2. Gram-negative organisms are more sensitive to lysis and digestion by strong alkali, acids, and lysozyme (a cell-lysing enzyme).
3. Gram-positive organisms are more fastidious; that is, they have complex nutritional requirements for growth.
4. Gram-positive organisms produce exotoxins, whereas Gram-negative bacteria form endotoxins.
5. A bacterium can lose its Gram positivity but not its Gram negativity. Gram-negative cells are found in Gram-positive slides, but not Gram-positive cells on a Gram-negative slide.

The first or second time you perform a Gram stain, you will find it easier to stain one slide at a time. After you are more familiar with the procedure, you will be able to stain a series of slides together on your staining rack.

Refer to Module 21, "Preparing a Bacterial Smear," if you wish to refresh your memory before you prepare your smears.

ACTIVITIES

Activity 1: Performing the Gram Stain

A. Prepare separate smears of *Bacillus subtilis, Escherichia coli, Staphylococcus aureus*, and *Neisseria sicca* using the respective stock cultures.
  - You can place two smears on a single slide if you wish.
  - Air-dry and heat-fix the smears and label the slides.

B. Place labeled slides with heat-fixed smears on a staining rack over a stainless steel pan or other receptacle.
  - *Reminder:* Once you begin staining, never let a smear dry before you have completed the procedure. By flooding the slide with excess stain, you will prevent drying and consequent precipitation of crystallized dye, which can obscure the bacterial cells.
  - *Caution:* Remember, stains are potentially hazardous. Handle slides with forceps and/or wear surgical gloves while staining.

C. Perform the Gram stain as follows, referring to Figure 23-1. Though you may be using different staining bottles, the procedure is the same.
  1. Flood the slide with crystal violet, and allow it to react for 1 minute.
  2. Handling the slide with a slide forceps, tilt it about 45° to drain the dye off into the pan or staining sink as shown in Figure 23-1.
  3. Continue to hold the slide at a 45° angle, and rinse it immediately and thoroughly with a gentle stream of water from a wash bottle.
  4. Replace the slide on the staining rack and flood it with iodine. Allow the iodine to react for 1 minute.
  5. With a slide forceps, tilt the slide and allow it to drain.
  6. Immediately *rinse* the slide thoroughly with water from your wash bottle.
  7. With the slide still held at 45°, decolorize it quickly by allowing the acetone alcohol to run over and off the smear. Do not decolorize it too much.
  8. Rinse immediately with water from the wash bottle to stop the decolorizing process.
  9. Replace the slide on the staining rack, and flood it with safranin counterstain. Allow the counterstain to react for 30 to 60 seconds.
  10. Drain the slide.
  11. Rinse the slide thoroughly with water from the wash bottle.
  12. Blot the stained slide in a booklet of bibulous paper. Do not rub, because you could rub off a thin smear.

D. Repeat the procedure until all four smears are stained.

E. Examine each smear microscopically. On the worksheet, draw several representative cells from each smear as they appear under oil immersion. Label the drawings with the Gram reaction, cell shape, and arrangement.

Repeat the activity if you need more practice to perform Gram stains consistently and with ease.

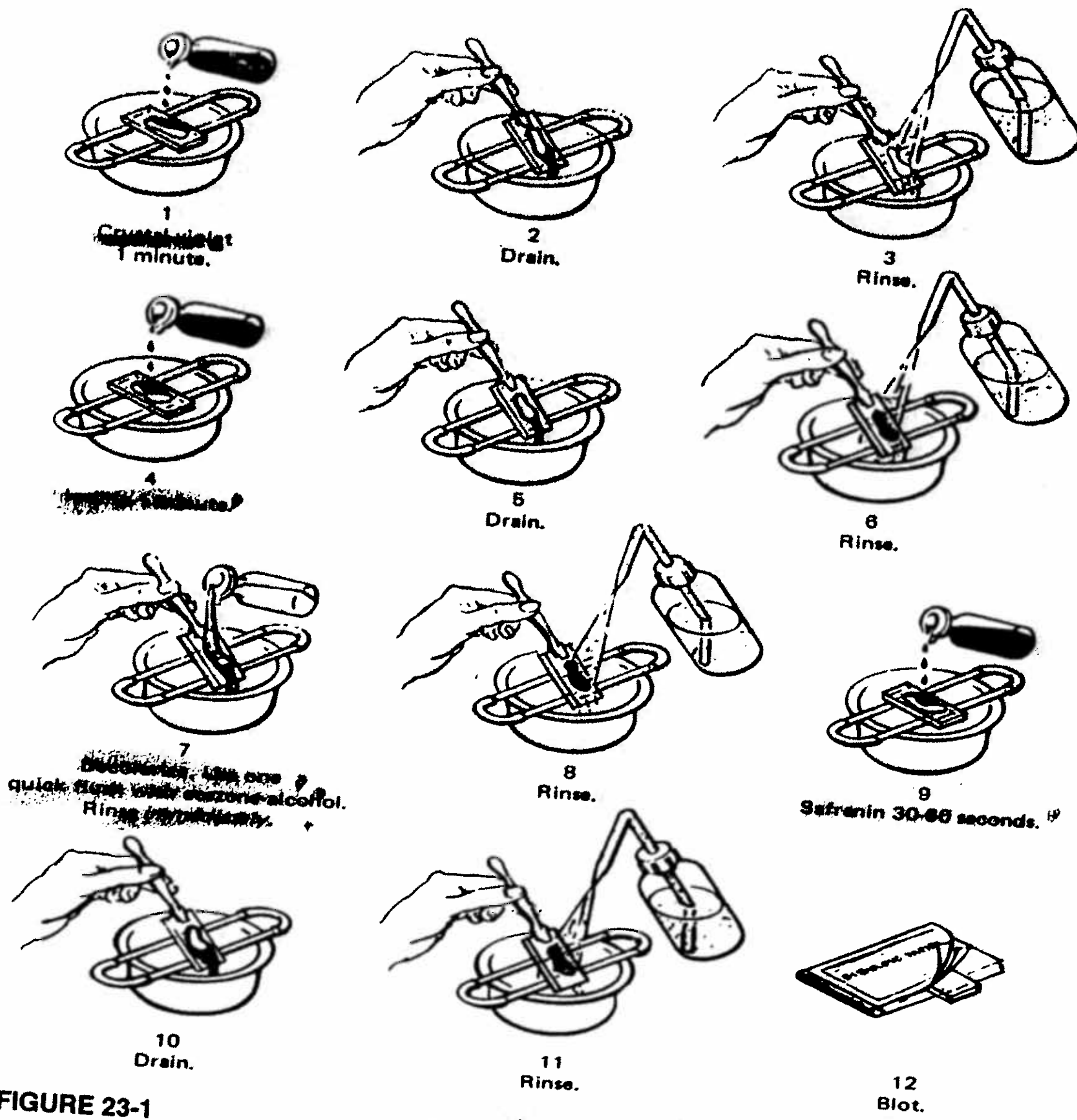


FIGURE 23-1
Procedure for performing a Gram stain.

Activity 2: Gram Staining Unknown Organisms

1. Prepare a smear of an unknown bacterium; air-dry and heat-fix it. Label the slide with the number of the unknown organism.
2. Prepare smears of the other unknown organisms.
3. Gram-stain the unknown smears, following the steps in Activity 1 (Figure 23-1).
4. Examine one of the smears microscopically, and sketch several representative cells as seen under oil immersion. List the Gram reaction, cell shape, and arrangement, if any.
5. Repeat step 4 with all unknown smears.
   - Many genera and species have the same Gram reaction and cell shape, so you cannot name unknown organisms from the Gram reaction only.

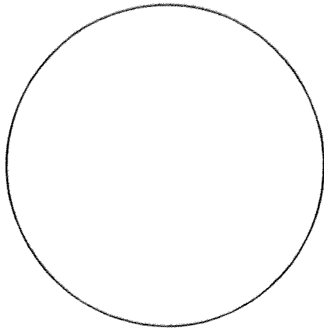
Check the numbers of unknowns against your lab instructor's master list of unknowns to determine the accuracy of your Gram reaction and microscopic observations. If the Gram stain reactions and descriptions you arrived at were not accurate, repeat the activity.

Name_______________________________

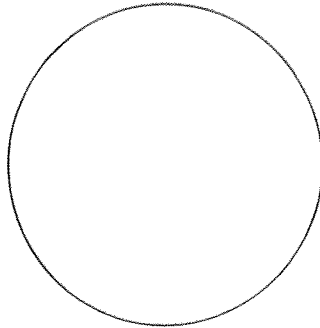
Lab Section_________________________

MODULE 23: GRAM STAIN

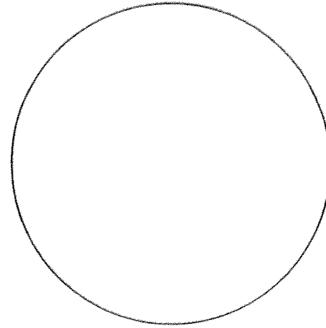
Activity 1: Performing the Gram Stain



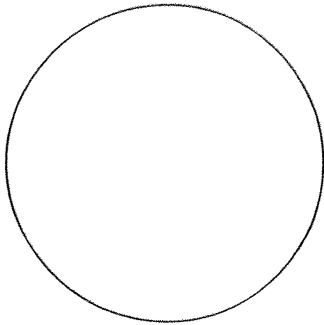
Bacillus subtilis



candida albicans

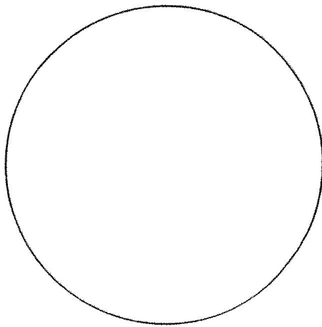


Staphylococcus aureus



Escherichia coli

Activity 2: Unknown Organisms



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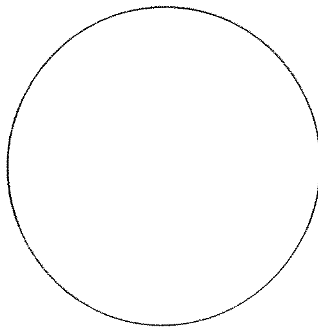

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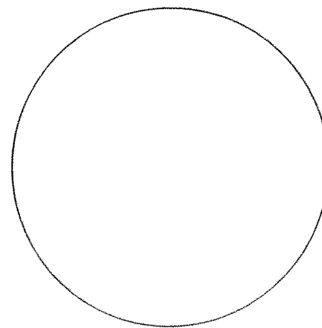

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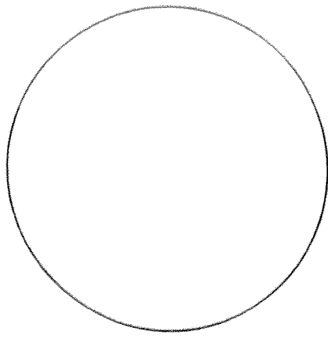

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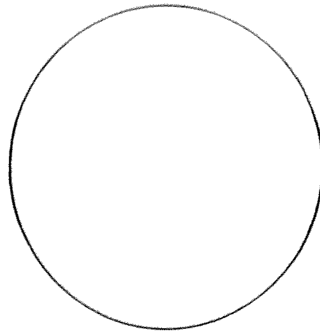

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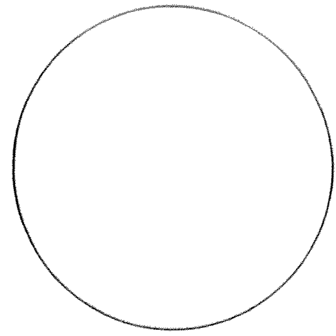

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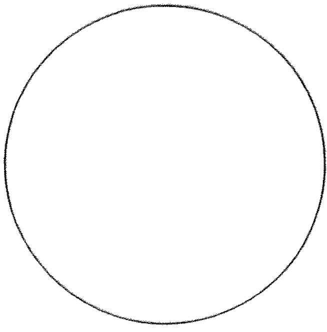

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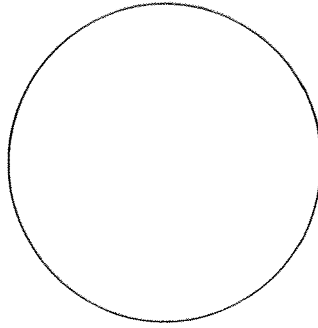


8

MODULE 24

Capsule Stain

PREREQUISITE SKILL

Mastery of Module 4, "Compound Microscope for the Study of Microbes;" Module 5, "Cleaning Microscope Slides and Preparing a Wet Mount;" Module 7, "Aseptic Transfer of Microbes;" Module 21, "Preparing a Bacterial Smear;" and Module 22, "Simple Stain."

