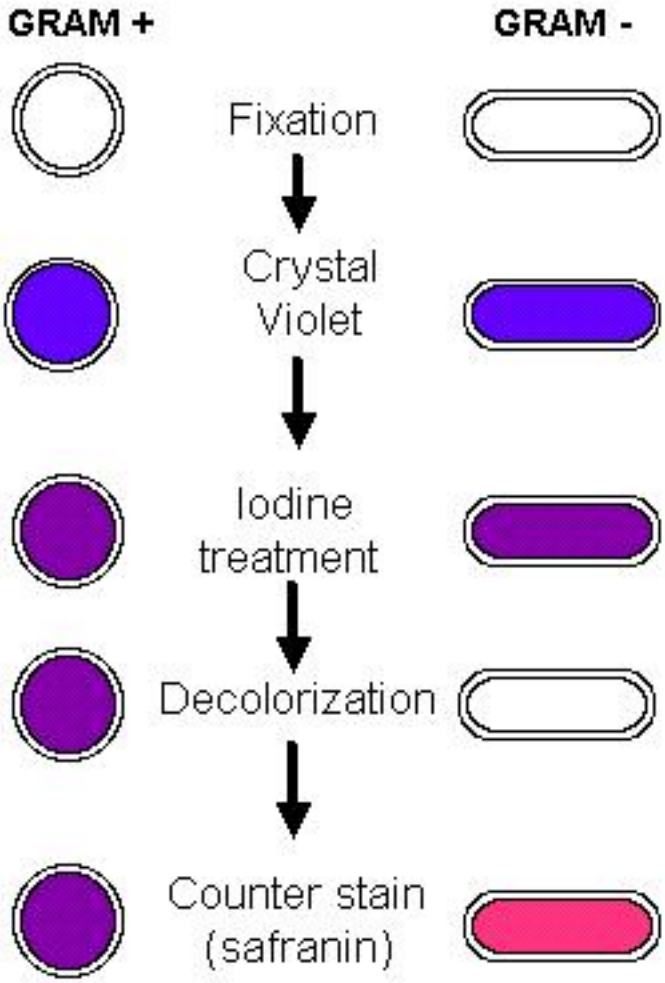
Overview

The Gram stain procedure was originally developed by the Danish physician Hans Christian Gram to differentiate pneumococci from Klebsiella pneumonia. In brief, the procedure involves the application of a solution of iodine (potassium iodide) to cells previously stained with crystal violet or gentian violet. This procedure produces "purple colored iodine-dye complexes" in the cytoplasm of bacteria. The cells that are previously stained with crystal violet and iodine are next treated with a decolorizing agent such as 95% ethanol or a mixture of acetone and alcohol. The difference between Gram-positive and Gram-negative bacteria is in the permeability of the cell wall to these "purple colored iodine-dye complexes" when treated with the decolorizing solvent. While Gram-positive bacteria retain purple iodine-dye complexes after the treatment with the decolorizing agent, Gram-negative bacteria do not retain complexes when decolorized. To visualize decolorized Gram-negative bacteria, a red counter stain such as safranin is used after decolorization treatment

Appearance of the Gram positive coccus and Gram negative bacillus at different stages of the gram staining procedure are illustrated below:



Preparation of the smear

The first consideration is the correct preparation of the smear. Make a **thin** film of the material on a clean glass slide, using a sterile loop or swab for viscous specimens. **Air dry**, then **heat fix** the slide by passing it several times through a flame (**the slide should not become too hot to touch**). Failure to follow these directions may cause staining artifacts and disrupt the normal morphology of bacteria and cells.

