

EXPERIMENT 1 – MICROORGANISMS IN THE ENVIRONMENT

Period 2

Materials

- 1 tube/person containing 1 ml of a soil suspension (a 1/10,000 dilution) **or** lake water (a 1/100 dilution)
- 1 bottle of melted Plate Count Agar (PCA; 80-100 ml/bottle) to share with your neighbors – in the 50°C water bath
- 1 empty, sterile petri plate per person

Procedure

1. Before observation of the plates prepared last period, the Pour Plate Method will be performed:
 - a. Dump the entire contents of your sample into the empty, sterile petri dish.
 - b. When your plates and those of your neighbors at your table have been inoculated, obtain a bottle of melted PCA from the water bath. One person of your group will pour the medium into all four plates according to the instructor's demonstration. Mix the sample and medium in each dish with a gentle, swirling motion.
 - c. Move the plates to a cool part of the bench and let the medium solidify. (This may take 15 minutes.)
 - d. Incubate the plates inverted at 30°C.
2. Be sure to discard the bottles and tubes in the proper places on the discard cart.
3. Observe the plates from Period 1, noting the various bacterial and mold colonies. At this point, **do not open the plates**, especially if molds are present. (Any fuzzy or hairy colonies are probably mold colonies. Their spores are very easily dispersed into the air, causing subsequent contamination problems and perhaps allergic reactions as well!) **As a rule, we always observe colonies through the top lids of the plates.** Very little information about colony characteristics and differences can be obtained by looking through the medium. Note the various shapes, sizes and colors of the colonies.
4. **Optionally** (as time permits) individual colonies can be studied: From one or more plates which do not contain molds, choose two or more different colonies and record their visible characteristics (shape, pigmentation, relative size, etc.) For each of these same colonies, prepare a smear and stain by the gram-stain procedure to note the gram reaction and shape of the cells.

Note: The medium will not re-melt if it is allowed to solidify and then put back into the 50°C water bath. As discussed in Appendix D about agar, it will only liquefy when it is heated to boiling temperature, and then it will remain liquid until cooled to around 43°C.

GENERAL OBSERVATIONS of the plates made in Period 1:

SOURCE MATERIAL	GENERAL DESCRIPTION OF GROWTH MADE FOLLOWING PERIOD
air	

EXPERIMENT 2 – ASEPTIC TECHNIQUE

Trying to study a mixed population is often difficult and in the tradition of the scientific method, researchers dissect a system and study each piece in isolation. For microorganisms this means separating the organisms and getting them into pure culture. A pure culture is defined as a growth of microorganisms (a culture) that contains one specific kind of organism. It is essential in microbiology to be able to obtain and preserve pure cultures. Over 100 years ago, Robert Koch devised methods to achieve this goal and the methods he developed are essentially still used today. The protocols used to maintain pure cultures are a major part of aseptic technique and are the subject of this experiment.

The goals of aseptic technique are two-fold. The first objective is to obtain pure cultures and secondly to prevent cross-contamination. Microorganisms in culture must not escape into the environment, and microbes in the environment must not get into the cultures we are studying. It is essential that aseptic technique be understood and practiced correctly. Contaminated cultures are worthless for diagnosis or for doing research on, because it is unclear what microbe is performing any action that is being observed.

Aseptic methods commonly used are flame sterilization, tube transfer, streak plates, spread plates and pour plates. Flame sterilization is an easy method to insure sterile transfer of a culture from a source to a growth medium. Tube transfer is useful for moving inocula from one tube to another. Mechanical dilution by making streak plates is the preferred method for obtaining a pure culture of a microorganism. Finally, spread plates and pour plates are common methods for enumerating microorganisms and are sometimes useful for obtaining isolated colonies.

Period 1

Materials

- 3 tubes/person of Nutrient Broth (6 ml)
- 2 plates/person of Nutrient Agar
- Bacterial cultures (grown in Nutrient Broth):
 - Staphylococcus epidermidis*
 - Bacillus cereus*

Be sure to have read Appendix B before starting.