MATERIALS

- clean microscope slides (2)
- inoculating equipment
- staining equipment
- fresh India ink
- Gram's crystal violet*
- slant culture of *Klebsiella pneumoniae* on tryptose phosphate agar†

* Prepared in advance by the student if the instructor so indicates. Formulas at the end of Module 23.
† Tryptose phosphate agar enhances capsule formation.

OVERALL OBJECTIVE

Perform Gin's method of capsule staining and understand the structure and functions of the capsule.

Specific Objectives

1. Discuss the bacterial capsule and why ordinary staining cannot be used to visualize it.
2. Stain *Klebsiella pneumoniae*, demonstrate bacterial capsules, and draw the capsules.
3. Define the terms negative stain, slime layer, phagocytosis, and ionic bond.
4. Cite the composition of most bacterial capsules.
5. Explain why the modified Gin's method is considered a differential stain.
6. Describe the role of the capsule in disease.
7. Explain why the modified Gin's method employs negative and positive staining.
8. Name the genus and species of an organism whose virulence depends on its capsule being present.

DISCUSSION

Most bacterial cells secrete a viscous substance that accumulates around the outside of the cell and coats the cell wall. This structure, depending on the thickness of the layer, its viscosity, and its demonstrability, is called either the capsule or slime layer. Most bacteria secrete at least some slime that is more soluble and less viscous than a capsule. The capsule appears as a larger structure and can be demonstrated more easily.

The environment in which the organism is cultured influences the size of the capsule. For example, tryptose phosphate agar induces the production of larger capsules than does nutrient agar. Disease-causing bacteria tend to produce large capsules.

The bacterial capsule is significant to both the bacteria and humans. The capsule protects the bacterium by acting as an osmotic barrier between the cell body and the environment. When encapsulated bacteria invade, the capsule appears to interfere with the phagocytic action of leukocytes. The bacterial capsule may be a reservoir of stored food or be involved in disposal of waste products.

For some disease-producing organisms, virulence and infectivity are increased by or depend on the presence of the capsule. For example, *Streptococcus pneumoniae* becomes avirulent when it loses the ability to produce capsules.

The bacterial capsule is composed of polysaccharides that are water-soluble and nonionic. As you learned in Module 22, "Simple Stain," most staining techniques are based on chemical bonding between ionized particles of the dye molecules and ionized areas on the surface of the cell. An ionic bond is formed by the attraction of unlike charges on the dye molecules and the cell surface, and the bacterium is stained. The bacterial capsule is nonionic, so it cannot be stained in the usual manner.

Since we cannot stain the capsules, techniques have been developed that allow us to stain the background and leave the capsule clear. This is called negative staining. A negative stain dyes everything except the structure you wish to visualize.

The phase microscope provides the best method of visualizing bacterial capsules. Phase equipment, however, is expensive and not always available. A similar effect can be achieved by preparing a wet mount of the bacterium and adding carbon particles (India ink) to the suspension. When this preparation is examined microscopically with reduced light intensity, a phase-like effect is achieved. The capsules appear to be halos or clear rings around the bacterial cell, and the background looks dark, as shown in Figure 24-1. The method can be completed quickly but is temporary. The difficulty of using a wet mount is that you must observe it with your high dry objective, which magnifies only 400 times.

A variation of this technique is the modified Gin's stain for capsules, which allows you to use an oil-immersion objective and magnify 1000 times. It differs from the wet mount demonstration of capsules in that it employs both a negative and positive stain. When examined microscopically, the background is dark, and the capsules appear as clear, unstained rings (negative stain), with the small purple cell body in the center of the rings (positive stain), as shown in Figure 24-2. This variation is not restricted by the shortcomings of the wet mount preparation. The counterstain makes the cell body more clearly visible and allows you to compare the size of the cell to that of the capsule.

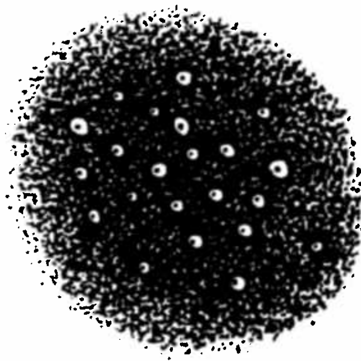


FIGURE 24-1
Phase contrast preparation of *Klebsiella pneumoniae.*

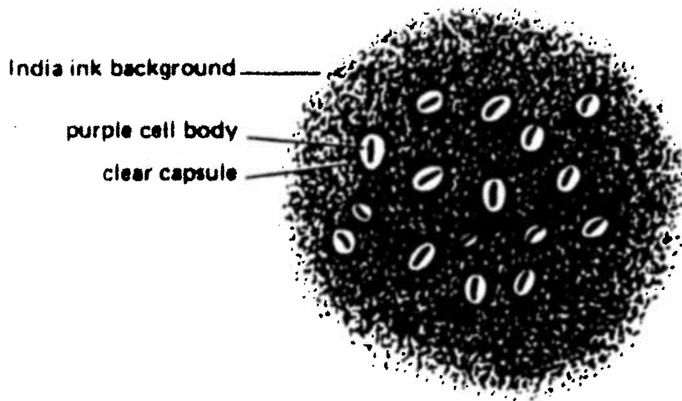


FIGURE 24-2
Gin's method capsule stain of *Klebsiella pneumoniae*. (See color plate 3.)

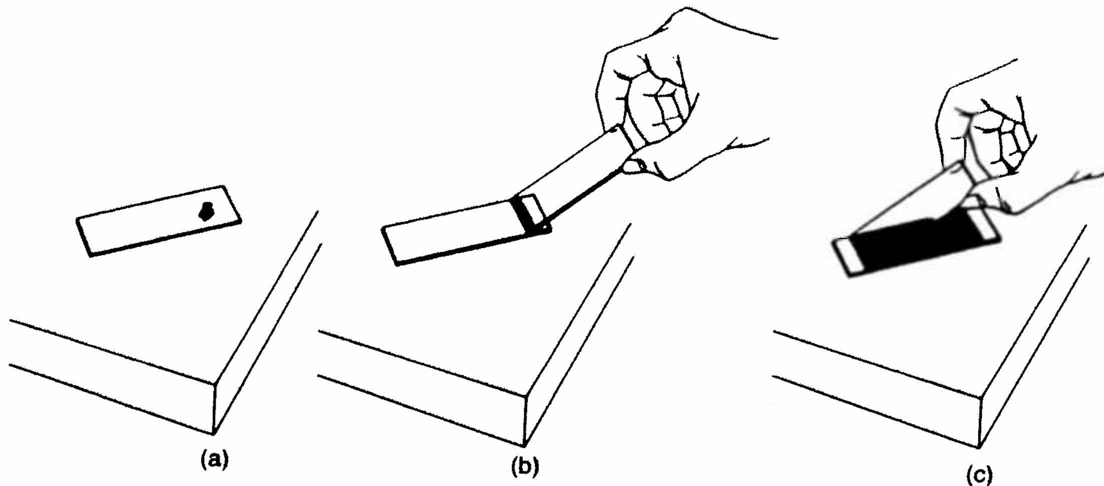


FIGURE 24-3
The three steps taken to prepare an India ink-bacteria film for the modified Gin's capsule stain.

Either capsule stain technique can be readily adapted for use with a broth culture by simply omitting water when you prepare the suspension of organisms and carbon particles. Be sure the organisms are well dispersed in the broth.

ACTIVITY

Modified Gin's Capsule Stain Using the Blood Smear Method

1. Mix India ink, *Klebsiella pneumoniae*, and water on the end of a clean slide, as shown in Figure 24-3a.
    - Use your inoculating loop to add the bacteria to equal amounts of India ink and water.
2. Place the edge of a second slide in the mixture. Allow the mixture to run across the base of the second slide, as in Figure 24-3b.
3. While holding the second slide at an acute angle, push the mixture toward the opposite end of slide 1, as in Figure 24-3c.
4. Allow to air-dry (do not heat-fix).
5. Using the staining equipment, flood the smear with crystal violet and let it react for 1 minute.
6. Drain and rinse the smear.

- Some of the smear may wash off as you rinse it. Do not be dismayed. Remember, this smear is not heat-fixed.

7. Place one end of the slide on a paper towel, and prop the slide up at a 45° angle.
   - Leave it to drain and air-dry. Do not blot or rub it.

When you make a capsule stain from a broth culture, you don't need to use a drop of water. Simply mix two parts of broth culture to one part of India ink and proceed as usual. Mix the broth gently to avoid disrupting any characteristic cell grouping, such as chains.

Examine the slide under oil immersion, and draw representative cells on the worksheet. Label the organism, cell body, and the capsule. Then take the post test.

Related Experience

Observe encapsulated and nonencapsulated bacteria using a phase-contrast microscope. Your lab instructor can set up a demonstration if a phase microscope is available. Draw the bacteria on the worksheet.

Phonetic Pronunciation

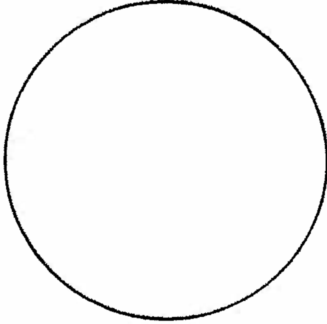
Klebsiella pneumoniae = Kleb-see-ell′-uh nyoo-moe′-nee-ee

Name___________________________

Lab Section_______________________

MODULE 24: CAPSULE STAIN

Gin's Stain



Klebsiella pneumoniae

1. Which component of Gin's method is the negative stain? ____________________
2. Why?____
3. Which is the positive stain?____
4. Why?____

MODULE 25

Bacterial Endospores

PREREQUISITE SKILLS

Mastery of Module 4, "Compound Microscope for the Study of Microbes," Module 7, "Aseptic Transfer of Microbes," Module 21, "Preparing a Bacterial Smear," and Module 23, "Gram Stain."

MATERIALS

clean microscope slides (2)
inoculating equipment
staining equipment*
Gram stain reagent set*

Schaeffer-Fulton stains:
- malachite green* †
- safranin (*not* Gram's safranin)*

28 hour slant culture of *Bacillus subtilis*

* Prepared in advance by the student if the instructor so indicates. Formulas at the end of Module 23.
† Formulas at the end of the module.

OVERALL OBJECTIVE

Recognize bacterial endospores, understand their functions, and perform the Schaeffer-Fulton staining method to visualize endospores.

Specific Objectives

1. Discuss sporulation and why it is thought to take place in some bacteria.
2. Explain the functions of sporulation in bacteria.
3. Name four pathogenic, sporulating bacilli and the diseases they cause.
4. List the two genera of most spore-forming bacteria.
5. Describe how spores appear in a Gram stain.
6. Describe the appearance of endospores in a Schaeffer-Fulton spore stain.
7. Define the terms vegetative cell, endospore, and germination.
8. Name the reagents and sequence in which they are used in Schaeffer-Fulton staining.

DISCUSSION

Bacterial endospores are small oval or spherical structures that are resistant to high temperatures, radiation, desiccation, and chemical agents such as disinfectants. Spores are produced intracellularly by some bacilli, the reason they are called endospores. The ordinary bacterial cell that gives rise to the spore is called the vegetative cell. Endospores are smaller than the parent cells and display different qualities, notably their resistance to hostile conditions which helps ensure survival.