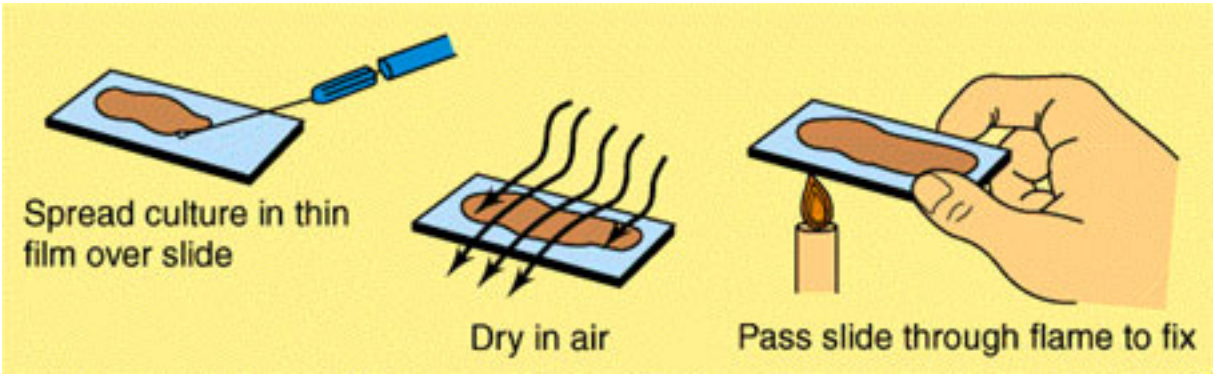
To be visible on a slide, organisms that stain by the Gram method must be present in concentrations of a minimum of 10^4 to 10^5 organisms/ml of unconcentrated staining fluid. At lower concentrations, the Gram stain of a clinical specimen seldom reveals organisms even if the culture is positive. **Smears that are not properly fixed tend to be washed away during staining and washing resulting in the absence of stained bacteria**.

In special situations, the following guidelines may be helpful:

When cerebrospinal fluid contains only a few organisms, they are more likely to be found if a concentrated "thick smear" is examined. To prepare a "thick smear" the specimen is spun at high speed and a large drop of sediment (or multiple drops, drying in between each drop) is placed in the center of the slide and allowed to air dry. The cytocentrifuge may prove to be useful in concentrating bacteria as well as in preserving cell morphology.

When fluid specimens such as urine or CSF seem to vanish after the staining procedure, a wax mark, placed near the smeared area on the slide (same side) after the staining procedure (to avoid introducing wax artifacts) will reduce frustration in locating the specimen under the microscope. The wax mark can be used for quick focussing.

In a grossly bloody specimen, it may prove difficult to distinguish microorganisms from artifacts. After air-drying and heat-fixing this type of specimen, the added preparatory step of covering it with distilled water, waiting five minutes, and then rinsing, may cause the red blood cells to lyse and float off.