Note that “**broth**” refers to a **liquid medium**, and a “broth culture” is simply a **population of cells** which have **grown** (that is, **increased in numbers** by binary fission) in a liquid medium.

Procedure

1. To help you learn aseptic technique and to test your skill, perform the following exercises.
 - a. **First we will practice the technique for transfer of microbial cultures.** Follow the directions in Appendix B carefully. Label 2 tubes of sterile Nutrient Broth *S. epidermidis* and *B. cereus* (or an abbreviation for each such as **SE** and **BC**). Aseptically transfer a loop of inoculum from each broth culture to an appropriately labeled tube of Nutrient Broth.
 - b. **Next, you will prove to yourself that the inoculating loop is being sterilized properly by your flaming technique.** Flame sterilize the loop, let it cool, and then dip it into one of the cultures (your choice). Take this loopful of culture and carefully flame-sterilize it. One it has cooled, place it into the third tube of Nutrient Broth. Label this tube “control.” If this is done correctly, would you expect any growth to occur in the tube?
2. Incubate all 3 tubes at 30°C for until the next period.
3. Often it is important to obtain a pure culture of an organism for subsequent analysis. One of the easiest methods to do this is by the streak plate procedure. Follow the directions in Appendix B to make a streak plate of the *Staphylococcus epidermidis* culture on **each of the two** Nutrient Agar plates provided. (We do not use the *Bacillus cereus* culture for these plates, as this species produces colonies which are very large and difficult for one to obtain isolated colonies.) After labeling the plates on the bottom (lids can be inadvertently switched, bottoms cannot), place them in an inverted position and incubate at 30°C until next period.

EXP. 3A – BASIC BACTERIOLOGICAL STAINING METHODS

Period 2

Materials

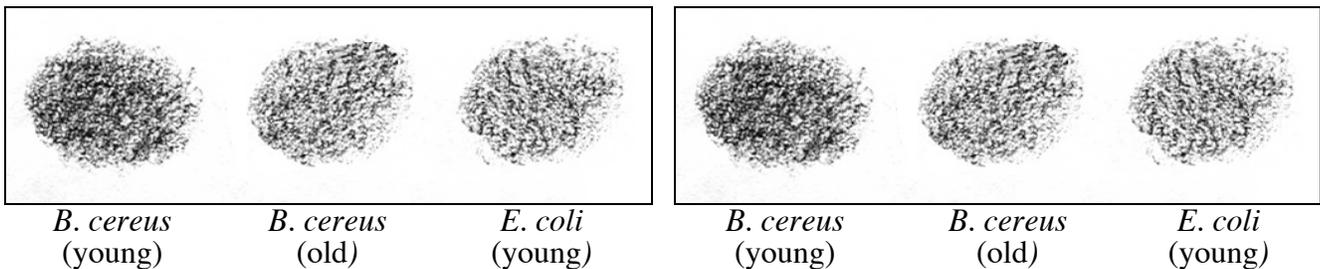
Broth cultures or saline suspensions of each of the following:
Escherichia coli – “young” culture, incubated 12-15 hours
Bacillus cereus – “young” culture, incubated 12-15 hours
Bacillus cereus – “old” culture, incubated 2-3 days

These cultures will be used individually. We generally pass out one set of cultures per pair.

Procedure

Smear-making.

1. The following will be performed **in duplicate** such that **two separate slides** will be prepared today: One slide is for use today in the gram stain procedure, and the other is to be saved and stained by the endospore stain procedure in a later period.
 - a. On **each** of two **clean** glass slides, prepare smears of the three cultures: See **page 91 (step 1)** regarding the making of smears, and note the instructor’s demonstration. All three cultures should be placed on each slide as shown in the diagram below – but do not place smears near the edge of the slide. **Do not use a separate slide for each smear.**



- b. When the smears have completely dried, **heat-fix** them according to the **instructor’s demonstration.**
 - c. Save **one** of the two slides in an empty petri dish for the **endospore stain** which will be performed in a later period.