Bacteria form spores as a survival mechanism in response to adverse conditions. The vegetative cell is stimulated by depletion of nutrients in the environment to induce sporulation through the expression of alternative genes. Inadequate carbon or nitrogen sources are especially effective triggers of bacterial sporulation. Complete transformation from a vegetative cell into a spore requires from 6 to 8 hours in most spore-forming bacilli.

Sporulation in bacteria is not a form of reproductive multiplication, as in some plants and fungi, because each bacterial cell produces only one spore which germinates into one vegetative cell, as shown in Figure 25-1. Reproduction, then, is by binary fission of the vegetative cell in spore-forming species the same as in other species of bacteria.

The vegetative cell is metabolically active and growing, whereas the spore is metabolically inactive, dehydrated and highly resistant to damage because it is encased in three heavy and impervious protein spore coats. Bacterial spores can remain dormant yet viable for thousands of years. Viable spores have been isolated from a 3000-year-old archaeological specimen. Spores break dormancy and germinate in the presence of water and a specific environmental chemical stimulus known as a germination agent. The germination agent varies among bacterial species, but is usually an amino acid or inorganic salt that stimulates production of hydrolytic enzymes by the spore membranes. The enzymes digest the cortex and expose the dehydrated core to water, which is quickly taken up. As the protoplast rehydrates and takes in nutrients it begins to grow out of the spore coats, eventually reverting to a fully active vegetative cell. Germination proceeds rapidly, in about 90 minutes.

The spore is the dormant, or resting, phase of the bacterial cell and in this respect is analogous to the seeds of higher plants or the cysts of protozoans. The bacterial spore is not an agent of sexual reproduction, whereas the seed is. The presence of spores in a culture is significant in identifying and differentiating bacteria because spore formation is primarily confined to the Gram-positive rod-shaped organisms in two genera, *Bacillus* and *Clostridium*.

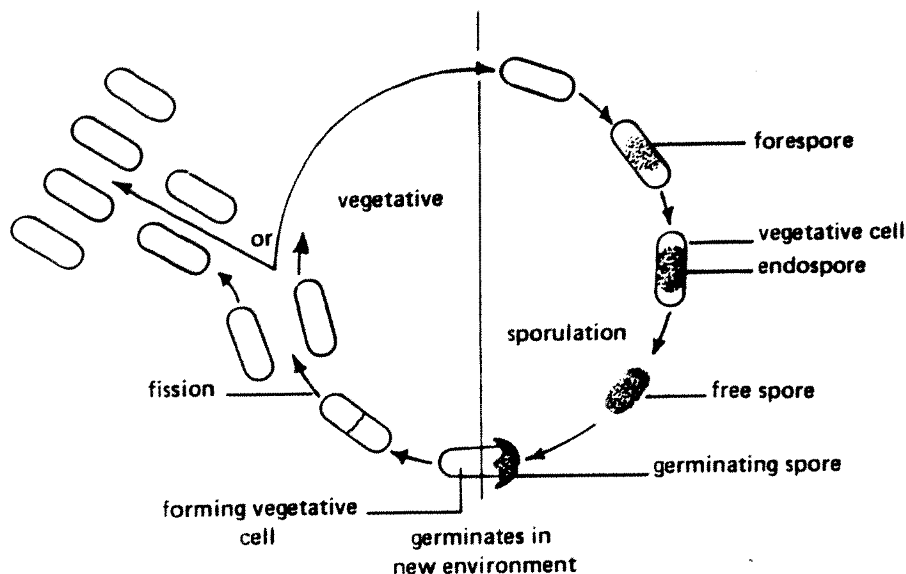


FIGURE 25-1
Life cycle of spore-forming bacteria.

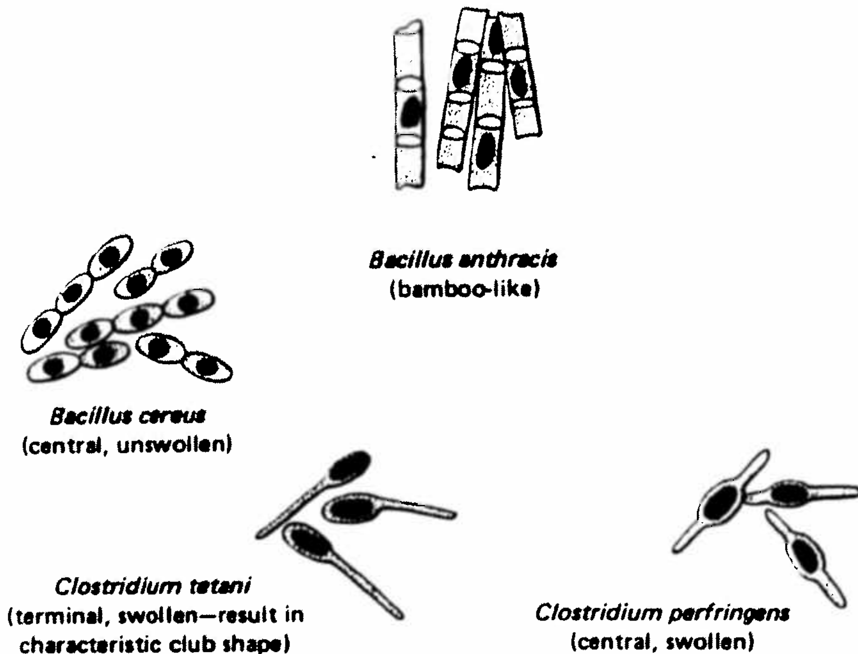


FIGURE 25-2
Size and location of four bacterial endospores within their vegetative cells. (See color plate 1(b).)

The size and location of the spore within the vegetative cell are also significant in differentiating organisms. For example, spores can be centrally, subterminally, or terminally located, and can be larger or smaller in diameter than the vegetative cell. When a spore is larger in diameter than the vegetative cell, a swelling or enlargement and distortion of the vegetative cell results, as shown in Figure 25-2. The sporulation characteristics of a species are constant each time sporulation occurs and are useful in identifying the organism.

Several spore-forming bacilli cause disease. The anaerobic clostridia are the most famous of these. *Clostridium botulinum* causes fatal food poisoning (botulism), *C. perfringens* causes gas gangrene, and *C. tetani* causes lockjaw (tetanus). These spore-forming clostridia all produce powerful exotoxins that can be fatal. The most powerful exotoxin is produced by *C. botulinum*. Ingesting a minute amount of food containing botulism toxin usually causes death. A 6 ounce bottle of botulism toxin could kill the entire human population of this planet.

The spores of *C. perfringens* and *C. tetani* are in the soil and hence on dirty objects and in food. In both diseases, the spores enter a wound with the soil or on objects with soil on them. The exotoxins of gas gangrene and tetanus are slower-acting than botulism and can be neutralized by antitoxins. Neutralization is followed by antibiotics to kill the toxin-producing bacteria and excision of the damaged tissue to remove the anaerobic environment. Early treatment is lifesaving in *C. perfringens* and *C. tetani* intoxications.

Most species of the genus *Bacillus* are harmless saprophytes. *Bacillus anthracis* is the only aerobic spore-forming pathogen. The spores of this organism are also found in the soil, where they remain viable for decades. If the spores are ingested by sheep, goats, or other animals, the disease anthrax is established. Although anthrax is primarily a disease of farm animals, it is transmissible to humans. It is an occupational hazard to farmers, veterinarians, and other handlers of animals because the organism can enter through a break in the skin. Workers handling animal products such as wool and goats' hair can contract the disease by inhaling the spores. When the infection begins in the respiratory tract, it is called woolsorter's disease.

The anthrax bacillus is easily recognized microscopically. It is a large, Gram-positive, spore-forming rod that forms characteristic chains. The ends of each bacillus are concave, and render a bamboo-like appearance, as shown in Figure 25-2.

Structures such as spores can be visualized by looking at certain peculiarities of the structure. For example, the bacterial endospore has resistant spore coats. When a spore-forming organism is stained by ordinary methods, the spore resists the stain and is seen through the microscope as an unstained area within the vegetative cell. If only a few spores are present or they have been released from the vegetative cells and are free in the smear, they can go undetected in a simple stain or a Gram stain.

The Schaeffer-Fulton stain is a differential stain developed to visualize both the endospore and the vegetative cell. Using this method, the spore itself is stained, and free spores are easily detected. Heat is used to drive the primary dye (malachite green) into the spore coats. The same characteristics of the spore that make it difficult to stain cause it to retain the dye tenaciously once it penetrates the spore coats. The malachite green rinses out of the vegetative cell readily because the cell wall has been disrupted by the heating process. The vegetative cell then accepts the counterstain, safranin. When examined microscopically, the spores appear as small, green ovals or spheres within the red vegetative cells (see Figure 25-3).

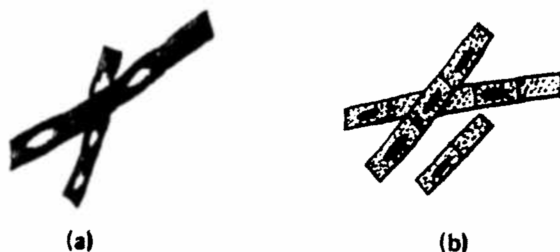


FIGURE 25-3
(a) Gram stain and (b) Schaeffer-Fulton stain of *Bacillus subtilis.* (See color plate 4.)

ACTIVITIES

Caution: Handle all stains with the care appropriate to a potentially hazardous chemical.

Activity 1: Visualization of Endospores by Gram Staining

1. Prepare a smear of *Bacillus subtilis.*
   - Air-dry and heat-fix it.
2. Gram-stain the heat-fixed smear.
   - Refer back to Figure 23-1 for a pictorial checklist.
3. Drain the slide and rinse it.
4. Blot it carefully.

Examine the slide under the oil-immersion objective. The spores appear as clear, unstained areas within the vegetative cells, as shown in Figure 25–3a. On the worksheet, draw several representative cells and sketch and label them. Go on to Activity 2, which will teach you a specific stain for endospores.

A[illegible]chaeffer-Fulton Spore Stain

1. Prepare a smear of *Bacillus subtilis* and heat-fix it.
2. Flood the slide with malachite green stain.
3. Heat the stain-flooded slide to steaming by inverting the Bunsen burner and passing the flame over the stain periodically, as shown in Figure 25-4. When you observe steam rise from the slide, remove the burner. When the steaming stops, pass the flame over

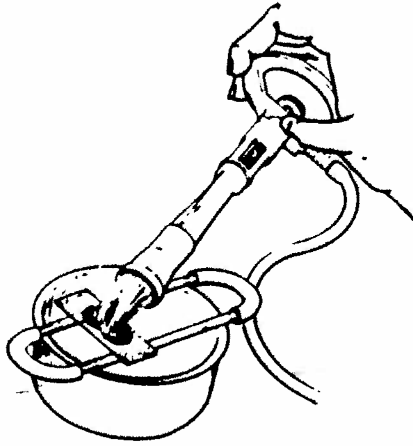


FIGURE 25-4
Heating process in the Schaeffer-Fulton stain.

the stain again briefly. Do not boil or allow the stain to dry. Steam for at least 3 to 5 minutes, replacing the malachite green if it evaporates from the slide.

4. Allow the slide to cool to prevent it from breaking. Continue to add stain as the slide cools, since the stain is still evaporating.
5. Drain the slide.
6. Rinse it with water for 30 seconds.
7. Replace the slide on the staining rack, and flood it with safranin counterstain. Allow the safranin to react for 1 minute.
8. Drain the slide, and wash it thoroughly with water.
9. Blot the slide carefully or allow it to air-dry.

Examine the Schaeffer-Fulton stain with the oil-immersion objective. You will see oval or spherical green spores and red, rod-shaped vegetative cells, as shown in Figure 25-3b. On the worksheet, draw several representative cells and label the spores and vegetative cells.

Practice the Schaeffer-Fulton stain procedure as many times as you need to. Then take the post test.

