STAINING AND BACTERIAL CELL MORPHOLOGY

I. OBJECTIVES

- To learn the technique of smear preparation.
- To learn the techniques of Gram staining, nigrosin staining and KOH test.
- To use and relate the Gram stain to the study of bacterial cell morphology, and as an important step in the identification of a bacterial species.

II. INTRODUCTION

Observations of bacteria with conventional bright field microscopy yield relatively little useful information. This is because of the lack of contrast coupled with the small size of the bacterial cell. The use of stains that react chemically with cell material will enhance the contrast between the cell and the background. A stain is a dye consisting of a colored ion (a chromophore) and a counter ion to balance the charge. Attachment of the chromophore part of the dye complex to a cellular component represents the staining reaction.

There are two types of dyes: cationic (basic) and anionic (acidic). Cationic dyes have a positively charged chromophore and high affinity for negatively charged cellular components. Since bacteria carry a net negative charge at pH 7, such dyes can be used to stain the cells directly. Some examples of cationic dyes are crystal violet, safranin, methylene blue and basic fuchsin.

The other type of dyes, the anionic dye, has a negatively charged chromophore. The surface of bacteria at pH 7 repels such dyes and thus only the background is stained. After staining, cells would be seen as clear and bright bodies against a dark background. Such staining procedure is called negative staining. Nigrosin and eosin belong to this group of dyes.

Simple staining implies the use of only a single stain, which is usually sufficient to reveal the basic morphological features of most microbial cells, including relative size, shape, and characteristic arrangements of groups of cells.

To obtain additional information, various specialized staining procedures have been developed. Stains that react differently with different cell types are known as differential stains, which play an important role in the identification of taxonomic groups. The most important and widely used differential stain for bacteria is the Gram stain. On the basis of their reaction to the Gram stain, bacteria can be divided into two large groups: Gram positive and Gram negative. The different response of the two groups to the Gram stain is based on fundamental differences in cell wall structure and composition.

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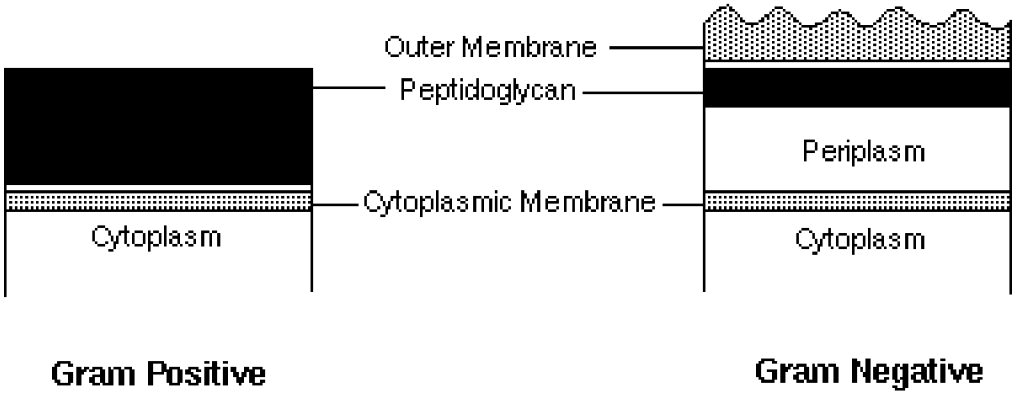
In a manner quite similar to the Gram stain, the acid-fast stain differentiates an important group of bacteria, the mycobacteria. Mycobacteria belong to the genus *Mycobacterium* which include important organisms like *Mycobacterium tuberculosis* (agent of tuberculosis) and *Mycobacterium leprae* (agent of leprosy). Due to high amounts of lipids in their cell walls, mycobacteria cannot be stained with Gram stain and remain colorless. In the acid-fast staining procedure, mycobacteria are first stained with the red dye basic fuchsin and the cells are then decolorized and re-stained with methylene blue. Basic fuchsin binds to lipids in the cell wall and turns mycobacteria red while other bacteria stain blue.

In a similar way as in acid-fast, in the Gram stain, a bacterial smear is dried and then heat-fixed to denature the cell proteins and to cause bacteria to adhere to the glass slide. The smear is then stained with crystal violet dye, which is rinsed off and replaced with an iodine solution. The smear is then decolorized with ethyl alcohol and counterstained with safranin. In Gram-positive organisms the purple crystal violet stain, treated with iodine solution, is not removed by the ethyl alcohol and the organisms therefore remain purple. On the other hand, the purple stain is removed from Gram negative organisms by the alcohol and so the colorless cells take up the red color of the safranin counterstain.