Note that we use an empty petri dish as a substitute slide box which can hold several slides. **Always** put your name on the petri dish lid.

The First Gram Stain.

2. Perform the **gram stain** procedure on one of the slides prepared above as described in Appendix F, page 117.
3. As with any stained smear, definitive observations are made with the 100X, oil immersion objective. Refer to the microscope directions already given, remembering the rule to **focus the slide initially with the 10X objective**, and then swing the oil immersion objective into place.
4. The young cultures of *B. cereus* and *E. coli* are your positive and negative **control** cultures, and as controls they are placed on the opposite ends of the slide. If these turn out to stain gram-positive and gram-negative, respectively, then the smear in the center is most likely stained correctly and will give a reliable result.
5. As you examine the smears, do you notice any difference seen between the young and old cultures of *B. cereus*? Recall from the introduction to Experiment 1 why we can refer to **cultures** as “old” or “young” but should not do so for **cells**. **Record your observations in the space below**, noting the **gram reaction** (positive if purple, negative if red) and the **shape** of the **vegetative cells** (page 122). **Endospores** should be seen in one or two of the smears and will show up as oval, unstained bodies – some within vegetative cells and some “free.” What is it about the **age** of a culture that may be relevant to **endospore formation**? Remember to discard the tubes and slides properly.

CULTURE	GRAM REACTION (+ or -)	DIAGRAM
<i>Bacillus cereus</i> (young culture)		
<i>Bacillus cereus</i> (old culture)		
<i>Escherichia coli</i>		

This page and the following four pages are taken directly from Appendices C and G in the Manual.

PROPER STERILIZATION OF LOOPS AND NEEDLES

1. The Bunsen burner should always be worked with at about an arm's-length distance away. (Be careful igniting it, especially if it hasn't been used for awhile! Note the instructor's demonstration.) Hold the inoculating loop or needle in an almost-vertical position as shown in Figure B1 such that its entire length (plus a little part of the handle) is sterilized efficiently; heat until the wire begins to glow. The instrument will be ready for use after cooling 15-20 seconds.
2. After the transfer procedure (the following parts of Appendix B), there are still viable organisms on the loop or needle. Again hold it almost vertically in the flame, but start heating the wire from the **base**, moving slowly to the tip as the inoculum dries. Heating the tip first can cause the inoculum to spatter, creating an aerosol of viable cells.



FIGURE B1. PROPER POSITION FOR INITIAL FLAMING OF LOOP OR NEEDLE

PREPARATION OF A BACTERIAL SMEAR FOR STAINING

Preparations of bacteria for staining (smears) are best when just a bit of growth from an agar plate or slant is emulsified to light turbidity in water and then spread on the slide. Satisfactory smears can also be made from broth cultures, although some "debris" can be visible. **Follow the directions above for flaming loops and needles** before and after transfer of the culture to the slide.

1. When preparing a smear from a **broth** culture, the sterilized **loop** is used to transfer a drop of the culture directly to the slide. No additional drop of water is used, as the cells are already in a liquid suspension. Spread the drop over a dime-sized portion of the slide.
2. When preparing a smear from a culture growing on a **solid** medium, **first place a drop of water on a slide with the loop**. Then, with the sterilized **needle**, transfer a **tiny** bit of the growth (not digging into the medium!) to the drop of water and rub the needle around a dime-sized area until the material is as evenly emulsified as possible.
3. Allow the preparation to air-dry completely. The smear should be **just barely visible**. **Heat-fix** the smear by quickly passing the bottom surface of the slide to the tip of the burner flame three times, until the slide is just comfortably warm when touched to your hand. **Observe the instructor's demonstration** of this process carefully, so that you neither under-fix (the smear will wash off) or over-heat (the cells will be ruptured or distorted). Next, cool and apply the stain.

