

## LAB 3 Bacterial Staining Techniques II

- I. Differential Stains: Gram Stain and Acid-fast Stain
  - II. Morphological Unknown
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### I. DIFFERENTIAL STAINS

- A. Gram Stain
- B. Acid-fast Stain

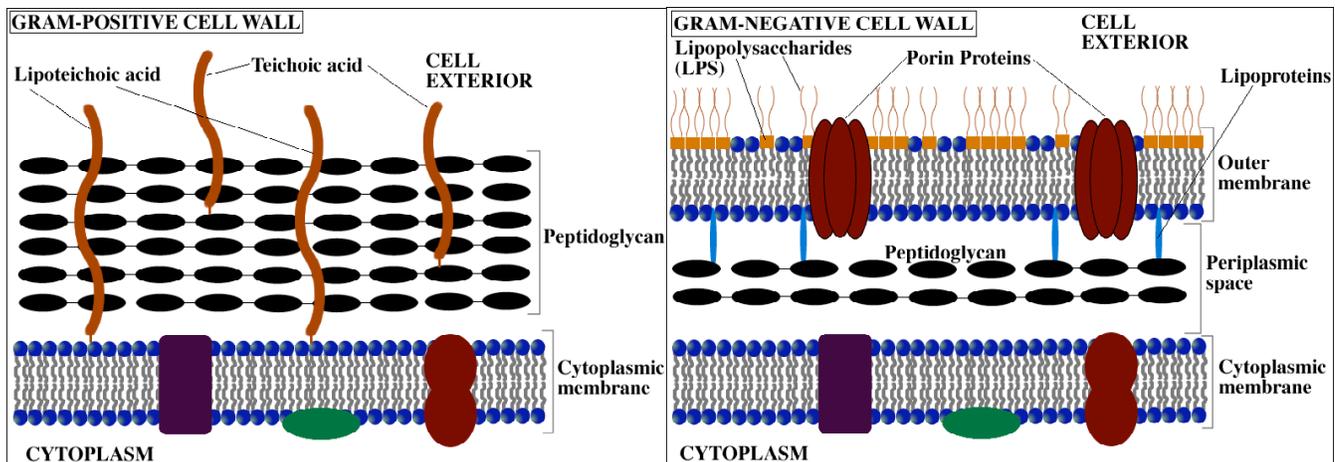
#### A. Gram Stain

The previous lab introduced simple staining techniques that enable microbiologists to observe the morphological characteristics of bacteria. Although simple stains are useful, they do not reveal details about the bacteria other than morphology and arrangement. The Gram stain is a differential stain commonly used in the microbiology laboratory that differentiates bacteria on the basis of their cell wall structure. Most bacteria can be divided into two groups based on the composition of their cell wall:

- 1) Gram-positive cell walls have a thick peptidoglycan layer beyond the plasma membrane. Characteristic polymers called teichoic and lipoteichoic acids stick out above the peptidoglycan and it is because of their negative charge that the cell wall is overall negative. These acids are also very important in the body's ability to recognize foreign bacteria. Gram-positive cell walls stain blue/purple with the Gram stain.
- 2) Gram-negative cell walls are more complex. They have a thin peptidoglycan layer and an outer membrane beyond the plasma membrane. The space between the plasma membrane and the outer membrane is called the periplasmic space. The outer leaflet of the outer membrane is composed largely of a molecule called lipopolysaccharide (LPS). LPS is an endotoxin that is important in triggering the body's immune response and contributing to the overall negative charge of the cell. Spanning the outer membrane are porin proteins that enable the passage of small molecules. Lipoproteins join the outer membrane and the thin peptidoglycan layer. Gram-negative cells will stain pink with the Gram stain.

This is **The Most Important** staining technique in Bacteriology.