Successful staining of bacteria and other microorganisms requires first of all that a suitable smear be prepared on a microscope slide. Cells from a culture are spread in a thin film over a small area of the slide, dried, and then fixed by heating or with a chemical fixative to make the cells adhere to the slide. A good smear preparation 1) will be of an appropriate thickness to view individual cells, 2) will withstand repeated washing during staining; and 3) will retain the original cell morphology after fixation and staining.

After you have stained your bacterial smears, you will examine them with the oil immersion lens, noting the morphological and staining characteristics of each species. The usual bacteria that you see range in size from 0.5 to nearly 10 microns (a few species are many times that long.) The bacteria may show the following shapes: spherical (coccus), rod-shaped (bacillus), curved (comma), or helical (spiral or spirilum).

After cell division occurs, the cells may assume a characteristic arrangement: singly (as most Gram negative rods), pairs (as the bacillus *Klebsiella pneumonia*, the coccus *Streptococcus pneumoniae*, and the cocci in the *Neisseria* genus), chains (as the *Bacillus* and *Streptococcus* genera), irregular clumps (as in *Staphylococcus*), cubical packets of four or eight cells (as in some *Micrococcus* species) or lying side by side or at sharp angles as in the palisade or Chinese character formation (as in *Corynebacterium* species).

Other deviations also may occur. For example, *Mycobacterium* species may assume clumps on a smear because their waxy cell walls make them difficult to emulsify in a smooth suspension in water; when grown in a broth medium they may form long cords of cells. Cocci of *Neisseria* show flattened sides, making them bean-shaped. *Streptococcus* species frequently do not show typical chains when the smear is taken from a colony on solid medium; growth in broth is required to reveal the chains. Some bacteria exhibit considerable variation in size and shape (pleomorphism), even in a single species and culture. *Mycoplasma* lack the rigid cell walls of most bacteria and may assume a variety of shapes: coccoid, rodlike, or even L-shaped. The rod-shaped *Mycobacterium* species and *Corynebacterium* species are often club-shaped or swollen at one end and do not have parallel sides; branching forms may occasionally be seen. Some bacilli show bipolar staining, with the ends staining deeply and the middle portion very little.

History of Gram Stain

The Gram stain procedure was developed in 1883 by Hans Christian Gram, a Danish physician, who was working as a pathologist on a method he hoped would differentiate all bacteria from mammalian cells. He applied aniline-gentian violet followed by Lugol's iodine as a mordant to a slide preparation; a mordant increases the affinity of the stain for the cells. The precipitate formed was so thick that he decided to use 95% ethanol to clear the excess stain. He found that tissue cells cleared much more quickly than the bacterial cells within them. It was not until a year later that he found that other bacteria decolorized as quickly as the tissue. Thus, Gram did not achieve the goal of differentiating bacteria from mammalian tissue, but the result of his work was a useful method for easily differentiating bacterial types.

Reagents of Gram Stain

Because the Gram stain reaction is more widely used and studied than any other stain, it is also the most open to variation in protocol. Four reagents are used in the Gram procedure:

1. A primary stain--originally aniline-gentian violet was used, but this has been replaced by the more stable crystal violet.

2. A mordant--a compound that allows for better complex formation between a dye and its target compound (Gram's iodine solution, for example).

3. A decolorizing agent--an organic solvent (alcohol or acetone) that is used to remove the primary stain from the cell.

4. A secondary or counterstain--a stain, such as safranin, that is used to recolor cells that have lost the primary stain after alcohol treatment; it should contrast in color with the primary stain.

The most important step in the procedure is decolorization. Some bacteria do not lose the primary stain when treated with the decolorizing agent; these are the Gram-positive organisms and appear purple in color after staining.

Other bacteria, the Gram-negative organisms, lose the primary stain after decolorization, and after counterstaining with safranin they appear pink. If Gram-positive cells are not treated with the iodine mordant after primary staining, they too will lose their primary stain at the decolorizing step and appear as Gram-negative cells.

The timing of the steps of the Gram stain is not critical but depends on the density of the cell suspension applied to the slide and the nature of the bacterial culture. If the decolorizer is left on the smear too long, some Gram-positive cells may appear Gram negative.