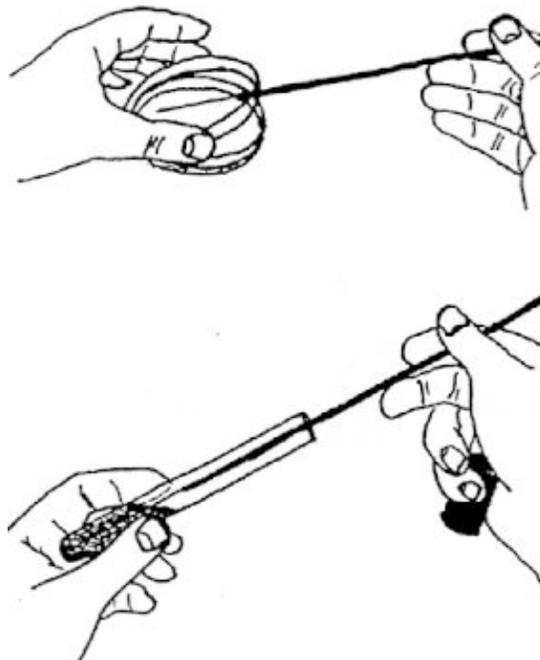


FIGURE B2. ASEPTIC TRANSFER FROM PLATE AND TUBE

When several organisms are to be examined by the same stain, smears of up to 3 or 4 organisms may be made on the same slide. Mark the location of each smear with a circle made by the wax pencil on the **underside** of the slide. Keep in mind that **smears may be kept indefinitely** after making them. They can then be heat-fixed, stained and observed whenever convenient.

TUBE-TO-TUBE TRANSFERS

To the bacteriologist, transfer of cultures is a common and simple procedure, but it requires careful attention to certain details. In **tube-to-tube transfers** by loop or needle, the tube containing the inoculum source and the tube with the recipient medium are held in the **same hand**. A procedure which avoids hand fatigue and the danger of dropping the tubes is as follows (the instructor will demonstrate):

1. Place the Bunsen burner in front of you (at about arm's length), and put all tubes and other equipment in a suitable location which will allow you to reach them without difficulty and without burning yourself!
2. Prior to picking up the loop or needle, the tubes are positioned in one hand as shown on the left in Figure **B3**. The plugs and caps should have been loosened (but not yet removed).
3. The transfer utensil is taken in the other hand, and the wire is flamed and allowed to cool (as shown previously).
4. The plugs or caps are removed with the **last two fingers** of the hand holding the inoculating needle or loop, leaving the thumb and index finger free to hold and manipulate the transfer utensil with the middle finger as a guide and support as shown on the right in Figure **B3**. Lightly flame the tops of the tubes.
5. Immerse the inoculating loop or needle into the broth culture to obtain inoculum (or scrape the needle across a small portion of surface growth to obtain inoculum if an agar slant culture is used). Make the transfer from one tube to the other.
6. Lightly flame the mouths of the tubes and return the stoppers to the tubes.
7. Flame the inoculating needle by moving it slowly through the flame – from the base of the wire to the tip – in order to avoid spattering. Put the needle down after it cools.

At first, this procedure for manipulating tubes and plugs may be awkward, but with practice it becomes the most rapid, least tiring and safest method.