#### Cell wall structure of Gram+ and Gram-



## GRAM STAIN

Procedure	Reagent	Cell Color	
		Gram Positive	Gram Negative
Fixed cells on slide		COLORLESS	COLORLESS
Primary stain	Crystal Violet	PURPLE	PURPLE
Mordant	Iodine	PURPLE	PURPLE
Decolorizer	Alcohol	PURPLE	COLORLESS
Counterstain	Safranin	PURPLE	RED

An easy way to remember the steps of the Gram stain is...

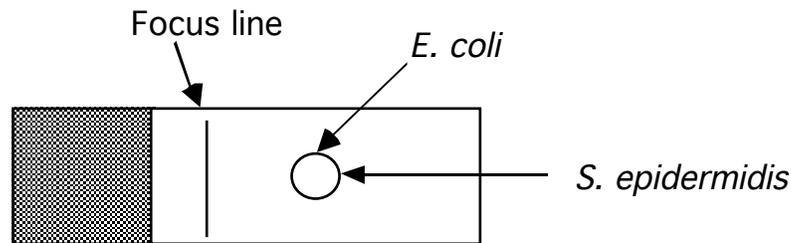
### Come In and Stain!

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### PROCEDURE: (EACH STUDENT)

1. Using a sterile inoculating loop, add 1 drop of sterile water to the slide. Prepare a mixed smear of *Escherichia coli* (G- rod) and *Staphylococcus epidermidis* (G+ coccus).
2. Air dry and Heat fix.
3. Cover the smear with Crystal Violet (primary stain) for 1 min.
4. Gently wash off the slide with water.
5. Add Gram's Iodine (mordant) for 1 min.
6. Wash with water.
7. Decolorize with 95% ethanol. This is the "**tricky**" step. Stop decolorizing with alcohol as soon as the purple color has stopped leaching off the slide (time will vary depending on thickness of smear). **Immediately** wash with water. Be sure to dispose of all ethanol waste in the appropriately labeled waste container.
8. Cover the smear with Safranin for 30 seconds.
9. Wash both the top & the bottom of the slide with water.
10. Blot the slide with bibulous paper.
11. Using the 10X objective lens, focus first on the line and then on the smear. Follow the focusing procedure in Lab #1. Use the focusing procedure in Lab #2 to view the smear using the 100X (oil immersion lens).



Note: *Escherichia coli* is a tiny pink (Gram-) rod. *Staphylococcus epidermidis* is a purple (Gram+) sphere or coccus.

Draw a picture of a typical microscopic field and identify both *Escherichia coli* and *Staphylococcus epidermidis*. Record this in the results section for this lab. Colored pencils are available throughout the room on the chalkboard trays.

## B. Acid-fast Stain

*Mycobacterium* and many *Nocardia* species are called acid-fast because during an acid-fast staining procedure they retain the primary dye carbol fuchsin despite decolorization with the powerful solvent acid-alcohol (95% ethanol with 3% HCl). Nearly all other genera of bacteria are nonacid-fast. The acid-fast genera have the waxy hydroxy-lipid called mycolic acid in their cell walls. It is assumed that mycolic acid prevents acid-alcohol from decolorizing protoplasm. The acid-fast stain is a differential stain.

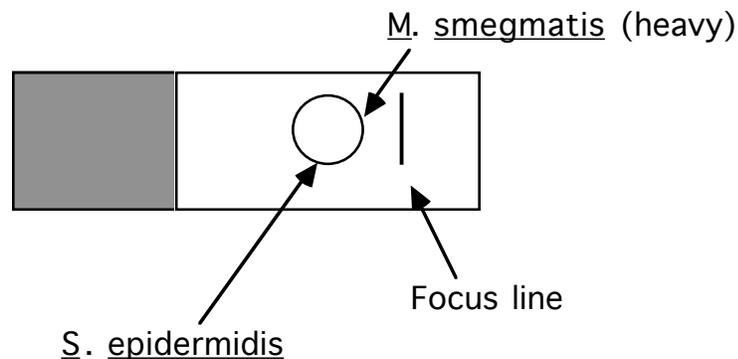
Ziehl Neelsen Acid-fast stain

### ACID-FAST STAIN

Procedure	Reagent	Cell Color	
		Acid-fast Bacteria	Nonacid-fast Bacteria
Primary dye	Carbolfuchsin	RED	RED
Decolorizer	Acid-alcohol	RED	COLORLESS
Counterstain	Methylene blue	RED	BLUE

