

Exercise 6-B

STAINING OF MICROORGANISMS

GRAM STAIN

Introduction

The **Gram stain**, developed by **Hans Christian Gram** in 1884, is a staining technique allowing different types of microorganisms (usually bacteria) to be distinguished from one another on the basis of the chemical and physical properties of their cell walls. Such staining techniques are referred to as **differential staining** techniques or **differential stains** because they cause different types of cells to look different. In the Gram stain, differences in cell wall structure typically manifest themselves as color differences. Bacteria that stain dark purple are said to be **Gram-positive**, while those that stain lighter and pink are **Gram-negative**.

The cell walls surrounding most bacteria used in this laboratory are rigid structures made up of **peptidoglycan**, a macromolecule of unique composition. As indicated in the illustration below, each layer of peptidoglycan includes polysaccharides composed of alternating **N-acetyl muramic acid** and **N-acetyl glucosamine** connected together by short peptide chains sometimes containing unusual amino acids (diaminopimelic acid, diaminobutyric acid and D-form amino acids).

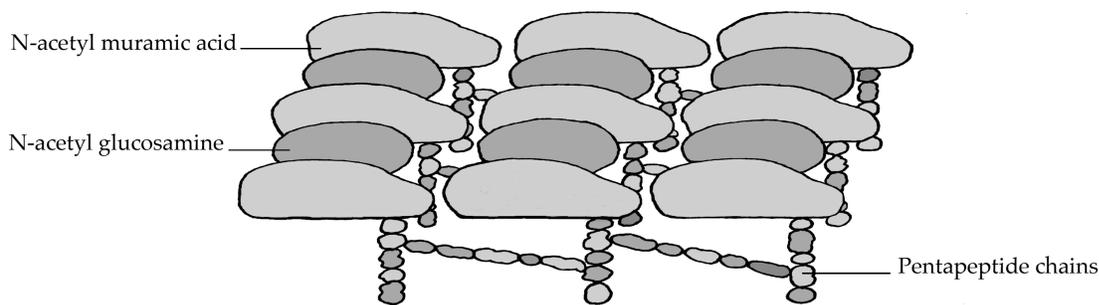


Figure 6.2 - A small section of one layer of peptidoglycan showing a portion of three polysaccharide chains connected together by pentapeptide chains. Some of the amino acids within the peptide chains are unique in nature, i.e., are not found in proteins.

On the surface of a typical Gram-positive bacterium, multiple layers of peptidoglycan form a thick wall permeated by **teichoic acid**, while on the surface of a typical Gram-negative cell, a few layers of peptidoglycan form a thin wall surrounded by an outer membrane of complex composition (often containing **lipopolysaccharides** toxic to mammalian cells). These differences in wall composition are significant because they can influence bacterial characteristics such as susceptibility to anti-microbial drugs, responses to growth retardant dyes, sensitivity to physical factors in the environment and the ability to cause disease symptoms in humans and other animals.

The Gram stain is one of the most useful staining procedures applied in microbiology. It requires the use of four different solutions; a basic dye or stain (primary stain), a mordant, a decolorizing reagent, and a counterstain. The steps involved in the Gram staining procedure can be summarized as follows:

1. Bacterial cells are **heat-fixed** and stained with a **basic dye** (Crystal violet). The crystal violet is sometimes referred to as the **primary stain**, and colors all of the cells purple.
2. The cells are treated with a **mordant** (iodine, potassium-iodide solution) to increase the affinity or attraction between the cells and the dye.

3. The cells are treated with a **decolorizing reagent** (alcohol-acetone mixture) that removes the previously applied stain either slowly or very quickly. Cells that decolorize slowly retain most of their purple color and are Gram-positive, while those that decolorize quickly, losing the purple, are Gram-negative. At the end of the decolorizing step, Gram-negative cells (those with thin cell walls) will tend to be colorless and very difficult to see.
4. The Gram-negative cells are restained with a **counterstain** (safranin) to render them visible in a contrasting color. The counterstain colors the Gram-negative cells pink.

Note - The division of bacteria into Gram-positive and Gram-negative types is an important first step in traditional identification procedures; however, it is important to remember that some bacteria having thick peptidoglycan in their cell walls are easily decolorized and will stain as though they were Gram-negative (pink). This is especially true if the cultures used for staining are old. Old cultures of Gram-positive bacteria typically contain some dead or dying cells that will stain pink. Some species of bacteria classified within Gram-positive genera (e.g., *Bacillus*), will almost always stain pink when subjected to a Gram-staining procedure as described below.