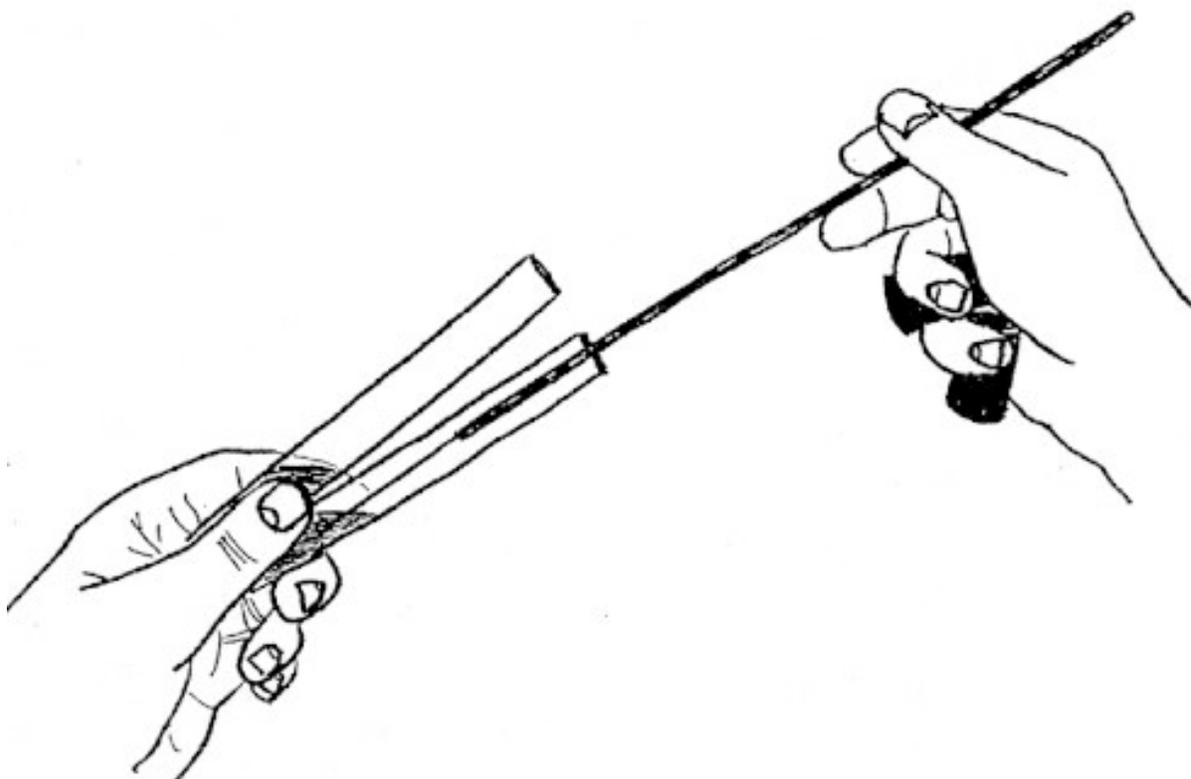


FIGURE B3. THE TUBE-TO-TUBE TRANSFER

PLATE-TO-TUBE TRANSFERS

Transfer from a colony on an agar plate to an agar slant or tube of broth may be made with an inoculating needle as follows:

1. As noted before, place the Bunsen burner in front of you at about arm's length, and put all other equipment in a suitable location which will allow you to reach them without difficulty and without burning yourself!
2. Take the inoculating needle with your hand and flame the entire wire to redness. Recall Figure **B1**.
3. When the wire cools (about 10-15 seconds), remove the lid of the plate with your other hand (as in Figure **B2**), and obtain an inoculum by removing a **tiny** portion of the surface growth on the agar plate. **Do not dig into the medium!** Replace the lid of the plate immediately.
4. Hold the tube to be inoculated with the free hand. (Note that the plate and tube are not to be handled simultaneously.) Remove the plug or cap of the tube with the little finger of the hand holding the needle (as in Figure **B2**). If a plugged tube is used, the mouth of the tube should be passed briefly through the flame to incinerate dust and lint particles in order to prevent their falling into the medium.
5. Introduce the inoculum into the tube. If a tube of broth is being inoculated, gently rub the wire on the moistened side of the tube just above fluid level; the tube is then slanted to wash the inoculum down and is shaken gently to distribute it. The **wire should not be rattled against the sides of the tube** to shake an inoculum into the broth. This is unnecessary and may actually create a dangerous aerosol. If the transfer is made to an agar slant, a single mid-line, lengthwise stroke over its surface is made with the wire.
6. Replace the cap or plug (the latter after re-flaming the mouth of the tube).
7. Flame the inoculating needle by moving it slowly through the flame – from the base of the wire to the tip – in order to avoid spattering. Put the needle down after it cools.