Factors Affecting Gram Stain

- If the smear is too thick, proper decolorizing will not be possible. Also, if the smear is overheated during heat fixing, the cell walls will rupture.

- Concentration and freshness of reagents may affect the quality of the stain.

- Washing and drying of the smear between steps should be consistent. Excess water left on the slide will dilute reagents, particularly Gram's iodine.

- In most cases, Gram stain is reliable only on cells from cultures that are in the exponential phase of growth. Older cultures contain more ruptured and dead cells. Cells from old cultures may stain Gram negative even if the bacteria are Gram-positive.

Mode of Action of Gram Stain

At this time the mechanism of the Gram stain reaction is not completely known. There are many theories, but none are completely satisfactory. They are based on the physical and chemical reactions of the cell to the staining reagents. The most prominent current theories are as follows:

- The cell wall of a Gram-positive bacterium is composed of a heteropolymer of protein and sugar called peptidoglycan. This peptidoglycan sheath provides a barrier through which the crystal violet-iodine complex cannot pass during decolorization. When this wall is removed enzymatically with lysozyme, Gram-positive cells no

longer retain the stain complex and become Gram negative. A Gram-negative bacterium contains less peptidoglycan and more lipid than a Gram-positive organism. These chemical characteristics cause more effective and rapid removal of dye complex when decolorizer is applied.

- There may exist a specific Gram-positive substrate within the cell. All major cellular macromolecules have been implicated, including lipids, carbohydrates, proteins, and nucleic acids. This theory is hard to prove because removal of any of these components from the cell vastly alters the chemistry of the cell wall. However, there is some evidence for a crystal violet-ribonucleic acid-iodine complex in Gram-positive cells.

- A combination of the two above theories suggesting that a Gram positive bacterium is not only less permeable but also contains a compound that reacts with and holds the stain complex tightly.

Precautions in Using Gram Stain

The Gram stain is especially useful as one of the first steps in the identification of a bacterial species, since it reveals both the morphology and the Gram reaction of the bacteria. In this exercise, both Gram-positive and Gram-negative rods and cocci of commonly encountered bacterial species will be stained. You will continue to use the Gram stain in the study and identification of other species throughout the course. As you use this stain, remember these precautions for achieving the best results:

- Use only fresh cultures, not more than 48 hours old.

- When making smear of a colony from an agar plate, mix the sample in a small amount of water and spread thinly so that the turbidity is just barely visible. Thick smears stain heavily and show little or no morphologic features. On the other hand, broth cultures should be made thick as they are usually quite dilute and the bacteria are frequently so spread out that beginners have difficulty locating them under the microscope.

- Never make smears from inhibitory media, such as MacConkey (MAC), eosin methylene blue (EMB), Salmonella-Shigella (SS) agar, etc. The bacteria may show variable staining reactions and atypical morphology.

- If possible, use both Gram-negative and Gram-positive organisms on the same slide as a convenient check on the quality of the staining.

- Use broth cultures rather than colonies from solid media when you need to see cell arrangement, as in the identification of *Streptococcus* (in chains) and *Neisseria* (in pairs).

III. LABORATORY SUPPLIES

Bacillus cereus, positive rod	1 plate/table
Escherichia coli, negative rod	1 plate/table
Staphylococcus aureus, positive coccus	1 plate/table
Neisseria flava, negative coccus	1 plate/table
KOH, 3% solution	1 bottle/table
Sterile toothpicks, beaker	1/table
Crystal violet	3 bottles/table
Gram's iodine solution	3 bottles/table
Alcohol:acetone mixture	3 bottles/table
Safranin	3 bottles/table
Nigrosin	1 bottle/table
Loops	6/table
Acid : alcohol jar	3/table
Glass-marking pencil	2/table
Staining bowl	6/table
Staining rack	6/table

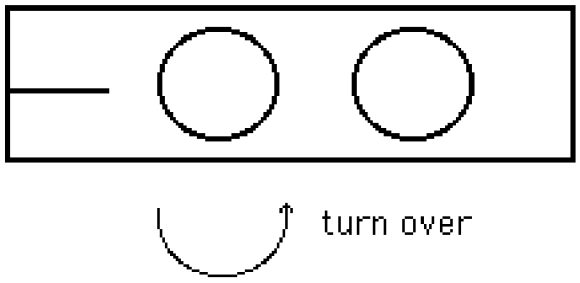
IV. PROCEDURE (Each student works independently for this exercise.)