#### PROCEDURE - (EACH STUDENT)

1. Add one loopful of sterile water to a microscope slide.
2. Make a heavy smear of *Mycobacterium smegmatis*. Mix thoroughly with your loop. Then transfer a small amount of *Staphylococcus epidermidis* to the same drop of water.  
You will now have a mixture of *M. smegmatis* and *S. epidermidis*.
3. Air dry and heat fix well.
4. Cover the smear with carbolfuchsin dye. Place a piece of paper towel on top of the dye. Be sure the paper towel is saturated with the dye. Carbolfuchsin is a potential carcinogen. Please wear gloves when working with this dye.
5. Steam for 5 min. over a steaming water bath in the hood.
6. Cool and rinse with water; then decolorize with acid-alcohol. As with the Gram-stain, rinse with acid-alcohol only until the run-off is uncolored. Be sure to dispose of all ethanol waste in the appropriately labeled waste container.
7. Wash the top and bottom of slide with water and clean the slide bottom well.
8. Counterstain with Methylene Blue for 30 seconds to 1 minute.
9. Wash and blot the slide with bibulous paper.
10. Focus 10X - then use oil immersion.



Draw a typical microscopic field and record in Results Lab 3.

Note: The acid-fast *Mycobacterium* retains carbolfuchsin and stains hot pink. The *Staphylococcus epidermidis* is decolorized and the counterstain colors them blue. See table for acid-fast stain.

**II. Morphological Unknown:** continue your investigation of your morphological unknown.

1. Collect your unknown from the side bench. Verify that the # matches that used for your direct stain.
2. Perform a Gram stain and an Acid-fast stain as described in the above sections.
3. Record your observations in the Results section of Lab #4.

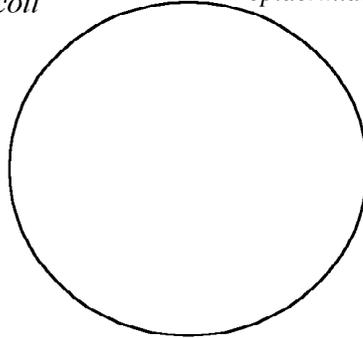
## NOTES

## LAB 3 RESULTS

### I. DIFFERENTIAL STAINS

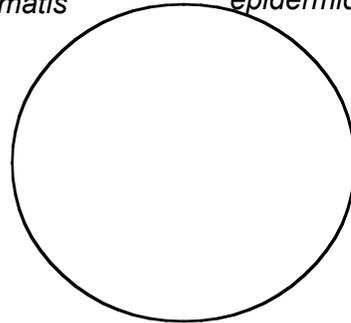
A. **Gram Stain** Draw and label examples of *Escherichia coli* and *Staphylococcus epidermidis*.

*Escherichia coli* & *Staphylococcus epidermidis*



B. **Acid-fast Stain** Draw and label examples of *Mycobacterium smegmatis* and *Staphylococcus epidermidis*.

*Mycobacterium smegmatis* & *Staphylococcus epidermidis*



### QUESTIONS:

1. What is the difference between a simple and a differential stain? \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

2. Describe the function of each of the following in the Gram stain

Mordant: \_\_\_\_\_

Primary stain: \_\_\_\_\_

Decolorizer: \_\_\_\_\_

Counterstain: \_\_\_\_\_

3. Which step in the Gram stain is most likely to cause poor results if done incorrectly? \_\_\_\_\_

\_\_\_\_\_

4. Why must fresh bacterial cultures be used in a Gram stain? \_\_\_\_\_

\_\_\_\_\_

5. Briefly describe the mechanism of Gram staining. \_\_\_\_\_

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6. What is the primary stain used in the acid-fast staining procedure? \_\_\_\_\_

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7. What is the purpose of the heat/steam during the acid-fast staining procedure? \_\_\_\_\_

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8. In a clinical microbiology laboratory, the acid-fast stain would be used for diagnosis of what diseases?

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9. What makes a microorganism nonacid-fast? \_\_\_\_\_

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Organisms introduced in this lab:

*Mycobacterium smegmatis*

*Mycobacterium tuberculosis*

*Mycobacterium leprae*

*Staphylococcus epidermidis*

## GENERAL MICROBIOLOGY 2210: GRAM STAIN REPORT (15 PTS)

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### ASSIGNMENT

Each student will have the opportunity to submit one example of a Gram stain with a brief written report describing the staining procedure and the stain itself. A good report will likely necessitate a minimum of 2 pages double-spaced and is limited to a 3-page maximum.

### OVERALL FORMAT

Please include the following in your report:

A. **Title Page** with the following information: (1 point) \_\_\_\_\_

--Title: A title should be brief, creative, but **descriptive** of the work that was done.

--Your Name

--Course and Lab Section

--Date

B. **Introduction:** (4 points) \_\_\_\_\_

An introduction should include pertinent background information. In this case, the history of the Gram stain and its significance in microbiology are relevant (1 point). A discussion of the mechanism of Gram staining and how it differentiates bacteria on the basis of their cell wall structure should also be included (1 point). The purpose and objectives of the experiment should be stated and a hypothesis should be made (2 points). Remember that a hypothesis need not be correct, but it must be tested by the procedure. Good hypotheses are also grounded in background knowledge and clearly state the predicted results. Hypotheses can be of several types.<sup>1</sup> We will discuss two types here:

1. Manipulated hypotheses

This type of hypothesis is used when one variable (the independent variable) is manipulated by the experimenter and the effects on a second variable (the dependent variable) are observed. This type of hypothesis is generally written as an if, then statement (e.g. If the smear of the unknown bacterium is decolorized as according to the above procedure (neither over- nor under-decolorized), then all the cells will stain a single color.) In this example, decolorization is the independent variable (the variable controlled by the experimenter) and the color of the cells is the dependent variable (the variable affected by the changes in the independent variable).

2. Observational hypotheses

States something about the nature of an organism (e.g. The unknown bacterium will stain Gram-positive.)

C. **Materials and Methods:** (4 points) \_\_\_\_\_

The Materials and Methods section should include a description of the procedure/steps in the Gram stain, including the preparation of a bacterial smear and heat fixation (3 points). This section must be written in complete sentences and in paragraph form (1 point). Illustrations may assist in clarifying points.

D. **Results:** (1 point) \_\_\_\_\_

In the results section, describe your Gram stain/s. **Include at least one illustration** of your own creation.

E. **Discussion:** (3 points) \_\_\_\_\_

What features of your Gram stain exemplify the Gram stain theory (1 point)? If there are features of the Gram stain that are not as expected, what possible sources or error may be responsible (e.g. poor quality of the smear preparation)? (1 point) Discuss whether the hypothesis made in the introduction was tested and if so was it supported? (1 point)

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<sup>1</sup> For a discussion of hypotheses formation please visit  
<http://www.utas.edu.au/sciencelinks/exdesign/HF2C.HTM>

**OVERALL QUALITY**

Students have the opportunity to earn points for reports that are well written, organized and edited.  
(2 points) \_\_\_\_\_

**DUE DATE: TAs will inform you of the due date.**

**\*Please tear this grading key out of your lab book and attach it to your lab report at the time of submission. Please also write your name and lab section on the frosted portion of your Gram stain slide and hand it in with your written report.**

TA comments: \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

TA's suggestions for improving Gram stain: \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**Points earned out of 15 possible: \_\_\_\_\_**