STREAK PLATES

The streak plate method is a rapid and simple technique of mechanically diluting a relatively large concentration of microorganisms to a small, scattered population of cells over the surface of the plated medium. The **primary purpose** is to **obtain well-isolated colonies** on a large part of the plate surface, each arising hopefully from a single cell, such that pure cultures of each desired species in a mixture can be established. Proper streaking of plates is a simple yet indispensable skill required of the bacteriologist.

The inoculating loop is the device used for streaking plates. The wire of the loop should not be badly oxidized or pitted or it will fail to “dilute” the inoculum and will scratch the surface of the agar.

A fluid suspension, well-shaken to homogenize the cell concentration, is prepared for streaking plates from materials with mixed microbial populations. After flaming the entire wire to redness in a Bunsen burner flame and then allowing it to cool without touching anything, the loop is tipped into the suspension. If the number of bacteria in the suspension is believed to be small, a loopful is transferred to the plate and the streaking process is begun immediately. If the suspension is believed to have a moderate to dense microbial population, the loop is drained first on the inside of the tube and then applied to a small area of the plate. After depositing this primary inoculum, the loop is flamed and cooled again before starting the streaking pattern. (Whenever desired, a flamed loop may be cooled rapidly by applying it to an uninoculated area of medium at the side of the plate.)

Success in obtaining isolated colonies is a function of the amount and density of the inoculum (often very difficult to judge) and the way in which it is streaked. The procedure becomes more of an art than a science, and consistent success is achieved with practice. Two basic patterns are illustrated in Figure **B5**. **The three-phase streaking pattern is recommended** for the beginning student, because it is most likely to give satisfactory results with suspensions having a wide range of microbial density.

To streak the plate, open the top lid just enough to allow the insertion of the loop from the right (from the opposite direction if you are left-handed). See Figure B4. With a wrist motion, move the loop handle back and forth over the surface of the medium, starting from the far left (from the opposite direction if you are left-handed) and moving toward the center of the plate. (Looking directly down at the plate, the loop should appear to be moving in an up-and-down direction.) Use a very gentle and even pressure. The loop should almost retrace its path with each swing as it moves across the agar surface. In using the multiphase streaking patterns, the loop is flamed after the first phase is completed, cooled, and recharged by streaking over the first phase a few times. **One does not go back to the culture tube for additional inoculum.** This is repeated for any subsequent phases. Do not keep the plate uncovered unnecessarily! Replace the lid immediately when you are through streaking the plate, and flame the loop – carefully, as discussed above – before setting it down.