Phonetic Pronunciation

Bacillus anthracis = buh-sill´-us an-thruh´-sis
Clostridium sporogenes = klo-strid´-ee-um spor-oj´-uh-neez
botulinum = boch-uh-ly´-num or bot-you-ly´-num
tetani = tet´-uh-nee
perfringens = per-frin´-junz

FORMULAS FOR REAGENTS

1. MALACHITE GREEN (5% aqueous)
   Dissolve 5.0 g of malachite green in 100.0 ml of distilled water.
2. SAFRANIN COUNTERSTAIN (0.5% aqueous)
   Dissolve 0.5 g of safranin in 100.0 ml of distilled water.

MODULE 12

Streaking for Isolation

PREREQUISITE SKILL

Completion of Module 7, "Aseptic Transfer of Microbes."

MATERIALS

unlined paper
pencil or pen
empty petri dish
felt pen
wax pencil
inoculating equipment:
  inoculating loop
  Bunsen burner
  burner striker
petri dishes containing nutrient agar (6 to 8)*
beaker of disinfectant
slant culture of *Escherichia coli*
broth culture of *Escherichia coli*
slant culture of *Serratia marcescens*
mixed broth culture of two different genera of bacteria:
  Serratia marcescens and
  Escherichia coli
sterile swab

* Prepared by the student if the instructor so indicates, otherwise, plates from Module 9, "Aseptically Dispensing Agar into Petri Dishes," may be used.

OVERALL OBJECTIVE

Streak a bacterial culture on a nutrient agar plate using a technique to separate individual bacterial cells. When you have applied the technique successfully, each isolated cell will develop into a pure colony after incubation.

Specific Objectives

1. Demonstrate the principle of streak dilution using paper and pencil.
2. Demonstrate the principle of streak dilution using an empty petri dish and a felt-tip pen.
3. Streak for isolation using living bacteria and a nutrient agar plate.

4. Define the terms confluent growth, original inoculum, pure colony, mother cell, daughter cells, streak dilution, and bacterial isolation.
5. Differentiate between bacterial growth and increase in bacterial size.

DISCUSSION

Beginning microbiology students find it difficult to streak for isolated colonies because:

1. They do not use enough streaking surface, which results in fewer dilutions.
2. They use too large an inoculum, meaning their streaking must dilute thousands and thousands of cells before individual cells can be separated from one another.

The practice activities in Module 12, "Streaking for Isolation," enable you to use the entire streaking surface and make the maximum number of dilutions. Module 12 will help you think small enough so you will not introduce too many bacterial cells in your original inoculum.

It is difficult to appreciate how minute bacterial cells are: each individual bacterium measures approximately 1/25,000 inch in size. When you introduce an inoculating loop of bacteria onto the surface of a nutrient agar plate, you are placing tens of thousands of cells on the medium. A large inoculum contains so many bacterial cells that they are too crowded to develop into individual colonies, resulting in a growth that is continuous over the entire surface. This continuous growth is called confluent growth. When you learn to use a small amount of inoculum and you learn to separate these thousands of cells so they do not touch one another, you have correctly streaked for isolation.

Proper streaking for isolation results in pure colonies, each arising from a single mother cell. After the nutrient agar plate is streaked, separating the bacterial cells from one another, the separated single cells are then called mother cells.

Upon incubation of the streak plate, each mother cell divides by asexual binary fission, that is, by splitting in half every 20 to 30 minutes and giving rise to two daughter cells. In the next 20 to 30 minutes, the daughter cells split in half, and another generation of four new daughter cells comes into existence (see Figure 12-1).

Cells divide in exponential numbers, resulting in billions of daughter cells. The billions of cells pile up on top of and around each other, and a pure colony is born. Remember, a colony is a pure colony only if it does not touch another colony.

For most bacteria after 24 hours of incubation, a pure colony consists of 50 to 72 generations of cells arising from a single mother cell. A colony is therefore composed of billions and billions of daughter cells. Bacterial growth, then, means an increase in cell numbers, not an increase in cell size.

After completing Module 12, you will be able to separate bacterial cells from one another, allowing them to develop into pure colonies.

When you have completed the practice activities, you will be able to streak for isolation, a basic microbiological technique critical to your success in the laboratory portion of the course.

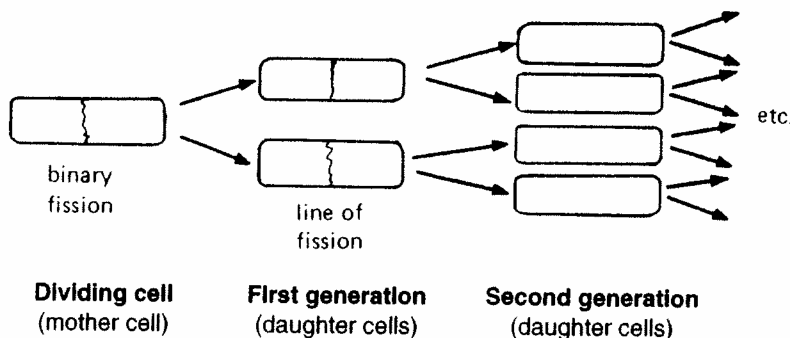


FIGURE 12-1
Asexual reproduction (binary fission) of bacterial cells.

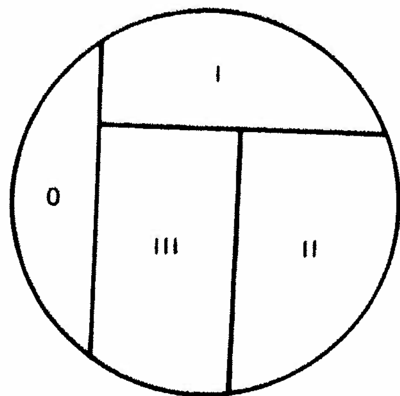


FIGURE 12-2
Dilution sectors as drawn on paper.

ACTIVITIES

Activity 1: Simulation of Streaking for Isolation Using Paper and Pencil

Using a pencil or pen, take the following steps, in which you will simulate the procedure you will use to streak for isolation on a nutrient agar plate with living bacteria. Keep in mind that when working with bacteria, you will be attempting to dilute the number of cells in each sector and you will be flaming the inoculating loop between sectors. Flaming the inoculating loop kills the remaining cells on the loop and aids in diluting the cells. Each sector represents a dilution or reduction in numbers of the thousands of bacterial cells in the original inoculum. Sector 0 is for the original inoculum and represents thousands of cells. Perform steps 1 through 4 in numerical order.

1. On a plain sheet of paper, draw a circle about 3 inches in diameter.
2. Line and label the circle as in Figure 12-2.
   - If you are left-handed, the 0 sector should be to the right; look for other reversals as you proceed through the steps.
   - Notice that the 0 sector is smaller in relation to sectors I, II, and III, which should be almost equal in surface area.
3. Begin step 1 (Figure 12-3). Using your pencil or pen, draw nearly solid lines in Sector 0.
4. Study step 2 (Figure 12-4), and draw lines from Sector 0 into Sector I as shown.
   - Be careful not to let the lines in Sector I cross or extend too near the boundary of Sector I. As you begin drawing the looping lines, be certain only the first two or three lines enter Sector 0. In doing so, you are simulating the dilution of the number of cells in Sector 0.

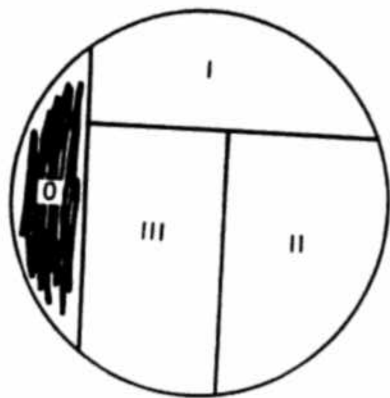


FIGURE 12-3
Step 1: Sector 0 represents the planting of a small amount of bacteria with your inoculating loop. This simulates the original inoculum.

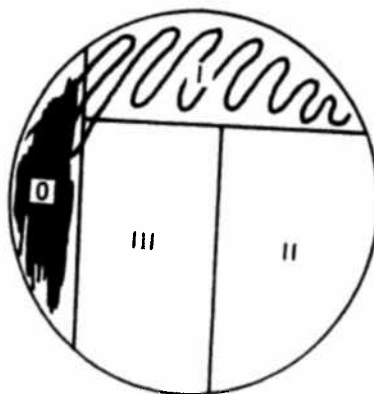


FIGURE 12-4
Step 2: Sector I represents the first dilution. Lines in Sector I should be as uniformly separated as shown here.

FIGURE 12-5
Step 3: Sector II represents the second dilution.

5. Rotate the paper counterclockwise one quarter turn so that Sector I is to the left.
6. Draw streak lines as shown in Figure 12-5 (step 3).
7. Rotate the paper a quarter turn to the left again, and imitate the final dilution by streaking Sector III as shown in Figure 12-6 (step 4).
8. Using another piece of paper, draw a circle similar to the one you just finished. Mark and label it in the same manner. Practice this activity several times, proceeding carefully through all four steps. When you are satisfied with your performance, show the final sketch to your lab instructor. If he or she approves your work, proceed to the next practice activity.

Dry Run Using Empty Petri Dish and Felt-tip Pen

1. Gather together a petri dish, a felt-tip pen, and wax pencil.
2. Turn the petri dish upside down, and mark it on the bottom with the wax pencil as shown in Figure 12-7.
   - Note the different position of the sectors compared with Figure 12-2. The position of Sector I is reversed and the petri dish is upside down. Sector I is on the bottom instead of on top.
3. Turn the unopened empty dish right side up.
   - The marking appears as shown in Figure 12-8, and the sector positions are exactly as in Figure 12-2.

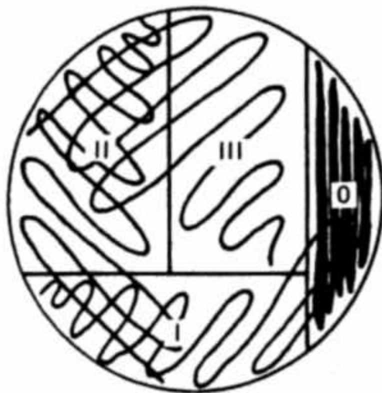


FIGURE 12-6
Step 4: Sector III represents the final dilution of the organisms. Leave space between each sector to allow for expanding bacterial growth.

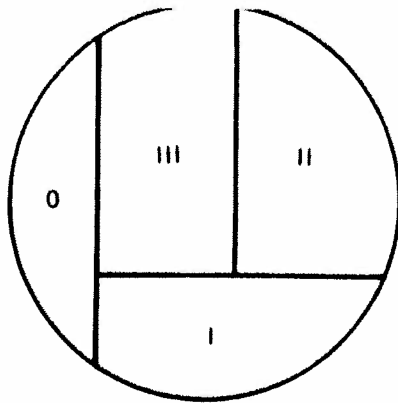


FIGURE 12-7
Dilution sector markings on the bottom of an empty petri dish.

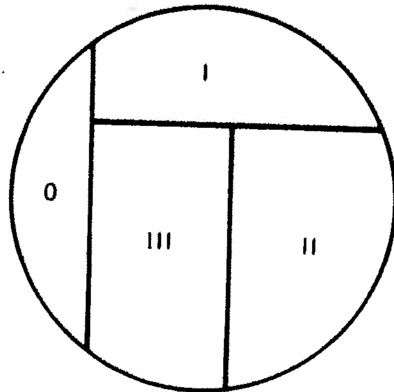


FIGURE 12-8
Dilution sector markings on the bottom of a plate as seen through the top of the empty petri dish. Sector I is again at the top and Sector 0 is to the left.

Now that you have learned to make a four-step dilution, pretend the felt-tip pen is your inoculating loop and your empty petri dish contains a solid medium. When you remove the petri dish lid, you will want to protect your medium from airborne bacteria. Remove the petri dish lid just enough to allow you to peek in and see where you are placing the pen and making your lines. Figure 12-9 shows you how to handle the lid with your left hand.

4. With the petri dish lying on the table in front of you, remove the lid as shown in Figure 12-9.
5. With Sector 0 on the left, draw the streak lines on the inside of the glass bottom.
   - Refer to the four steps in Activity 1 and follow them exactly.
6. Proceed through all four steps, reading the instructions as you go.
7. Close the petri dish and ask your laboratory instructor to check your streaking patterns.

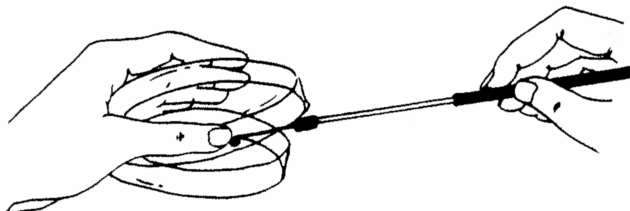


FIGURE 12-9
Proper handling of the lid of the petri dish. Open the dish slightly, but keep the lid over it to protect the sterile medium from airborne bacteria.

Streaking for Isolation Using Living Bacteria

1. Label and line the bottom of the agar plate as you did in Activity 2.
2. Follow the steps shown in Activity 1. Using your inoculating loop, transfer a small amount of bacteria from a slant culture of *Escherichia coli* to Sector 0 of the agar plate.
3. Proceed through the dilutions numerically (see Activity 1).
4. After you finish, invert the plate, label it, and incubate it at 30°C for 48 hours.

Only after incubation will you know if you have successfully streaked for isolated colonies. If you have mastered the technique the streak plate should look like one of the plates in Figure 12-10. Isolated colonies often grow before Sector III. The sector in which isolation takes place is not important as long as the colonies are separated. Show the plate to your lab instructor for approval.

Precautions: The inoculating loop can cut the agar, so use a light but definite touch. Keep the loop as horizontal to the agar as possible. The inoculum need not be so large that you can see it macroscopically; simply touch the loop to the bacterial growth. Flame the loop and let it cool between dilutions. If colonies appear on any area of the agar surface that you did not streak, they are airborne contaminants and must not be used for subculturing. Examine the location of the colonies carefully.

Using a Cotton Swab to Apply Original Inoculum

1. Divide the bottom of the agar plate into the same sectors you used in Activities 2 and 3.
2. Hold both the *E. coli* broth tube and the tube containing the sterile swab in your left hand.
3. Using your best aseptic technique, remove the closures with your little finger and the one next to it.
4. Remove the sterile swab from the sterile tube with the thumb and forefinger of your right hand.
5. Flame the necks of the tubes.
6. Dip the swab into the broth culture tube, saturating it with *E. coli*.
7. Flame the necks of the tubes again, recap them, and put them back in the tube rack.
8. You should still be holding the swab in your right hand, so carefully lift the lid of the agar plate and streak Sector 0 with the *E. coli*-saturated swab.

FIGURE 12-10
Incubated petri dish. After 48 hours of incubation the plate shows isolated colonies. Isolation can occur in any sector except Sector 0, depending on the number of cells in the inoculum.

9. Discard the swab in a container of disinfectant or 10% bleach.
10. Streak for isolation with the inoculating loop, using the same technique you developed in the preceding activities.
11. Incubate the plate with the plate from Activity 3.

Take the post test when you feel ready.

At your next lab session, show all your isolation plates to your lab instructor and sketch them on the worksheet.

SUMMARY OF STREAKING TECHNIQUES FOR ISOLATION

Following is a review for reference in future laboratory work. If you find you do not get pure colonies when you streak for isolation, the summary will be of help. Follow it until you become so adept at getting pure colonies you no longer need to divide the plate into sectors.

When you can make three streak dilutions of the original inoculum that result in isolated colonies, discontinue marking the bottom of the plate in sectors. You have mastered obtaining pure colonies and no longer need this aid.

1. Mark the bottom of the nutrient agar plate with a wax pencil, dividing it into sectors as in Figure 12-11.
   - If you are left-handed, the 0 sector will be on the right.
2. Turn the marked plate right side up. The markings will be reversed and will look like those in Figure 12-12.
3. Hold the lid in your left hand,* and use the lid to protect the nutrient from airborne bacteria.

*Reverse if you are left-handed.

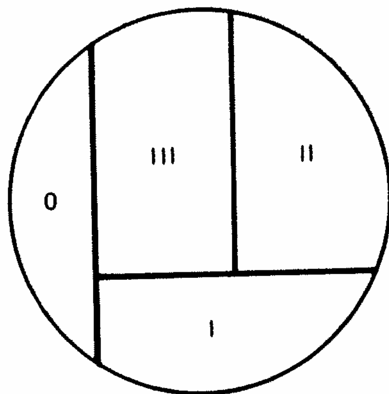


FIGURE 12-11
Sector markings on the bottom of an empty petri dish.

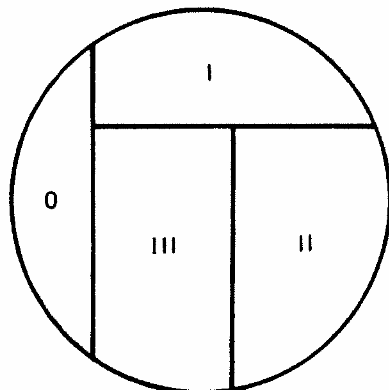


FIGURE 12-12
Sector markings on the same petri dish turned right side up.

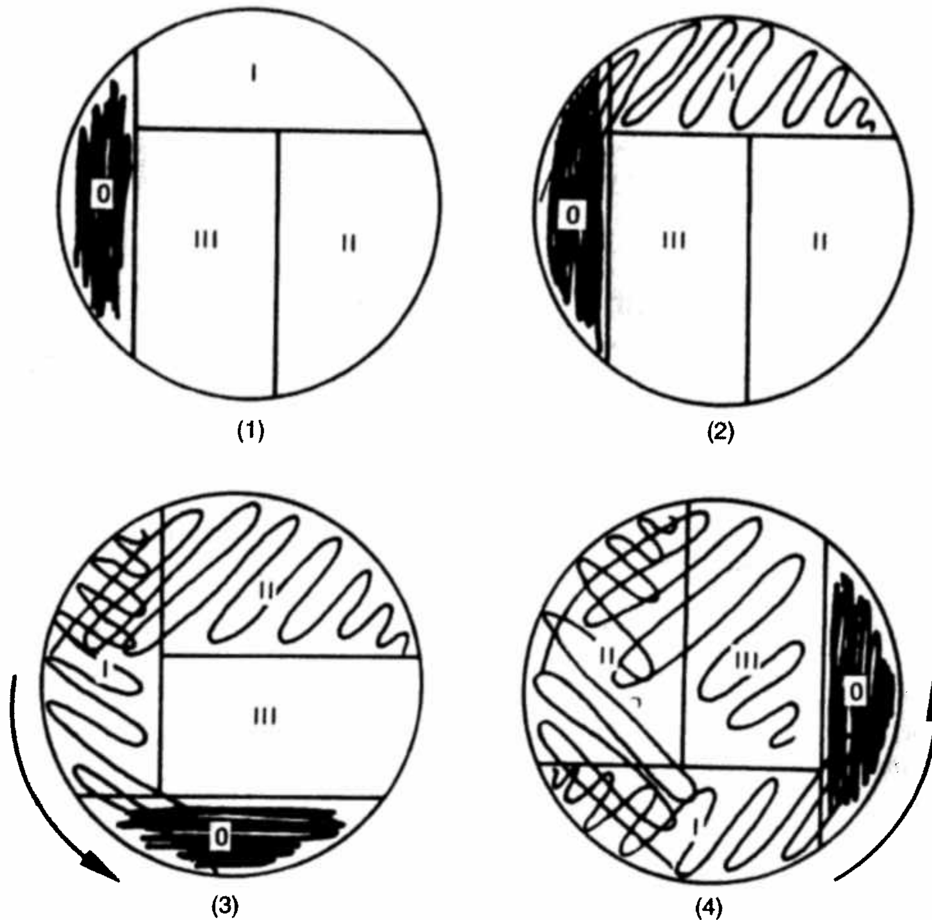


FIGURE 12-13
Summary of the steps used to streak for isolation.

4. Using the inoculating loop and living bacteria, streak each sector in numerical order, simulating the four steps shown in Figure 12-13.
   - Use light but definite touch to create uniform streaks without digging into the agar.
   - Flame and cool the inoculating loop before each dilution.

Keep the sector from which you are streaking on the left by rotating the plate a quarter turn counterclockwise.* Note in Figure 12-13 that the arrow follows the correct position of Sector 0. Note also that Sector 0 (for the original inoculum) is small compared with the other sectors (I, II, and III). The difference in size of the sectors allows you to use the major portion of the plate to dilute the numbers of bacteria in the original inoculum.

MODULE 13

Cultural Characteristics of Bacteria

PREREQUISITE SKILL

Completion of Module 2, "Preparing and Dispensing Media," Module 7, "Aseptic Transfer of Microbes," and Module 12, "Streaking for Isolation."

MATERIALS

48 hour streak plates*
hand lens or dissecting microscope
sterile nutrient broth tubes (4)†
sterile nutrient agar 1.5% plates (2)†
sterile tryptic soy agar plates (2)
sterile nutrient agar plates (2)†
slant cultures of *Escherichia coli*, *Bacillus subtilis*, *Proteus vulgaris*, *Streptococcus pyogenes*, and *Staphylococcus aureus*
nutrient broth culture of *Pseudomonas aeruginosa*

* You may use the streak plates from the post test Module 12; otherwise, plates will be prepared for you.
† Prepared by the student if the instructor so indicates.

OVERALL OBJECTIVE

Recognize, name, and describe the growth patterns of different bacteria using several media preparations.

Specific Objectives

1. Draw and name the colonial morphology of four different-appearing colonies from streak plates.
2. Inoculate two nutrient broths with two bacteria. Draw, name, and use a descriptive term or phrase for the differing patterns of growth of the two bacteria.
3. Define the terms comparative control and pure culture.
4. Draw and distinguish between a soluble and nonsoluble pigment, and name the organism that produces each type.

5. Describe differences in colony size between *Streptococcus pyogenes* and *Staphylococcus aureus.*
6. In the table on the worksheet, supply descriptive names for features of growth patterns you will observe.

DISCUSSION

Some microbes have characteristic growth patterns but they aid in the identification of a species only if they are distinctive. Although some bacteria grow in distinctive patterns, others look alike. Most bacterial colonies are circular in shape with an entire (smooth) margin or edge and are raised, or convex, in elevation. It is tempting to overemphasize cultural characteristics, but many types of bacteria share growth characteristics. To avoid confusion, you will observe organisms with distinct growth patterns. Compare Figures 13-1 and 13-2 with figures in other microbiology lab manuals, and you will see that only the most common cultural types are included. Figures 13-1 and 13-2 provide enough information for you to complete all the activities in the module, and they introduce the cultural characteristics you will encounter in this course. Study Figures 13-1 and 13-2 and refer to them as you perform Activities 1, 2, and 3.

After studying Figures 13-1 and 13-2, you will see that many features are used in categorizing bacteria by growth pattern. Three types of culture patterns are used:

1. An isolated colony on the surface of a nutrient agar plate
2. A nutrient broth culture
3. An agar slant

Bacterial growth on these three different preparations has become standard for studying cultural characteristics.

A single species of isolated bacteria is a pure culture. A single isolated colony on a streak plate, a single species on a slant, and a single species in a broth are all pure cultures.