Procedure:

1. On a **single** clean glass slide, prepare **four small smears** as directed by your instructor, using the bacteria cultures provided (one smear each of known Gram-positive and Gram-negative bacteria, one smear containing a mixture of Gram-positive and Gram-negative bacteria and one smear of your morphological unknown culture). Allow the four smears to air dry, and then heat-fix them.
2. Place the slide on a stain rack over a sink, and stain each smear by covering it with crystal violet and allowing the stain to act for 60 seconds.
3. Rinse the slide (held down in the sink) thoroughly with tap water. Be careful to avoid contact between crystal violet and skin surfaces.
4. Return the wet slide to the stain rack and flood the smears with Grams iodine. Allow this mordant to act for at least 60 seconds.
5. Rinse the excess iodine from the smears with tap water.
6. Decolorize each smear with acetone/ethanol decolorizing reagent as demonstrated by your instructor. Remember to continue the decolorizing process only as long as you see color flowing from the smear, and when the color stops moving, rinse immediately and thoroughly.
Use caution – Excessive application of decolorizing reagent will remove color from both Gram-positive and Gram-negative cells.
7. Rinse the slide thoroughly with water. **Note** – It is important to accomplish the water rinse as quickly as possible following the decolorizing step in order to stop the action of the acetone/alcohol.
8. Counterstain the smears by covering the slide surface with safranin and allowing the stain to act for 60 seconds.

9. Rinse the slide thoroughly with tap water, remove excess water from the slide bottom and allow the smears to air dry (place near the base of a lit Bunsen burner).
10. Examine each smear under oil immersion (focus with the 10X objective first) and compare the results. If the Gram-staining procedure has been carried out correctly, the Gram-positive smear will contain dark, purple-colored cells, the Gram-negative smear will contain lighter, pink-colored cells, and the mixed smear will contain a combination of the two.
11. Make sketches illustrating the characteristic appearance of each smear (cell shape, color and arrangement), and record results (interpretations of what has been observed). Use this information to determine the Gram-staining characteristic of your morphological unknown culture.

Note - If your first Gram stain attempt is unsuccessful, try it again. This is an important staining technique and will be used on numerous other occasions throughout the semester.

Questions:

1. What is a differential stain and when might such a stain be used?
2. What is peptidoglycan, what is its composition and where is it found?
3. How does the cell wall of a typical Gram-positive bacterium differ from that of a typical Gram-negative bacterium?
4. What is an important feature of the lipopolysaccharides (LPS) found in association with Gram-negative cell walls?
5. What is the function of a mordant?
6. What would you expect to observe if you forgot to apply a counterstain while making a Gram stain of a Gram-negative bacterial culture?
7. What were the Gram stain characteristics and morphological features of the cultures you observed?

Exercise 6-B Supplement

THE KOH TEST: DIFFERENTIATION OF GRAM POSITIVE & NEGATIVE BACTERIA WITHOUT STAINING

Introduction

The KOH (potassium hydroxide) test, as a differential technique, was originally described in the late 1930's by Dr. Ryu in Taipei, Taiwan. It was later published in an American Journal (Am. J. Vet. Clin. Path, 1967, 1:31-35), where the authors said they had been using the 3% KOH technique for years in conjunction with routine Gram-staining procedures. Gram-positive cells, with their thick peptidoglycan walls, are resistant to the reagent used (3% KOH), but Gram-negative cells are lysed by it. The viscosity observed is due to the release of nucleic acids from the Gram-negative cells. The KOH test is simple to perform and the results obtained with it have correlated closely with those obtained from standard Gram-stained preparations. When applied to certain types of bacteria, e.g., *Microbacterium* species, the KOH test will provide a more accurate indication of wall composition than will a Gram stain.

Procedure:

1. Apply one drop of 3% KOH solution to the surface of a clean glass slide.
2. Using a sterile loop, transfer a visible sample of a pure culture or isolated bacterial colony from the surface of solid media (plate or slant) into the KOH solution on the slide.
3. Stir the mixture thoroughly, occasionally lifting the loop from the slide surface in order to observe the results. If the mixture becomes markedly viscous and slimy (snot-like) within 60 seconds, the result is **KOH-positive**, and the bacteria present have thin cell walls (will most likely stain Gram-negative). If the bacteria fail to produce such viscosity (are **KOH-negative**), they have thick cell walls (will most likely stain Gram-positive).

Note - Young cultures of Gram-negative bacteria often form slimy gels within 5 to 15 seconds, while some older cultures required up to 60 seconds.

Cultures containing mixed growth of Gram-positive and Gram-negative bacteria typically yield positive reactions (slime formation) when subjected to the KOH test. This indicates that well-isolated colonies and pure cultures of bacteria must be used in order to make this a reliable test.

The only bacterial culture mentioned by the authors as having failed to yield a proper response was *Moraxella bovis* (a Gram-negative). James Singeltery, another investigator, mentioned that he had trouble getting the expected slime formation with *Azotobacter* and *Rhizobium* species when they had formed gummy colonies on plates. Both of these genera are also Gram-negative.

We will apply the KOH test to all bacteria isolates being used for semester projects, as it is necessary to determine the wall composition of these organisms prior to the extraction of chromosomal DNA (and the KOH test is often more reliable than a Gram stain for environmental isolates). KOH-positive cultures with their thin cell walls are easily lysed by boiling, while KOH-negative cultures (thick-walled cells) require additional treatments (we will beat them with glass beads in order to break them open).