Smear Preparations

1. Assemble the materials necessary for making the smears: a rack with the stock cultures to be used (these will be shared with other students working at your table), glass slides that have been soaked in acid alcohol or cleansed with a nonabrasive cleanser, an inoculating loop, a Bunsen burner and a glass-marking pencil.

2. Mark the first glass slide with a glass-marking pencil on the back of the slide. Draw a single line to the left and then two round large circles, leaving a margin on the right side. The left-hand line indicates where numbering begins. Turn the slide over, so that the crayon marks are on the underside.

3. Follow the proper steps below depending upon the source of your cultures (see the next two pages). We will be using plates in today's lab but you will need the broth procedure when you are trying to determine the Gram reaction as well as the spatial cell arrangement of your unknown.



4a. **Preparing a smear from a plate culture**

a. Sterilize your loop (from the hub of the handle along the entire length) in the Bunsen burner flame by holding it almost perpendicular in the flame until it glows. Remove the loop and air-cool.

b. Place a drop of water in the middle of each circle with your loop or a dropper. The water need not be sterile.

c. Sterilize your loop again and after it has cooled, remove a very small amount of inoculum from a single representative colony on the plate.

NOTE: If too much inoculum is taken, you will not obtain a good smear due to flaking of cell aggregates upon drying. If you see a clump of cells on your loop, you are taking too much.

d. Spread the inoculum in the first circle, filling the circle and mixing with the water.

e. Flame the loop by placing it in the flame at the hub of the holder where the wire is attached and then gradually move the wire through the flame, until the wire glows. Finally the loop itself is flamed until it is glowing. (If the wet loop is put immediately in the flame, it may splash out and contaminate the area.)

f. Repeat the above steps for the second sample.

g. Let the slide air dry. You may speed up the drying process by placing the slide in a warm spot near a lamp or a Bunsen burner.

NOTE: When the slide is dry, the specimen may be hardly visible especially if the culture was taken from a broth; however, the surface of the slide will be dull and not shiny.

h. Fix the bacteria onto the slide by passing the slide, smear side up, quickly through the flame of the burner two or three times. Avoid getting the slide too hot. This fixation process coagulates the proteins and fixes the bacteria onto the slide so they will not get washed off.

i. Your smear is now ready to be stained (see next steps).

4b. **Preparing a smear from a broth culture**

a. Sterilize your loop (from the hub of the handle along the entire length) in the Bunsen burner flame by holding it almost perpendicular in the flame until it glows. Remove the loop and air-cool.

b. Choose two of the cultures for the first slide. Holding one culture tube in your left hand at an angle (in the right hand if you are left-handed), remove the cap with your right hand. Flame the mouth of the slanted broth tube.

c. Insert the sterile, cooled loop into the culture and remove a loopful from the tube carefully to avoid touching the sides of the tube.

d. Flame the mouth of the tube, and quickly return the cap to the tube. Replace the tube in the rack.

e. Spread the inoculum in the first circle, filling the circle. Do not spread out the inoculum too thinly, as the bacterial cells will be widely separated in the broth medium to begin with, making it difficult for you to locate and focus on the cells during microscopic examination.

f. Flame the loop by placing it in the flame at the hub of the holder where the wire is attached and then gradually moving the wire through the flame, until the wire glows. Finally the loop itself is flamed until it is glowing. In this way the broth dries out gradually before incineration. If the wet loop is put immediately in the flame, it may splash out and contaminate the area.

g. Repeat steps c and d several times, each time waiting for the inoculum to dry on the slide. This procedure is especially needed if your sample is dilute.

h. Repeat steps a to g for the second sample.

i. Let the slide air dry. You may speed up the drying process by placing the slide in a warm spot near a lamp or a Bunsen burner.

   NOTE: When the slide is dry, the specimen may be hardly visible especially if the culture was taken from a broth; however, the surface of the slide will be dull and not shiny.

j. Fix the bacteria onto the slide by passing the slide, smear side up, quickly through the flame of the burner two or three times. Avoid getting the slide too hot. This fixation process coagulates the proteins and fixes the bacteria onto the slide so they will not get washed off.

k. Your smear is now ready to be stained (see next steps).