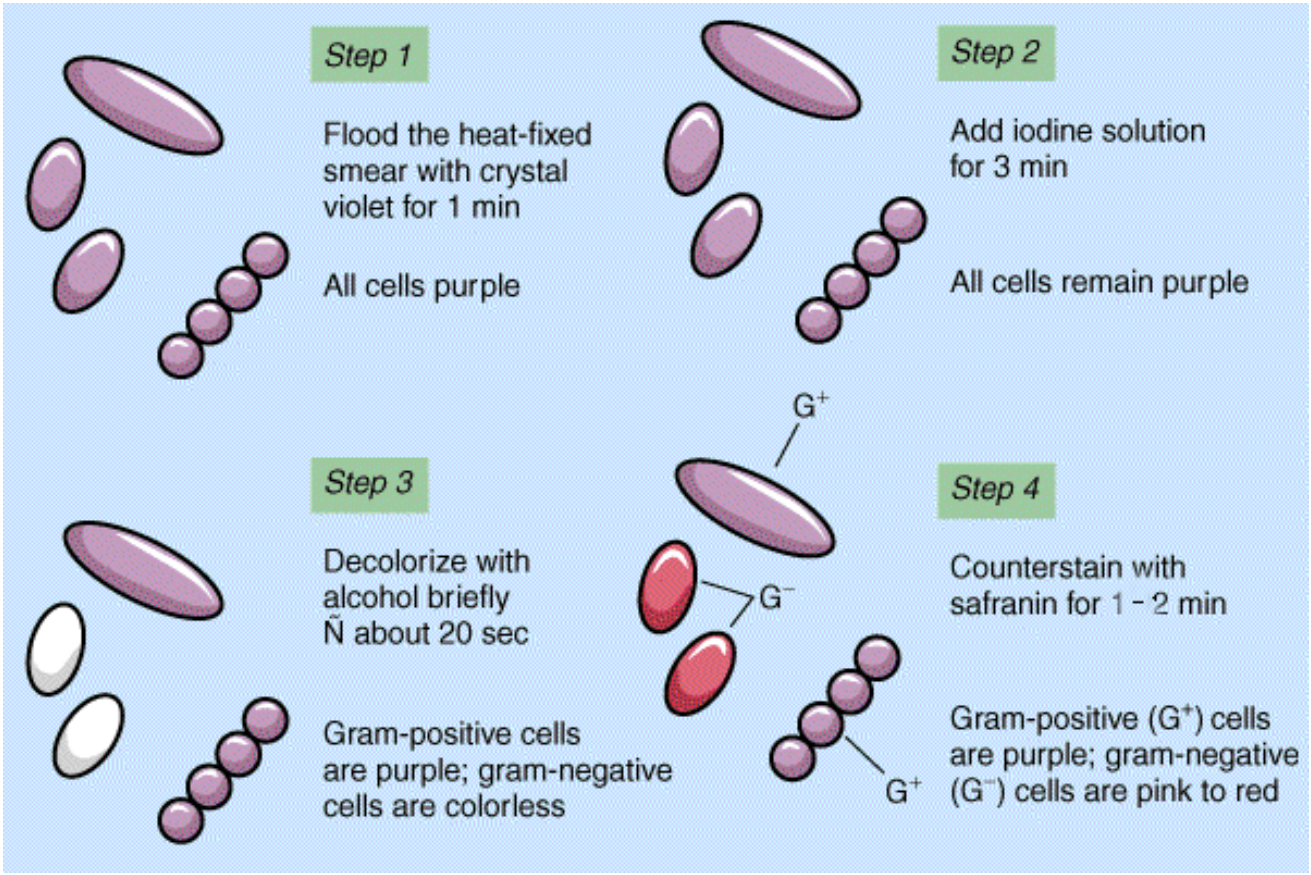


Staining procedure

1. Flood slide with **crystal** (or **gentian) violet**- 60 seconds.
2. Flood with **Gram's iodine** - 180 seconds.
3. Carefully decolorize with **95% ethanol** until thinnest parts of the smear are colorless. (Wash with water).

   This third step is the most critical and also the one most affected by technical variations in timing and reagents.

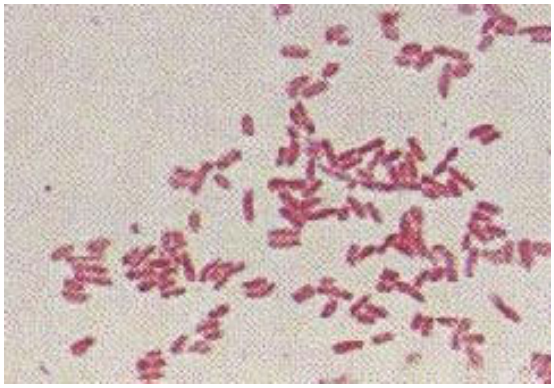
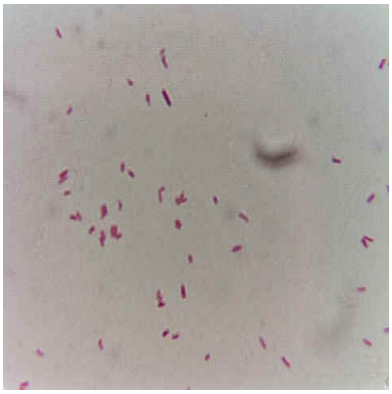
4. Flood with **safranin** (pink color) (10% Fuchsine) - 60 seconds. (Wash with water).
5. Air dry, or blot with absorbent paper.