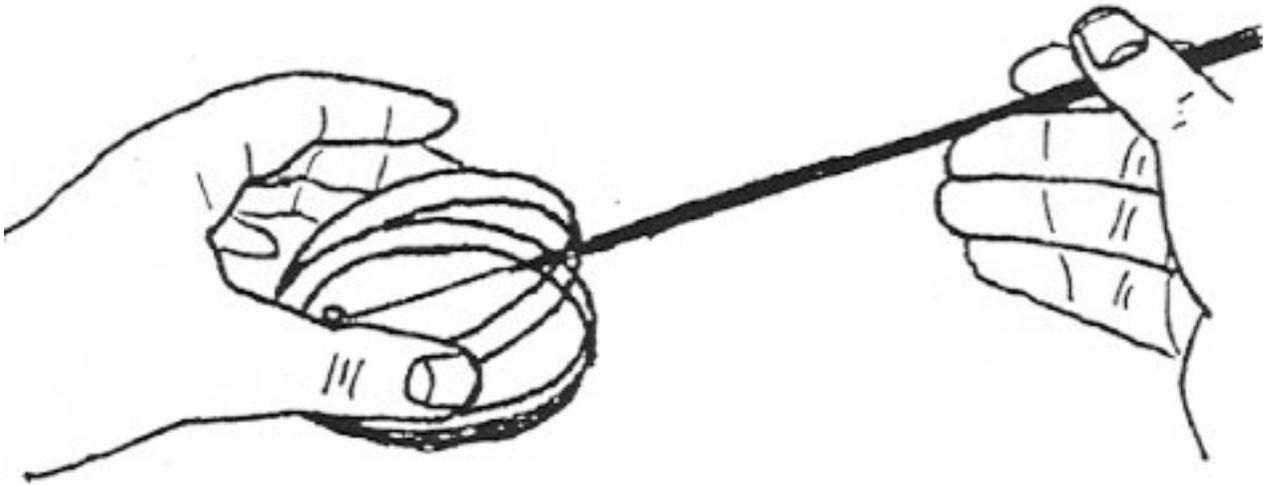
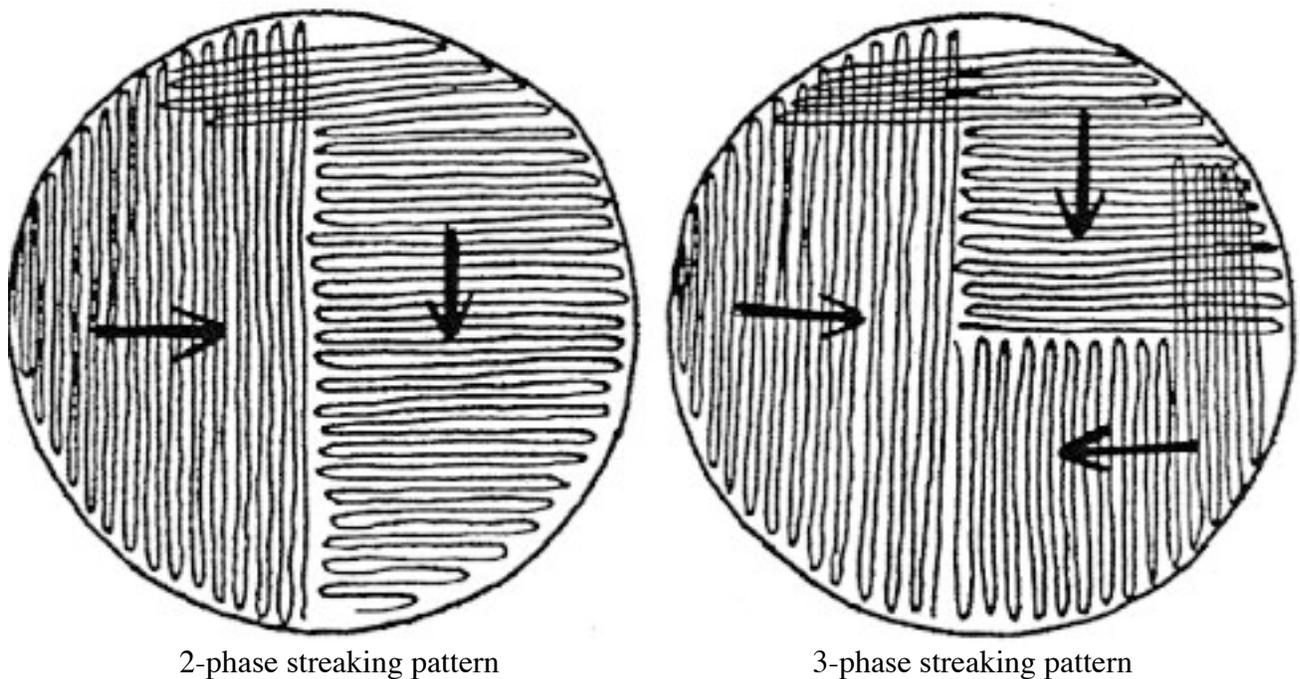