Gram Staining

1. Place the slide on a staining rack, smear side up, crayon markings down. If more than one slide is placed on the rack at one time, do not allow the slides to touch each other.

2. Add 2-3 drops of crystal violet stain directly on the smear. Stain for one minute.

3. Rinse the slide by washing the stain off with a gentle jet of water from a wash bottle (at this stage all bacteria will be stained purple by the crystal violet).

4. Drain off the rinse water. Add 2-3 drops of Gram's iodine solution. Let the slide stand for one minute.

5. Rinse with water as described in step 3 above.

6. Decolorize the stain by letting the alcohol:acetone mixture run down over the slide, which should be held at an angle, until the stain is no longer being removed from the slide. Quickly rinse the slide with water.

   NOTE: A thick stain takes longer to decolorize than a thin one, so the exact time cannot be specified. It is usually not more than 20 seconds. Too little or too much decolorization can affect your results. At this stage the Gram-positive organisms will remain purple. If the smear is thick, they may appear so visually, but a thin smear from a broth culture may not be visible at all. Gram-negative bacteria will be colorless at this stage.

7. Add 2-3 drops of safranin stain and let stain for 30 seconds.

8. Rinse the slide with water as described in step 3 above.

9. Let the slide air-dry, or blot (not rub) it dry with bibulous or blotting paper. The slide should be completely dry before adding oil for microscopic examination.

Negative Staining

1. Place a small drop of nigrosin at one end of a slide.

2. Sterilize your loop and aseptically transfer some cells to the drop of nigrosin, and mix to make an even suspension.

3. Sterilize your loop again and set it aside.

4. Use the end of a clean slide to spread the mixture to a thin film. Allow the slide to air dry and examine it microscopically.

Microscopic Examination of Your Stained Slides

1. Examine the stained slides with low power objectives and finally use the oil immersion lens. For this you will need maximum light by opening the diaphragm, raising the condenser as much as possible, and adjusting the light source intensity.

2. Examine the bacteria in each circle and observe the size, shape (rod, spherical or curved), Gram staining (positive: purple; negative: pink), and arrangement (singly, in pairs, in chains, irregular clusters, or regular packets of four or eight). Compare the Gram negative organism(s) with the Gram positive and note the difference in color. If not clearly evident, check your procedure with the instructor.

   NOTE: Achieving a good focus with oil immersion is frequently difficult and time-consuming for the beginner; be patient and get help!

3. Sketch a few of the organisms found in each circle (on the page provided for this purpose at the end of this handout), enlarging your drawing enough so that the arrangement, shape and relative size of the organisms can be shown. Do not try to sketch the entire field.

Use of KOH as an Indicator of Gram Stain Reaction

A relatively new and easy technique has recently been developed by Ryu which can also characterize the Gram stain reaction to a large extent. We use this new test in our lab to complement the results of the Gram test and not as a replacement for the commonly used Gram stain. The procedure is as follows:

1. Place one drop of 3% potassium hydroxide (KOH) solution on a clean slide. Transfer a good amount of bacteria from the culture medium with a sterile toothpick to the drop.

2. Mix the bacteria into the solution rapidly and in a circular motion.

3. After 5-8 seconds, raise and lower the toothpick just off the slide surface to determine the stringing effect. If there is stringing (increased viscosity) within 15 seconds, the KOH test is positive, while the bacteria are considered to be Gram negative. See if your results from this test agree with those from the Gram stain and record your observations.

Results of the Staining Lab Exercise

NAME _____DATE _____ GROUP NAME _____

Gram Stain and KOH Reactions: Record the color taken up by the cell and the Gram stain reaction (Gram positive: purple; Gram negative: pink). Try the KOH method the organisms and see if the results agree with those of Gram stain.

Bacterial Species	Cell Color	Gram Positive/Negative	Degree of Stringiness
Species #1			
Species #2			
Species #3			
Species #4			

Nigrosin Stain: Sketch a few cells from each smear so that some detail can possibly be seen. Sizes (record size in microns) should be in relative proportions. Be sure to indicate any special features of arrangements: pairs, chains, packets, and so on.

Bacterial Species	Sketch of Cells and Size Measurements
Species #1	
Species #2	
Species #3	
Species #4	

Based on above data, write the scientific name of each species below:

Species #1 is: Species #3 is:

Species #2 is: Species #4 is: