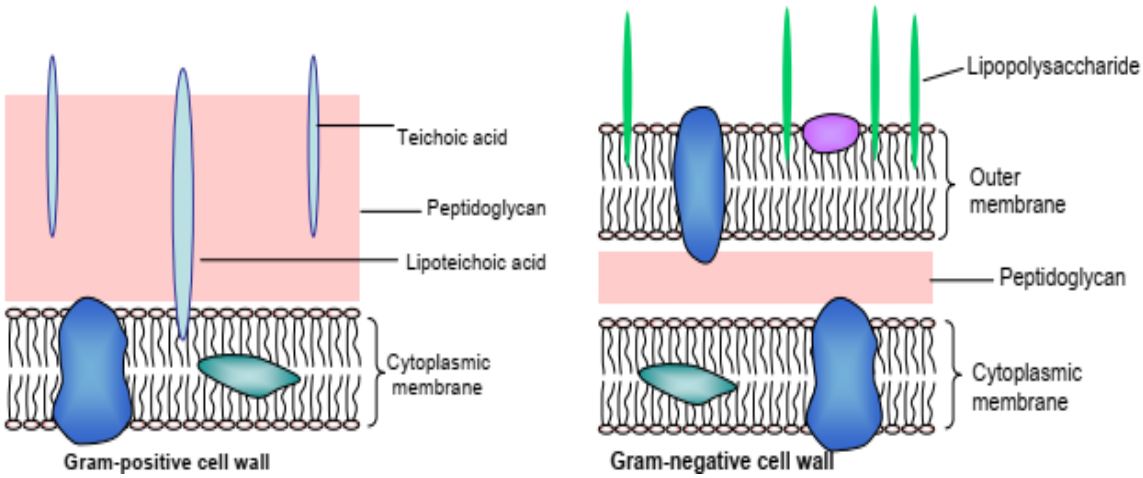
PW 02: GRAM STAIN

1. Introduction

The **Gram stain** is a differential stain which distinguishes bacteria based on cell wall properties. Bacterial cell walls are composed primarily of **peptidoglycan** and bacteria can be classified into two main groups dependent on the amount of peptidoglycan present in their cell wall. **Gram-positive** organisms have a thick layer of peptidoglycan, whereas **Gram-negative** organisms have a thin layer of peptidoglycan, plus an additional **outer membrane** that is absent in Gram-positive organisms.



In the gram staining procedure, the **primary stain** is crystal violet, and all cells take up the purple crystal violet stain. Following the primary stain, Gram's Iodine is applied to the bacterial smears. The iodine acts as a **mordant,** enhancing the ability of the stain to enter and bind to the bacteria. Specifically, the iodine binds with crystal violet and locks it into peptidoglycan of bacteria. It also intensifies the purple color. The **decolorizing agent** used in the gram staining procedure is 95% ethanol, which is a lipid solvent that melts the Gram-negative outer membrane and leads to decolorization of Gram-negative cells. It also dehydrates proteins, helping the primary stain to remain in Gram-positive cell walls. The **counter stain** then used is Safranin, which stains the decolorized Gram-negative cells pink. Thus, at the end of the staining procedure, Gram-positive cells are purple and Gram-negative cells are pink.

Note: It is preferable to use fresh cultures for the Gram stain. Old cultures may stain "Gram-variable" (a mix of purple and pink) because they decolorize easily.

2. Cultures needed

Nutrient broth tubes or plates of the following bacteria: *Escherichia coli, Staphylococcus aureus, Bacillus sp. and Pseudomonas aeruginosa.*

3. Procedure

1. Prepare a bacterial smear with a mixture of all 4 organisms (i.e. 1-2 loopfuls of each) listed above and **heat fix**;

2. Place the slide on a staining tray, and cover the smear with crystal violet. Allow to stain for 60 seconds;

3. Tilt the slide and gently rinse with distilled water until the stain is removed;

4. Cover the smear with Gram's Iodine, and allow to sit for 60 seconds;

5. Tilt the slide and gently rinse with distilled water;

6. **IMPORTANT STEP**: Tilt the slide and let 2-3 drops of Decolorizer run over the slide. If the last drop is still purple, continue decolorizing, 2-3 drops at a time, until the decolorizer runs clear. Rinse with distilled water;

7. Cover the smear with Safranin, and stain for 45 seconds;

8. Tilt the slide and rinse with distilled water;

9. Place the slide in a book of Bibulous paper and blot to dry. You do not need a cover slip! Observe the slide under oil immersion, and draw what you see in the results section;

10. Clean your microscope with lens cleaner, paying extra attention to the 40X and 100X objectives. Have your instructor check your microscope to make sure it is clean.

4. Results

Using colored pencils, draw and label *Escherichia coli, Staphylococcus aureus, Bacillus sp.* and *Pseudomonas aeruginosa.*

5. Study questions

1. Describe several advantages of differential staining procedures compared with simple staining techniques;

2. Give the purpose of each of the following reagents in a differential staining procedure: a. Primary stain; b. Counter stain; c. Decolorizing agent; and d. Mordant;

3. Why is it important for the counter stain to be a lighter color than the primary stain?