Name _____

Lab Section _____

WORKSHEET
Exercise 6B & 6B-Supplement
Staining of Microorganisms: Gram Stain & KOH Test

Goals: _____

Materials & Methods:

1. Gram Staining

Age of Morphological Unknown: _____

Reagents used: _____

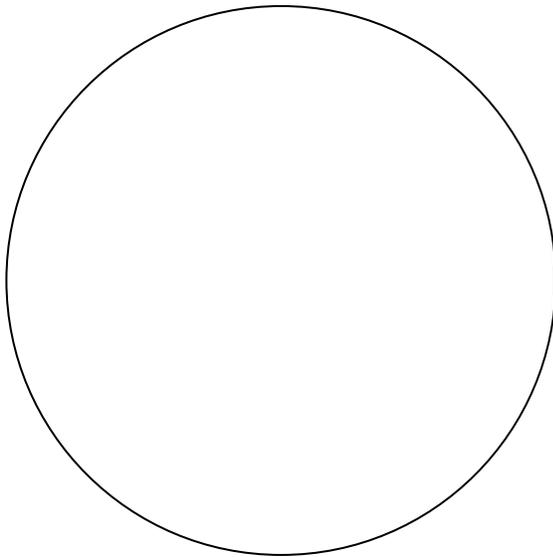
2. KOH Test

Age of Morphological Unknown: _____

Reagent used: _____

Data & Results:

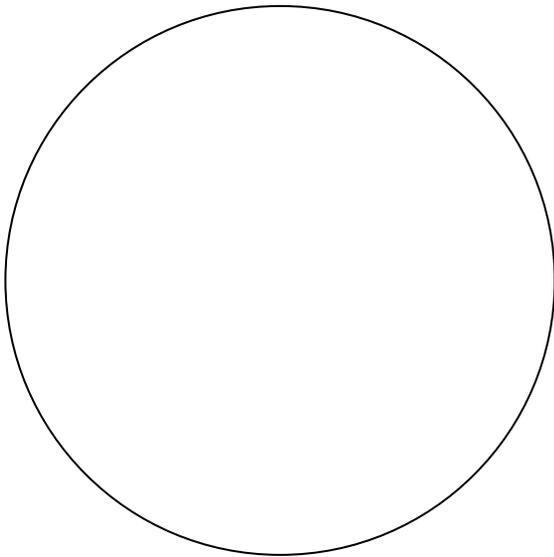
1. Gram Staining



A) Gram-positive Control

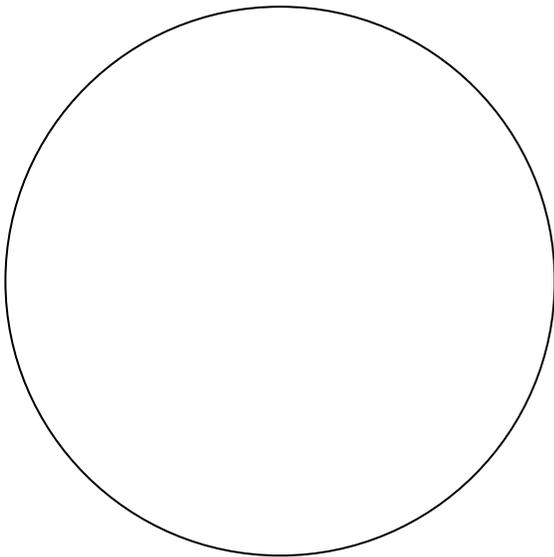
Specimen:	_____
Total Magnification:	_____
Length:	units x $\mu\text{m}/\text{unit} = \mu\text{m}$
Width:	units x $\mu\text{m}/\text{unit} = \mu\text{m}$
Notes:	_____

B) Gram-negative Control



Specimen:			
Total Magnification:			
Length:	units x	$\mu\text{m}/\text{unit} =$	μm
Width:	units x	$\mu\text{m}/\text{unit} =$	μm
Notes:			

C) Morphological unknown



Specimen:			
Total Magnification:			
Length:	units x	$\mu\text{m}/\text{unit} =$	μm
Width:	units x	$\mu\text{m}/\text{unit} =$	μm
Notes:			

Gram Stain Data & Results Summary:

Specimen	Data	Result

NOTE: If your controls do not appear as expected, **SOMETHING IS WRONG**. Consult your instructor.

2. KOH Test

Specimen	Data	Result

Conclusions:

Based on your results, what can you conclude about the cell walls of your Morph. Unknown? _____

Does the conclusion from the Gram stain match the conclusion from the KOH test? _____

Does the cell morphology of your Morphological Unknown in a Gram stain match what you saw for the indirect stain (Exercise 6A)? _____

Additional Comments: _____