FIGURE B4. BEGINNING THE STREAK-PLATE PROCEDURE



2-phase streaking pattern

3-phase streaking pattern

FIGURE B5. TWO COMMON STREAK-PLATE PATTERNS

THE GRAM STAIN

The **gram stain** is one of the most useful differential stains in bacteriology, including diagnostic medical bacteriology. The differential staining effect correlates with certain fundamental differences in the structure of bacterial cell walls as the instructor will explain.

For the most reliable results when performing the gram stain, certain precautions should be taken:

1. The cultures to be stained should be “**young**” – incubated in broth or on a solid medium until growth is just visible (no more than 12 to 15 hours old if at all possible). With our lab periods being two or more days apart during the regular school year, we realize that short incubation times are not always possible. However, most of our organisms will still give reliable results for their gram reactions. One notable exception concerns organisms of the genus *Bacillus*: After about a day of incubation of the culture, the normally gram-positive (purple) reaction tends to give way to a gram-negative (pink) reaction as cell walls weaken and increasing numbers of cells become more easily decolorized. This “gram-variable” appearance should not occur if the effort is made to stain the culture as early in its lifetime as possible.
2. The cultures to be stained should be **grown on a sugar-free medium**, if at all possible. Many organisms produce substantial amounts of capsular or slime material in the presence of certain substrates – especially carbohydrates. Capsular material can interfere with decolorization, and certain gram-negative organisms such as *Klebsiella* may give a “gram-variable” appearance. This is a reason why organisms taken from MacConkey Agar give unreliable results in the Gram stain.
3. Smears must be **thin**, just **barely visible** (when dry) to the naked eye. Thick smears are not decolorized efficiently by the alcohol-acetone, and gram-negative cells can stain gram-positive.

Following is the traditional gram-stain procedure which we find reliable, as long as the precautions listed above are heeded.

1. Prepare and heat-fix a smear of the organism to be studied. Cover the slide with **crystal violet**. Allow one minute for this **primary stain** and then wash off (thoroughly and quickly, but gently) with a **minimum** amount of tap water, as an excess application of water tends to decolorize. Drain off most of the water onto a paper towel.
2. Cover the slide with **iodine** solution for one minute. The iodine acts as a **mordant** (fixer) and will form a complex with the crystal violet, fixing it into the cell. Rinse briefly with tap water, and then drain off most of the water.
3. Tilt the slide lengthwise over the sink and apply the **alcohol-acetone** solution dropwise – such that the solution washes **evenly over the entire slide** from one end to the other. Continue in this manner for about 10-15 seconds and then rinse **immediately** with tap water. If applied properly, the alcohol-acetone should **decolorize** cells with a gram-negative type of cell wall but not those with a gram-positive type of cell wall. Drain off most of the water.
4. Any decolorized, gram-negative cells need to be stained in order to be visible and differentiated from gram-positive cells. Cover the slide with **safranin** for one minute and then rinse briefly. Safranin serves as the **counterstain** in this procedure; a “counterstain” stains the decolorized cells differently than those which had retained the primary stain throughout the procedure. **Gently** (without rubbing) blot the slide dry.
5. For each smear, focus with the 10X objective, and then switch immediately to the 100X (oil-immersion) objective for the “official” observations, making sure you added a generous drop of immersion oil to the smear before moving the 100X lens into position. **Going from 10X to 100X (skipping the intermediate objective lens) is our standard operating procedure.** Observe the cells for morphology and gram reaction. Regarding the latter, record each culture as “gram-positive” (purple cells) or “gram-negative” (pink cells).