Study the morphology of an isolated colony by examining Figure 13-1. You will see that there are three aspects of a single colony used to study the characteristic growth features of that colony:

1. Colony shape
2. Margin of the colony edge
3. Elevation of the colony

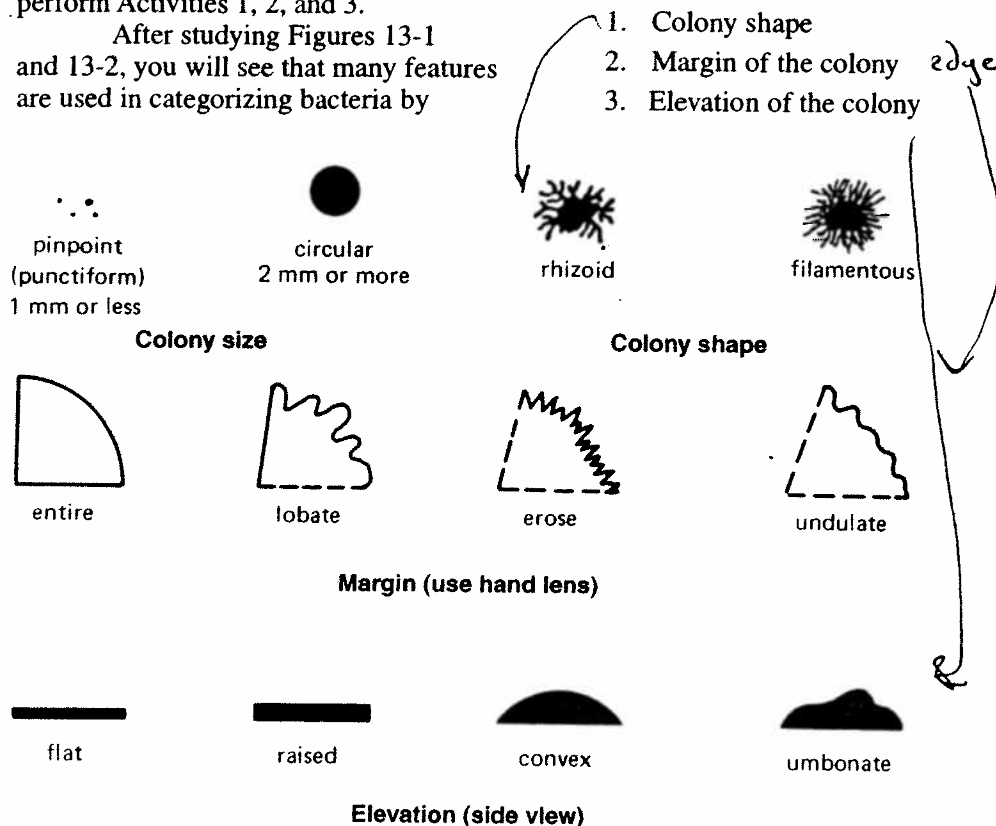


FIGURE 13-1
Characteristic growth features of an isolated colony.

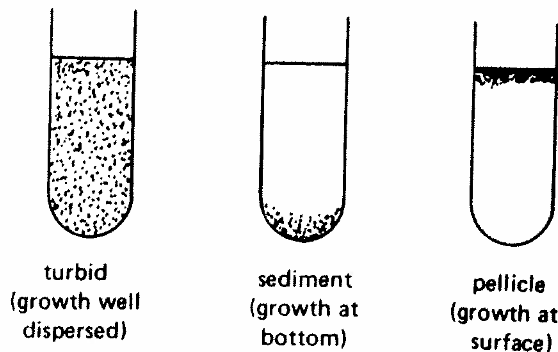


FIGURE 13-2
A few growth patterns in nutrient broth.

Activity 1 details how to study and name these three features of a colony.

Pigment production and patterns of bacterial growth in broths are included when studying growth characteristics. See Activity 4 for further explanation of pigment production by bacteria.

You will be able to complete Activity 1 during the present lab session, but you will be able to make inoculations only for Activities 2, 3, and 4. Complete them during the next lab session after they have had a chance to grow. Completing Activities 1 to 4 will allow you to perform Activity 5 with ease.

ACTIVITIES

Activity 1: Determining Colony Morphology from Previously Prepared Streak Plates

Using the plates provided or those saved from the post test in Module 12, draw and name the following features of the colony morphology of two different colony types:

1. Shape: form of colony.
2. Margin: edge of colony. (Use a hand lens or dissecting microscope. You may need to magnify the edge of the colony to determine its type.)
3. Elevation.

Refer to Figure 13-1 for names of the colony features to label on your drawings. You were given two genera of bacteria, so draw and name a colony type that arises from each genus.

Draw on the worksheet. Save your best *Serratia marcescens* plate for Activity 4.

Activity 2: Determining Colony Morphology from Self-Streaked Plates

1. Streak one nutrient agar (NA) 1.5% plate for isolation using *Bacillus subtilis*.
2. Incubate at 30°C for 48 hours.
3. Streak a second NA 1.5% plate with *Proteus vulgaris*.
   - The 1.5% refers to the concentration of agar in the medium. It differs from regular NA in the addition of 0.8% NaCl so it can easily be used as a base for blood agar. Including NaCl causes *Proteus* to swarm over the surface of the agar in a thin film.
4. Streak a tryptic soy agar (TSA) plate for isolation using *Streptococcus pyogenes*.
5. Streak another TSA plate for isolation using *Staphylococcus aureus*.
6. Incubate the last three streak plates at 37°C for 48 hours.

After incubating, examine the plates and draw colony morphology as you did in Activity 1. You may need to drag the loop across the surface of the *Proteus vulgaris* plate to detect the spreading growth.

Activity 3: Detecting Growth Patterns in Nutrient Broth

1. Use a slant culture of *Escherichia coli* and the same culture of *Bacillus subtilis* you used in Activity 2 to make aseptic transfers of each organism to a separate nutrient broth tube.
2. Transfer *Streptococcus pyogenes* and *Staphylococcus aureus* from the slant cultures to separate tubes of nutrient broth.
   - Use your best aseptic technique because strep and staph can be pathogens.
3. Incubate *B. subtilis* at 30°C and all other broth cultures at 37°C for 48 hours.
   - Since *B. subtilis* is not usually found in the body, its optimum growth temperature is lower than body temperature (37°C).

After incubating, draw all organisms grown in nutrient broth. Refer to Figure 13-2 for names and descriptions of growth types to accompany the drawings.

The pellicle of *B. subtilis* is heavy and may break loose from the top of the tube and sink to the bottom of the broth. Look for remnants where the pellicle was attached to the sides of the tube at the surface of your broth.

Activity 4: Production of Pigment by Bacteria

Pigment production is best studied from isolated colonies since a separated colony allows you to see whether the pigment is soluble or nonsoluble. A soluble pigment is water-soluble and can diffuse out of the bacterial cells into the surrounding medium. This soluble pigment colors the medium surrounding the colony. If the pigment is nonsoluble, it remains inside the bacterial cells and only the colony is colored, not the medium.

Most bacteria are not chromogenic, that is, they do not produce pigments. Their colonies are white or gray. The few bacteria that are chromogenic produce most colors of the spectrum, for example, green/blue, yellow/orange, red/violet, and shades of these. A few microbes produce a black pigment. Most pigments are not water-soluble.

As you proceed through the course, it will become clear to you that the pigment produced by an organism can be used as a clue leading to the identification of that organism. As you study the growth features of a colony, observe the pigment production that can occur.

This activity demonstrates the difference between a soluble and a nonsoluble pigment.

1. Streak a nutrient agar plate for isolation using a broth culture of *Pseudomonas aeruginosa*.
2. Incubate at 37°C for 48 hours.
3. Use the second nutrient agar plate as a comparative control to detect pigment production after incubation.
   - A comparative control is an uninoculated medium that is subjected to the same physical conditions or reagents as the inoculated medium.

In your next lab session, draw and describe the growth on this and the *Serratia marcescens* plate. *Pseudomonas aeruginosa* should produce a greenish water-soluble pigment. When comparing *P. aeruginosa* with the control plate, place both plates on a sheet of white paper for a neutral background.

Activity 5: Summary of Significant Cultural Characteristics

Complete the table on the worksheet to distinguish between a soluble and a nonsoluble pigment. Use the descriptive names for growth patterns as you did in the activities in this module. Write these descriptive names in the appropriate blanks. The chart reinforces the fact that the same organism must be grown several different ways to study its cultural characteristics.

You have finished the module. Be sure you completed all the work required of you in each activity, along with the worksheet table. Some blocks have been shaded in the table because only organisms with distinctive characteristics were chosen.

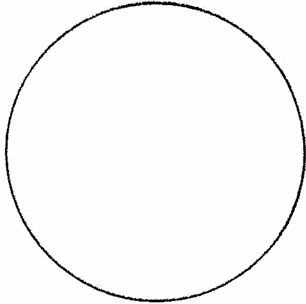
Take the post test.

Name______________________________

Lab Section__________________________

MODULE 13: CULTURAL CHARACTERISTICS OF BACTERIA

Activity 1: Determining Colony Morphology from Previously Prepared Streak Plates



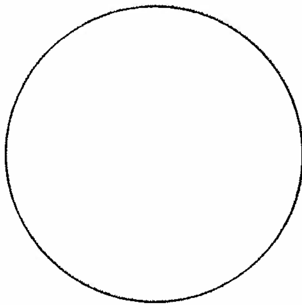
Serratia marcescens

Colony characteristics:

____

____

____



Escherichia coli

Colony characteristics:

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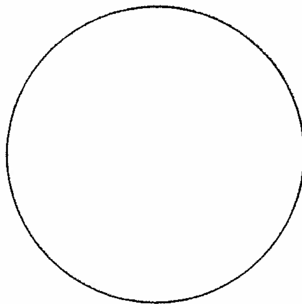
Activity 2: Determining Colony Morphology from Self-Streaked Plates



Bacillus subtilis

Colony characteristics:

____

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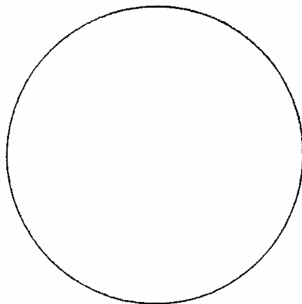

Proteus vulgaris

Colony characteristics:

____

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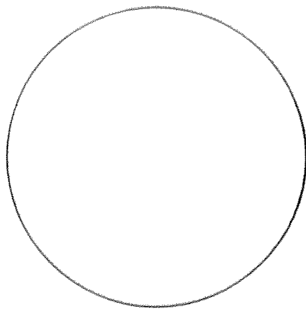
____



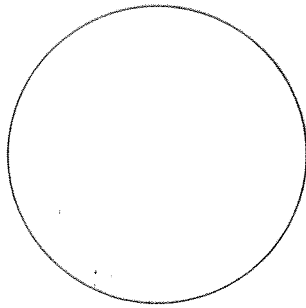
Streptococcus pyogenes

Colony characteristics:

____

____

____



Staphylococcus aureus

Colony characteristics:

____

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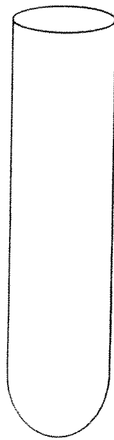
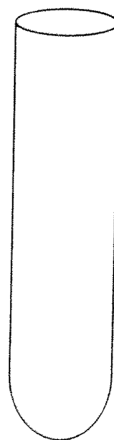
____

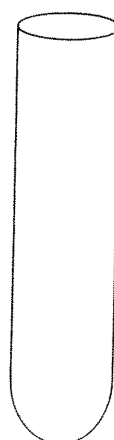
Activity 3: Detecting Growth Patterns in Nutrient Broth



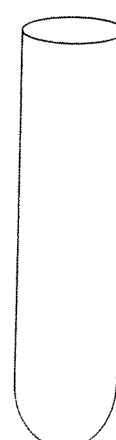
E. coli



B. subtilis



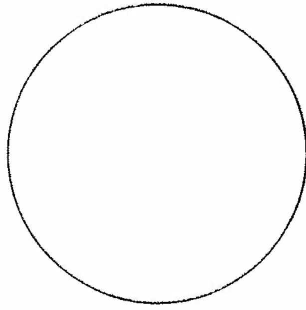
S. pyogenes



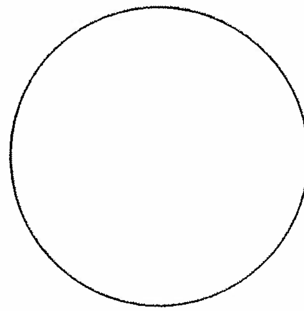
S. aureus

Growth type: ______ ______ ______ ______

Activity 4: Pigment Production by Bacteria



P. aeruginosa



S. marcescens

Compare a water-soluble pigment to a nonsoluble pigment.

____

____

____

____

Activity 5: Summary of Significant Cultural Characteristics

Microbe	Plate growth					Broth growth
	Shape of colony	Margin of colony	Elevation of colony	Size of colony (mm)	Pigment color	Nutrient broth
Activity 1						
S. marcescens						
E. coli						
Activity 2						
B. subtilis						
S. pyogenes						
P. vulgaris						
S. aureus						
Activity 3						
B. subtilis						
E. coli						
S. aureus						
S. pyogenes						
Activity 4						
P. aeruginosa						
S. marcescens						

Name: ____ Block: ___ Date: ________________________

Lab: Selective and Differential Media

MATERIALS:

2 EMB plates
1 MacConkey plate
2 CNA plates
1 MSA plate
1 ssA plate
4 tubes SIM medium

E.coli
Salmonella sp.
E.faecalis
P.mirabilis
C.albicans
Candida glabrata

S.aureus
S.epidermidis
S.pyogenes
S.pneumoniae
Permanent marker
Inoculation loop

Bunsen burner
Inoculating needle
Kovac's Reagent

BACKGROUND:

Selective media are designed to suppress the growth of unwanted bacteria and encourage the growth of desired organisms. This is accomplished by the addition of antibiotics, high concentrations of salt or high acidity. **Differential media** make it easier to distinguish colonies of the desired organism from other colonies growing on the same plate. The colonies have different colors or cause changes in the surrounding medium. Sometimes selective and differential functions are combined in one medium.

ACTIVITY 1

EOSIN METHYLENE BLUE (EMB) MEDIA

PURPOSE:

EMB Agar is selective media used for the isolation and differentiation of enteric bacilli, especially coliforms, in clinical specimens, water, and dairy products. It is also used to differentiate *Candida albicans* for other yeast species.

PRINCIPLE:

Holt, Harris, Teague first developed Eosin Methylene Blue agar. The eosin dye inhibits growth of gram-postive bacteria and combines with the methylene blue indicator to produce a color change whenever lactose or sucrose are fermented. The modified formulation of Holt, Harris and Teague further balances eosin and methylene blue to optimize differentiation between organisms which ferment these carbohydrates and those that do not. This medium does not allow discrimination between which carbohydrate is fermented. *Yersinia enterocolitica*, which ferments sucrose, but not lactose, will produce the same purple-black colony as lactose-fermenting bacteria.

The Levine5 formula eliminates the sucrose and doubles the lactose concentration. As in the HHT-modified formulation, lactose fermenters appear as colonies with blue-black centers and non-lactose fermenters appear as clear to opaque colonies. Because the Levine formulation contains lactose as the only fermentable carbohydrate, reactions are more comparable with MacConkey Agar.

Candida albicans can also be differentiated using the Levine formula from other *Candida* and *Cryptococcus* species by its ability to produce germ tubes within 3 hours, and pseudohyphae and budding cells at 18-24 hours when incubated at 35oC in 5-10% CO2. The addition of tetracycline to the Levine formulation aids in the selection of *C.albicans* from clinical sources that are contaminated with bacteria.

FORMULAS:

Approximate, per liter deionized filtered water.

(1) EMB Agar, Levine:

Pancreatic Hydrolysate of Gelatin ....10.0 g
Lactose 10.0
Dipotassium Phosphate 2.0
Agar 15.0
Eosin Y 0.4
Methylene Blue ....65.0 mg
Final pH 7.1 ± 0.2 at 25°C

PROCEDURE:

Method of Use: Prior to inoculation, the medium should be brought to room temperature. Inoculate the specimen onto the media surface using standard microbiological procedures to obtain isolated colonies. Obtain 2 EMB plates, using a permanent marker apply lines to divide plate into four quadrants. Inoculate each of the seven organisms below, one per quadrant. Incubate aerobically at 35oC for 18-24 hours.

Interpretation:

Escherichia coli Blue-black, dark centered colony with green, metallic sheen
Salmonella species Colorless or transparent, light-purple colonies
Klebsiella species Mucoid brownish colony with blue-black center
Proteus species Smooth, translucent, colorless colonies

Enterococcus faecalis Small, pin-point, clear colonies
Candida albicans (in CO_2) At 2-4 hours incubation, germ tubes can be observed when plate is examined under low magnification using a microscope. At 24 hours the characteristically feathery colonies will demonstrate pseudohyphae and budding cells under low magnifi cation.
Candida species No germ tube production. Colonies will appear as smooth, circular, and cream colored.

DATA TABLE:

	Salmonella Sp.	***E. coli***	***E. faecalis***	***P.mirabilis***	***Candida albicans***	***K. pneumoneae***	***Candida, not albicans***
Growth							
Colony Description							

ACTIVITY 2

MACCONKEY MEDIA

PURPOSE:
MacConkey media are selective, differential media used to isolate enteric microorganisms from mixtures of bacteria. MacConkey media and is especially useful for the recognition of enterococci in the presence of coliforms and nonlactose-fermenting organisms. MacConkey broth is an enrichment media and meets the U.S. Pharmacopeia (USP) standards in performing microbial examination of nonsterile products.
PRINCIPLE:
In 1905 MacConkey first described the selective, differential media that he used to isolate enteric gram-negative bacilli. It consisted of a nutritious base media that also contained crystal violet and bile salts which inhibited the growth of gram-positive microorganisms. The original formula has been modified by an addition of sodium chloride and a modification of the concentration of bile salts, agar, and neutral red. These changes have enhanced the recovery of *Shigella* and *Salmonella* species, the differentiation of coliforms from enteric pathogens, and the inhibition of the swarming of *Proteus* species. Microorganisms capable of growing on MacConkey Agar and capable of metabolizing lactose, produce acid by-products that lower the pH of the media close to the colony. The lowering of the pH causes the neutral red indicator to turn red, and if sufficient acid is produced, a zone of precipitated bile develops around the colony. Microbes that do not metabolize lactose appear colorless and translucent.
FORMULAS:
Approximate, per liter of deionized fi ltered water.
(1) MacConkey Agar:
Pancreatic Digest of Gelatin .... 17.0 g
Peptic Digest of Animal Tissue 1.5
Pancreatic Digest of Casein 1.5
Lactose 10.0
Bile Salts Mixture 1.5
Sodium Chloride 5.0
Agar 13.5
Neutral Red .... 30.0 mg
Crystal Violet 1.0
Final pH 7.1 ± 0.2 at 25°C
PROCEDURE:
Method of Use for MacConkey Agar: Prior to inoculation, the medium should be brought to room temperature. Inoculate the specimen onto the media surface using standard microbiological procedures to obtain isolated colonies. Obtain 1 MacConkey plate, using a permanent marker apply lines to divide plate into four quadrants. Inoculate each of the four organisms below, one per quadrant. Incubate aerobically at 35oC for 18-24 hours. Strong lactose and sorbitol fermenters will form deep red colonies on the respective media. Weak fermenters will form light pink colonies or colonies that have pink centers with a clear periphery. Nonfermenters will form colorless, translucent colonies.
Interpretation:
MacConkey Agar
Escherichia coli Pink, smooth, circular, with zone of precipitation and entire edge.
Salmonella species Colorless, translucent to opaque, smooth, circular, with entire edge.
Shigella species Colorless, moderately transparent, smooth, circular, with entire edge.
Proteus species Colorless, translucent, circular, smooth, some strains will show signs of spreading, but spreading is usually inhibited.
Enterobacter species Colorless to pink with pink centers, mucoid, thick, smooth, with entire edge.
Pseudomonas species Large, colorless to grayish-green with dark centers, translucent, with irregular edge.

DATA TABLE:

	E.coli	*P.mirabilis*	*K.pneumoneae*	*S.epidermidis*
Growth				
Colony Description				

ACTIVITY 3

COLUMBIA AGAR (CNA) MEDIA

PURPOSE:
Columbia agar is nutrient media used for the cultivation of fastidious and nonfastidious microorganisms from clinical and nonclinical specimens. Sheep or horse blood is added to enhance the growth of bacterial species by providing the X factor (heme) necessary in the preliminary identification of hemolytic strains. The media becomes selective for streptococci and staphylococci with the addition of colistin and nalidixic acid (CNA). Various combinations of antibiotics are used as additives to further select for fastidious microorganisms. Columbia Agar (P8233) preparation meets the U.S. Pharmacopeia (USP) standards for use as an isolation media in performing microbial examination of nonsterile products.
PRINCIPLE:
Columbia agar base (CAB) is a general purpose media with the basic ingredients necessary for microorganisms to replicate and grow. The media contains peptones, which provide a mixture of nitrogeneous compounds and amino acids, and yeast and beef extracts to provide additional nitrogenous compounds, carbohydrates and vitamins. Ellner et all discovered peptones from both animal and vegetable protein to be complementary, and the growth of the microorganisms to be better than on the then more frequently used base media (casein hydrolysate or meat infusion media). In addition, yeast and beef extracts were added and appeared to increase the growth of *Neisseria* species, while cornstarch, by neutralizing the inhibitory effects of glucose, decreased the formation of a green coloration (alpha hemolysis) by beta-hemolytic streptococci.
Columbia agar base (CAB) was made a selective media by adding colistin and nalidixic acid (CNA), which inhibit gram-negative microorganisms. CNA was found to be more effective in suppressing *Proteus*, *Klebsiella*, and *Pseudomonas* species than Phenylethyl
FORMULAS:
Approximate, per liter of deionized filtered water.
Columbia Agar + 5% Sheep Blood:
Peptic Digest of Animal Tissue ....5.0 g
Pancreatic Digest of Casein..............................5.0
Yeast Enriched Peptone..................................10.0
Pancreatic Digest of Heart Muscle 3.0
Cornstarch 1.0
Sodium Chloride.. 5.0
Agar..14.0
Sheep Blood..50.0 ml
Final pH 7.3 ± 0.2 at 25°C

PROCEDURE:*
Method of Use: Prior to inoculation, the medium should be brought to room temperature. Inoculate the specimen onto the media surface using standard microbiological procedures to obtain isolated colonies. Obtain 2 CNA plates, using a permanent marker apply lines to divide plate into three sections. Inoculate each of the six organisms below, one per section. Incubate aerobically at 35°C for 18-24 hours.
Interpretation:
Organism: Colony Morphology:
Streptococcus pyogenes Small, beta-hemolytic, transparent to opaque, domed, smooth and entire edge
Enterococcusfaecalis Small, gamma-hemolytic, transparent to opaque, domed, smooth and entire edge
Streptococcus pneumoniae Small, alpha-hemolytic, round and mucoid with entire edge
Staphylococcus aureus Average, ± hemolysis, opaque, circular, smooth, raised, white to golden yellow pigment
Staphylococcus epidermidis Average, ± hemolysis, opaque, circular, smooth, raised, usually white to colorless
Escherichia coli ±growth. Large, grayish colonies
For other clinically significant organisms, a reference such as Murray et al.5 should be consulted.

DATA TABLE:

	S.pyogenes	*E.faecalis*	*S.pneumoniae*	*S.aureus*	*S.epidermidis*	*E.coli*
Growth						
Colony Description						

ACTIVITY 4

MANNITOL SALT AGAR

PURPOSE:
Mannitol Salt Agar is a highly selective medium designed for the recovery and isolation of pathogenic staphylococci. This medium meets the U.S. Pharmacopeia (USP) standards in performing microbial examination of nonsterile products.

PRINCIPLE:
In 1942 Koch described the tolerance of *Staphylococcus aureus* to high concentrations of sodium chloride. Chapman formulated a medium which incorporated 7.5% sodium chloride into an agar containing mannitol and a phenol red indicator for the recovery of pathogenic staphylococci. Most strains of coagulase-positive staphylococci grow on the medium, producing colonies with yellow zones as a result of the fermentation of mannitol. Coagulase-negative strains may be inhibited or produce small colonies with no color change in the surrounding medium. Other bacteria are generally inhibited, so that a heavy inoculum of a culture containing mixed flora will not result in an overgrowth.

FORMULA:
Approximate, per liter deionized filtered water.
Beef Extract .... 1.0 g
Peptic Digest of Animal Tissue 5.0
Pancreatic Digest of Casein 5.0
Sodium Chloride 75.0
D-Mannitol 10.0
Agar 15.0
Phenol Red ....25.0 mg
Final pH 7.4 ± 0.2 at 25°C

PROCEDURE:
Method of Use: Prior to inoculation, the medium should be brought to room temperature. Inoculate the specimen onto the media surface using standard microbiological procedures to obtain isolated colonies. Obtain 1 MSA plate, using a permanent marker apply lines to divide plate into three sections. Inoculate each of the three organisms below, one per quadrant. Incubate aerobically at 35°C for 18-24 hours. Most strains of *S. aureus* capable of fermenting mannitol will do so within 24 hours. However, delayed fermentation of mannitol may occur with a few strains of *S. aureus*, so negative plates should be incubated for an additional 24 hours before being discarded.

Interpretation:
Positive: Growth of smooth, raised colonies; yellow color change in the medium. (Possible growth of *Staphylococcus aureus*; further identification of cation required.)
Negative: Growth of smooth, raised colonies; no color change in the medium; or inhibition of growth.

DATA TABLE:

	S.aureus	*S.epidermidis*	*E.coli*
Growth			
Colony Description			

ACTIVITY 5

SULFIDE-INDOLE-MOTILITY (SIM) MEDIA

PURPOSE:
SIM media is a semisolid agar used for the identification of members of the family *Enterobacteriaceae* by detecting indole formation, sulfide production, and motility.

PRINCIPLE:
Green et al. first described the use of SIM medium, suggesting that a reduced amount of medium would improve the incubation times for motility detection. SIM medium is designed to detect three biochemical characteristics of bacteria through the following mechanisms:

Motility: When organisms are stabbed into the semisolid agar with a straight wire, the bacteria will migrate by means of their flagella away from the stab line. This produces turbidity throughout the medium. Nonmotile organisms grow only on the stab line, leaving the surrounding medium clear.
Triphenyletrazolium chloride (TTC) is a soluble compound incorporated in the medium. When taken up by the bacterial cells, the substance is reduced releasing the acid formazan, a highly pigmented red, insoluble compound. SIM with TTC demonstrates motility by means of a diffuse pink color throughout the medium. Nonmotile organisms will produce a straight pink line.

Hydrogen Sulfide (H_2S): Sulfur is incorporated into the medium in the form of sodium thiosulfate, with the indicator ferric ammonium citrate. If H_2S is produced, it reacts with the sodium thisulfate, to produce ferrous sulfide which is precipitated in the medium, producing a blackish color.

Indole: Tryptophan is incorporated in the medium in the form of peptones. If the organism produces tryptophanase, tryptophan will be broken down into by-products; in particular, indole. This compound reacts with the aldehyde in Kovacs reagent to form a red or purplish-red color. A negative reaction will show no pink color change after the addition of the reagent. **(**H_2S and motility must be observed before Kovacs reagent is added!)**

FORMULAS:
Approximate, per liter deionized filtered water.
SIM Medium
Pancreatic Digest of Casein..............................20.0g
Peptic Digest of Animal Tissue..........................6.1g
Ferrous Ammonium Suflate..............................0.2g
Sodium Thiosulfate.......................................0.2g
Agar..3.5g
Final pH 7.3+/- 0.2 at 25°C

PROCEDURE:
Method of Use: Prior to the inoculation, the medium should be brought to room temperature. Obtain 4 tubes of SIM medium. Inoculate each tube with one of the organisms listed below, obtain colonies from a pure 18-24 hour culture. Using a straight needle, stab the center of the medium to about one half its length. Incubate the tubes with caps loose at 35°C for 18-24 hours. After incubation observe for production of H_2S and motility. After reading the above reactions, add a few drops of Kovacs reagent and observe for indole production.
Interpretation: Motility: turbidity or fuzzy growth throughout medium denotes motility. Growth only on stab site is indicative of a nonmotile organism.
H_2S Production: Blackening of the medium is a positive result. Absence of blackening denotes a negative test.
Indole: A pink to red color after the addition of Kovac's Reagent is a positive result. A yellow color denotes a negative test.

DATA TABLE:

	Control	***E.coli***	***Salmonella sp.***	***K.pneumoneae***
Hydrogen sulfide production				
Motility				
Indole				

ACTIVITY 6

BBL. Group A Selective Strep Agar with 5% Sheep Blood

INTRODUCTION
Group A Selective Strep Agar with 5% Sheep Blood (*ssA*) is a selective medium for use in the isolation and presumptive identification of group A streptococci from throat cultures and other specimens. ***Trypticase*** Soy Agar with 5% Sheep Blood (TSA II) is used for the growth of fastidious organisms and for the visualization of hemolytic reactions.
PERFORMANCE TEST PROCEDURE
1. Inoculate representative samples with the cultures diluted to contain 103.104 CFU/0.01 mL.
a. To each plate, add 0.01 mL of the dilution and streak for isolation. Make a stab in the primary streak area before streaking the rest of the plate.
b. Incubate plates at 35 ± 2°C in an aerobic atmosphere supplemented with carbon dioxide.
c. Include ***Trypticase*** Soy Agar with 5% Sheep Blood (TSA II) plates as nonselective controls for all organisms.
2. Examine plates after 18.24 h for beta hemolysis in the stabbed area and for amount of growth, inhibition, colony size and hemolytic reactions.

PRODUCT INFORMATION

INTENDED USE

Group A Selective Strep Agar with 5% Sheep Blood (*ssA*) is recommended as a primary selective plating medium for the primary isolation of group A streptococci (*S. pyogenes*) from throat cultures and other specimens in which the presence of *S. pyogenes* is suspected. Group B streptococci will also grow on this medium; most other streptococci, neisseriae, staphylococci and gram-negative bacteria are inhibited. The medium is designed for use in conjunction with ***Taxo*** A (bacitracin, 0.04 unit) discs for presumptive identification of *S. pyogenes*.

Trypticase Soy Agar with 5% Sheep Blood (TSA II) is used for cultivating fastidious microorganisms and for the visualization of hemolytic reactions produced by many bacterial species.

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SUMMARY AND EXPLANATION

Infection with Lancefield group A streptococci (*S. pyogenes*) may produce serious sequelae such as rheumatic fever and acute glomerulonephritis. Therefore, early detection and identification are important.

The nutritional composition of ***Trypticase*** Soy Agar has made it a popular medium, both unsupplemented and as a base for media containing blood. ***Trypticase*** Soy Agar with 5% Sheep Blood (TSA II) is extensively used for the recovery and cultivation of fastidious microbial species and for the determination of hemolytic reactions which are important differentiating characteristics for bacteria, especially *Streptococcus* species.

Because of the overgrowth of normal flora present in throat culture specimens plated on routine blood agar plates, selective ingredients have been added to sheep blood agar to enhance the detection of group A streptococci.

Evaluation of various antimicrobial agents in our laboratories resulted in a combination with improved selectivity over other selective media tested. This medium (*ssA*) allows presumptive identification of group A streptococci, based on bacitracin susceptibility and beta hemolysis, within 24 h after inoculation with the specimen when the medium is incubated in a CO_2-enriched atmosphere.[1]

The divided bi-plate, containing the nonselective blood agar (TSA II) in the sector marked "I" and the selective blood agar (*ssA*) in the sector marked "II," permits the recovery of group A streptococci and evaluation of the total specimen microbiota with one dish.

PRINCIPLES

The combination of casein and soy peptones in the ***Trypticase*** Soy Agar base renders the medium highly nutritious by supplying organic nitrogen. The sodium chloride maintains osmotic equilibrium.

Defibrinated sheep blood provides proper hemolytic reactions of streptococci. In addition, growth of *Haemophilus hemolyticus*, a nonpathogen whose hemolytic colonies are indistinguishable from those of beta-hemolytic streptococci, is inhibited.

Trypticase Soy Agar with 5% Sheep Blood (TSA II) provides excellent growth and beta hemolysis by *Streptococcus pyogenes* (Lancefield group A) and also provides excellent growth and appropriate hemolytic reactions with other fastidious organisms.

Group A Selective Strep Agar with 5% Sheep Blood (*ssA*) incorporates a unique combination of selective ingredients in ***Trypticase*** Soy Sheep Blood Agar (TSA II) to suppress normal throat flora for improved recovery of *S. pyogenes*. Defibrinated sheep blood supplies enrichment for the growth of such fastidious organisms and allows detection of the typical beta hemolysis of *S. pyogenes*.

FORMULA:

Group A Selective Strep Agar with 5% Sheep Blood (ssA)		***Trypticase Soy Agar with 5% Sheep Blood (TSA II)***	
Approximate Formula* Per Liter Purified Water		Approximate Formula* Per Liter Purified Water	
Pancreatic Digest of Casein	14.5 g	Pancreatic Digest of Casein	14.5 g
Papaic Digest of Soybean Meal	5.0 g	Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g	Sodium Chloride	5.0 g
Agar	14.0 g	Agar	5.0 g
Growth Factors	1.5 g	Growth Factors	14.0 g
Selective Agents	40.2 mg	Defibrinated Sheep Blood	1.5 g
Sheep Blood, defibrinated	5%		5%

*Adjusted and/or supplemented as required to meet performance criteria.

PROCEDURE:

Method of use: Prior to inoculation, the medium should be brought to room temperature. Inoculate the specimen onto the media surface using standard microbiological procedures to obtain isolated colonies. Obtain 1 ssA plate, using a permanent marker apply lines to divide plate into three sections. Inoculate each of the three organisms below, one per section. Incubate aerobically at 35oC for 18-24 hours.

Interpretation:

After 18.24 h of incubation in an atmosphere enriched with carbon dioxide, group A streptococci (*S. pyogenes*) on ***ssA*** will appear as translucent or opaque, white to gray, small (1.2 mm) colonies surrounded by a zone of beta hemolysis. A decrease in size as compared to the nonselective control, ***Trypticase*** Soy Agar with 5% Sheep Blood, is typical. Pinpoint or very small colonies of alpha- , nonhemolytic or other beta-hemolytic streptococci may grow in small numbers, but they should not interfere with the recovery of group A streptococci or interpretation of the results. *Neisseria* species, viridans streptococci, staphylococci, gram-negative rods and most beta-hemolytic streptococci other than groups A and B are inhibited on the ***ssA*** medium. Bacitracin susceptibility may be used to differentiate group A streptococci from group B. Fair to heavy growth of

beta-hemolytic colonies demonstrating a zone of inhibition around the ***Taxo*** A disc may be presumptively reported as *S. pyogenes.* A PYR (pyroglutamic acid) test may also be performed. It is more specific and as sensitive as the bacitracin test for this purpose.7 Gram stains should be made and examined.

DATA TABLE:

	S.pyogenes	***S.pneumoniae***	***E.coli***
Growth			
Colony Description			

DISCUSSION:

1. Complete the following table indicating whether the media is selective or differential by placing check mark in the appropriate column.

	Selective	Differential
Eosin Methylene Blue (EMB) media		
Macconkey media (MAC)		
Columbia media (CNA)		
Mannitol Salt Agar (MSA)		
Sulfide-Indole-Motility (SIM) media		
Group A Selective Strep Agar (ssA)		

2. Complete the following table by recording the inhibitive/selective agent, if any, of the listed medias and what organism(s) are inhibited.

	Inhibitive/Selective Agent	Type of Organism(s) Inhibited
Eosin Methylene Blue (EMB) media		
Macconkey media (MAC)		
Columbia media (CNA)		
Mannitol Salt Agar (MSA)		
Sulfide-Indole-Motility (SIM) media		
Group A Selective Strep Agar (ssA)		

3. Complete the following table by recording the method or type of differentiation, if any, for each of the medias listed.

	Method or type of differentiation
Eosin Methylene Blue (EMB) media	
Macconkey media (MAC)	
Columbia media (CNA)	
Mannitol Salt Agar (MSA)	
Sulfide-Indole-Motility (SIM) media	
Group A Selective Strep Agar (ssA)	