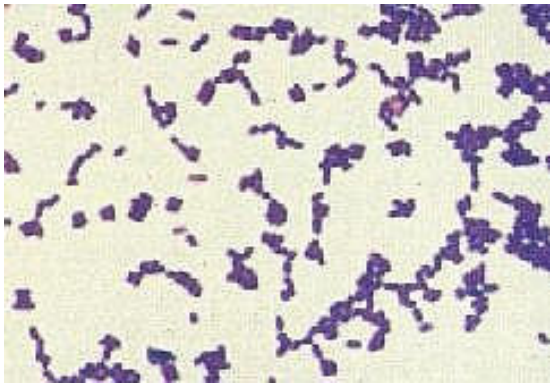
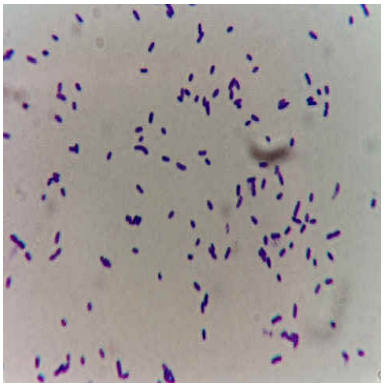
Results

As shown below, organisms that retain the violet-iodine complexes after washing in ethanol stain purple and are termed **Gram-positive**, those that lose this complex stain red from the safranin counter stain are termed **Gram-negative**.

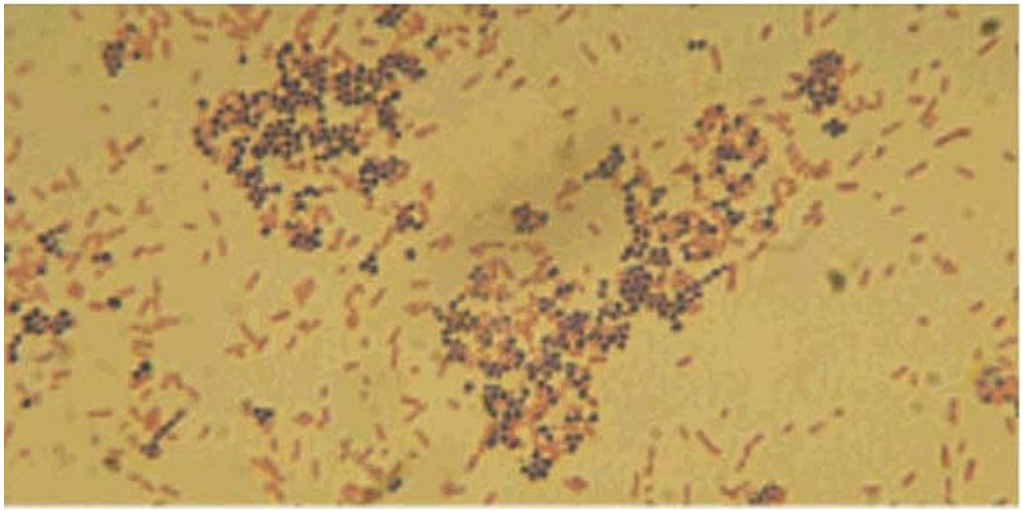
Typical Gram stain

negative

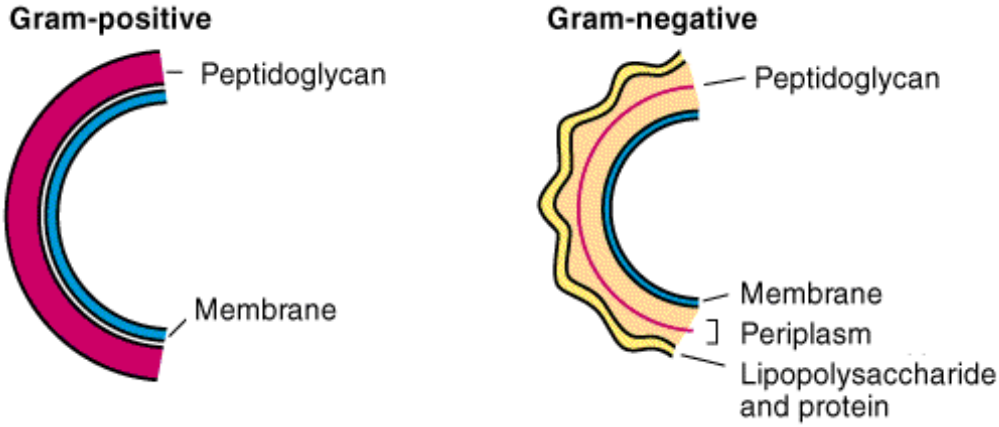
positive



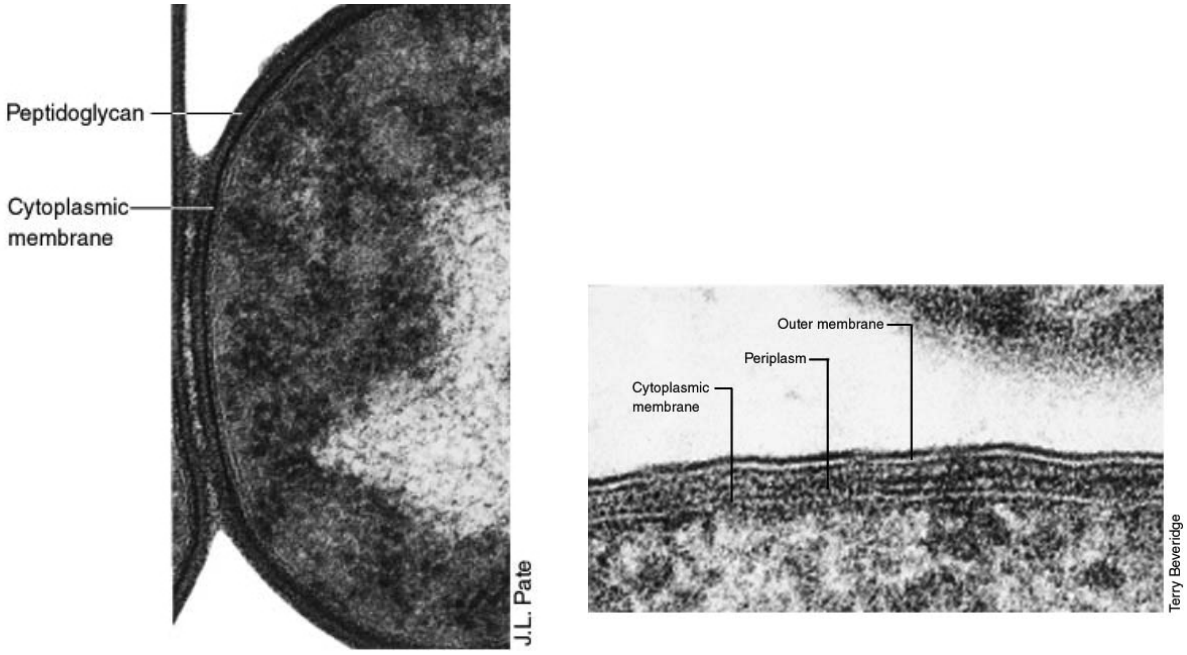
Mix

Relationship of Cell Wall Structure to the Gram Stain

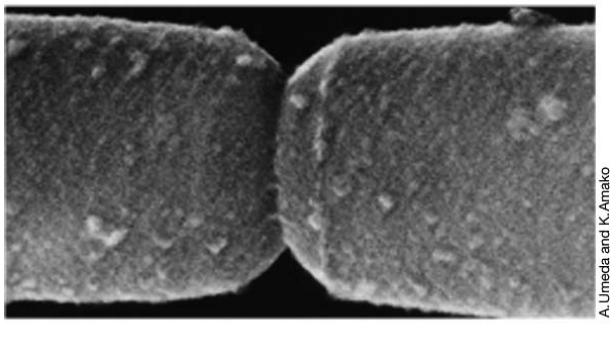
Are the structural differences between the cell walls of gram-positive and gram-negative Bacteria responsible in any way for the Gram stain reaction?
In the Gram stain, an insoluble crystal violet-iodine complex is formed inside the cell, and this complex is extracted by alcohol from *gram-negative b*ut not from gram-positive Bacteria. The alcohol dehydrates Gram positive Bacteria, which have very thick cell walls consisting of several layers of peptidoglycan. This causes the pores in the walls to close, preventing the insoluble crystal violet-iodine complex from escaping. In gram-negative Bacteria, alcohol readily penetrates the lipid-rich outer layer, and the thin peptidoglycan layer also does not prevent solvent passage, thus, the crystal violet-iodine complex is easily removed.

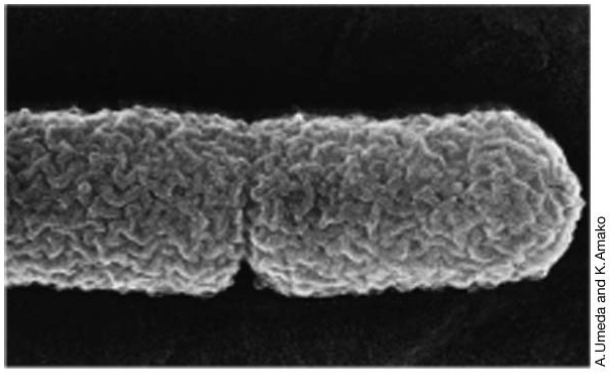


Schematic diagrams of gram-positive and gram-negative cell walls.

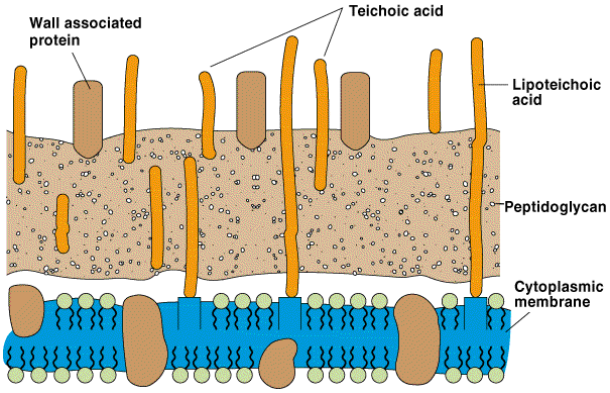


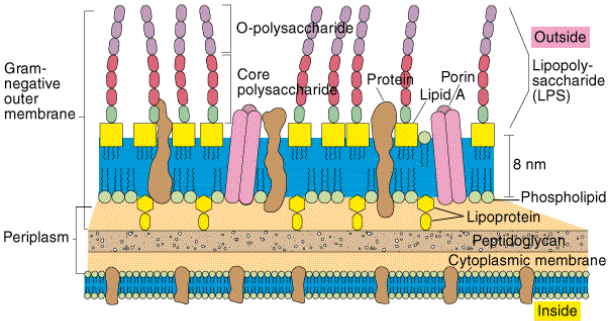
Electron micrograph showing the cell walls.



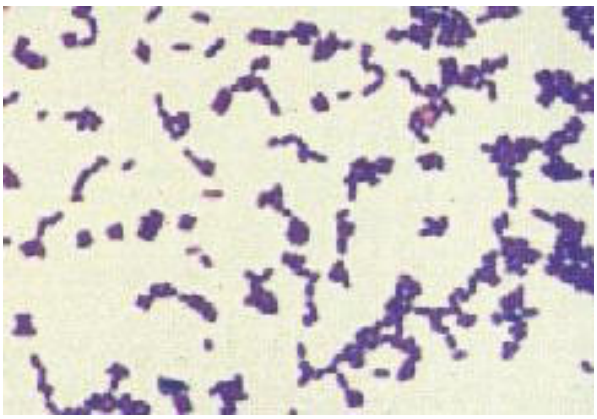


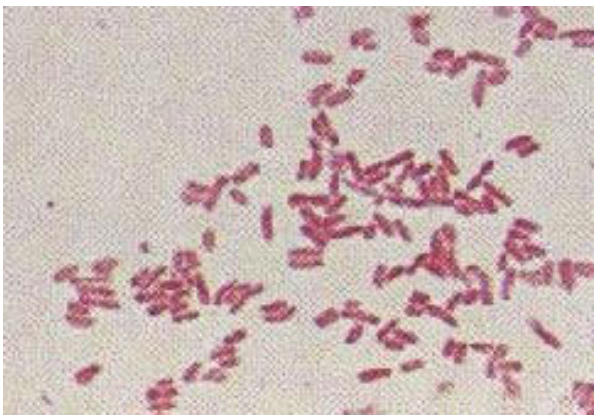
Scanning electron micrographs of gram-positive (Bacillus subtilis) and gram-negative (Escherichia coli) bacteria. Note the surface texture in the cells shown.





Gram-positive cell wall Gram-negative cell wall.





Result of the Gram stain method