Science Education Collection

Gram Staining of Bacteria from Environmental Sources

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Overview

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The spectrum of research in environmental microbiology is broad in scope and application potential. Whether the work is bench-scale with known bacterial isolates, or in the field collecting soil or water samples containing unknown bacterial isolates, the ability to quickly and visually discern culturable populations of interest remains of great import to environmental microbiologists even today with the abundance of molecular techniques available for use. This video will demonstrate one such technique, known as Gram staining.

Principles

The Gram stain is a classical and important staining technique that remains widely used by environmental microbiologists. Similar to a simple stain, it allows for assessment of bacterial cell morphology (*e.g.*, cocci, rods, spore-formers), size, and arrangement (*e.g.*, chains or clusters). In addition, it allows for differentiation of bacteria into two principle distinct groups — Gram-negative and Gram-positive — according to cell wall composition and structure (**Figure 1**).

Gram staining is a multi-step process. Prior to staining, a bacterial smear is prepared using a plate, slant, or broth culture. The smear prep is dried and fixed onto a clean glass slide. A primary stain of crystal violet is then applied to the fixed smear. Crystal violet is a basic stain comprised of positively charged colored ions (*i.e.* chromophores) that form weak ionic bonds with negatively charged functional groups present in the bacterial cell wall. After gently rinsing the slide with water, Gram's iodine is applied, and forms insoluble complexes with the crystal violet in the cell wall. The crystal violet-iodine complexes further bind with peptidoglycan, a principle component of bacterial cell walls. Following a second water rinse, a decolorizing agent is briefly applied to the smear. For Gram-negative bacteria, the crystal violet-iodine complex is washed away during the decolorizing step, with Gram-positive bacteria retaining the purple stain. A third and final water rinse is followed by a counterstain of safranin that colorizes Gram-negative bacteria pink or red.

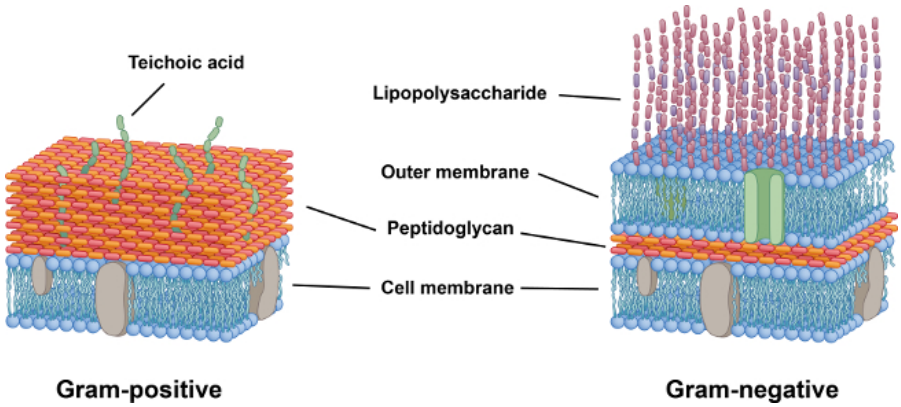

Figure 1. Comparison of the cell wall of Gram-positive and Gram-negative bacteria.

Procedure

1. Sample Collection

1. Collect soil sample and transport to the laboratory for microbial analysis.
2. In the lab, weigh a 10 g sample using an analytical balance.
3. Dilute the sample 1:10 into 95 mL of phosphate-buffered saline (10 parts soil is equivalent to 5 parts aqueous liquid), and vortex to mix (**Figure 2**, Step 1).
4. Perform subsequent 1:10 dilutions up to at least 10^{-5} g soil per mL, and spread-plate selected dilutions in replicates of two or three onto a low nutrient agar medium (*e.g.*, R2A) (**Figure 2**, Steps 2-3).
5. Incubate the plates for one week at room temperature (**Figure 2**, Step 4).
6. Select one or two colonies for isolation, and streak onto fresh agar plates (**Figure 3**, Steps 1-3).
7. Incubate the streak plates for two to three days at room temperature (**Figure 3**, Step 4).

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2. Preparation of Bacterial Smears

1. Observe the streak plates for isolated colonies.
2. To prepare each smear prep, dip an inoculating loop into ethanol, flame-sterilize, and place 1 to 2 loopfuls of sterile distilled water onto the center of pre-cleaned glass slides.
3. Sterilize the inoculating loop again as previously described. Once cooled, remove a small amount of culture from a single isolated colony and mix it with the water droplets on the slide (the smear should resemble diluted skim milk). The inoculating loop must be cooled prior to colony isolation. A loop that is too hot will cause the colony and/or medium to splatter, which may lead to aerosolization of bacteria. Generally, when the loop is too hot for use, a “hissing” sound will be heard when applied to the agar or colony. Improper cooling of the loop may also result in less efficient culture-to-slide transfer, and distortion of cell morphology.
4. Spread the smear over the surface of the slide measuring approximately 2.5 cm x 2.5 cm, and allow it to air dry. It is important for air drying to occur under laminar flow conditions. Slides should not be blown dry so as not to disrupt the smear. Also, slides must not be flame-dried, in order to maintain cell morphology.
5. After drying, heat fix the smear by passing the slide quickly through a flame 2-3x. The slide should not be held stationary in the flame, to prevent distortion of cell morphology and/or damage to the glass slide.

3. Gram Staining

1. Secure the slide at one end using a clean clothespin.
2. Cover the smear with crystal violet (primary stain) and hold for 2 to 3 min.
3. Carefully wash the slide with distilled water. The water stream should not be directed at the smear in order to prevent damage and/or detachment from the glass slide.
4. Cover the smear with Gram’s iodine and hold for 2 min, then gently rinse the slide with water.
5. Decolorize the smear using 95% ethanol until stain no longer washes from the slide (this usually takes no more than 20 s depending on the thickness of the smear), then immediately rinse with distilled water. This step is critical to avoid over decolorizing the slide, which may lead to a false Gram stain designation (*i.e.,* Gram-variable).
6. Add the counterstain (safranin) to the smear and hold for 30 s. Then gently rinse the slide with distilled water and blot dry using absorbent paper.

4. Microscopic Observation of Slides

1. Observe the slides using low (*e.g.*, 4X or 10X), high-dry (*e.g.*, 40X), and oil immersion (100X) objectives. For oil immersion, add the oil directly to the smear.
2. For representative results of Gram-positive and Gram-negative soil bacteria, see **Figures 4** and **5**.

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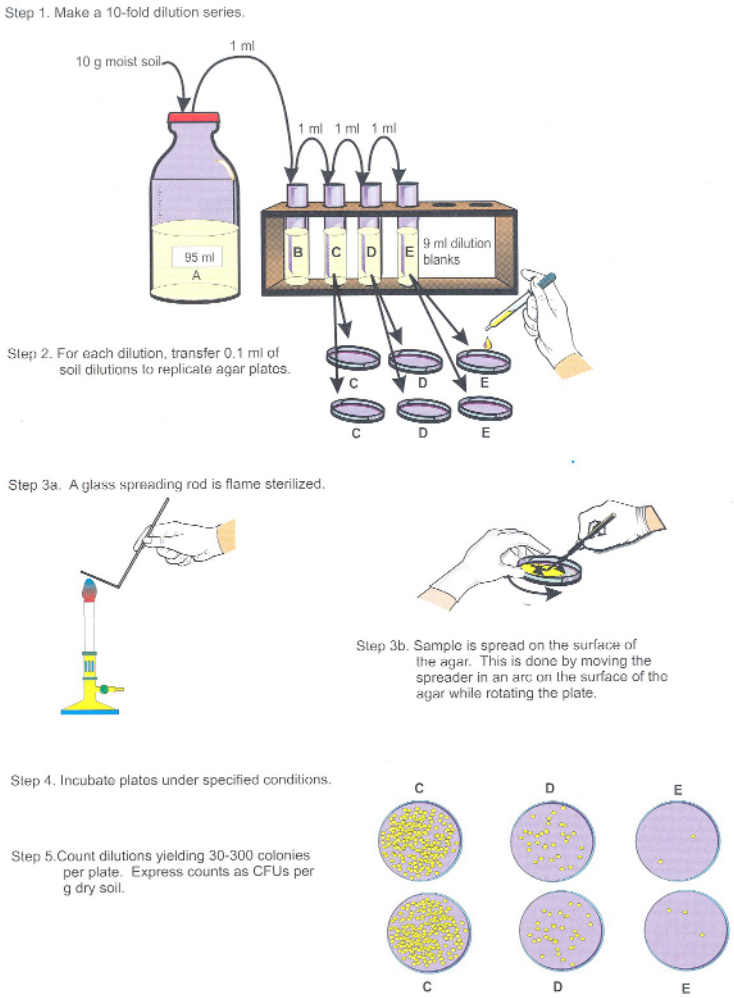

Figure 2. Dilution and Spread-Plating Technique. Please click here to view a larger version of this figure.

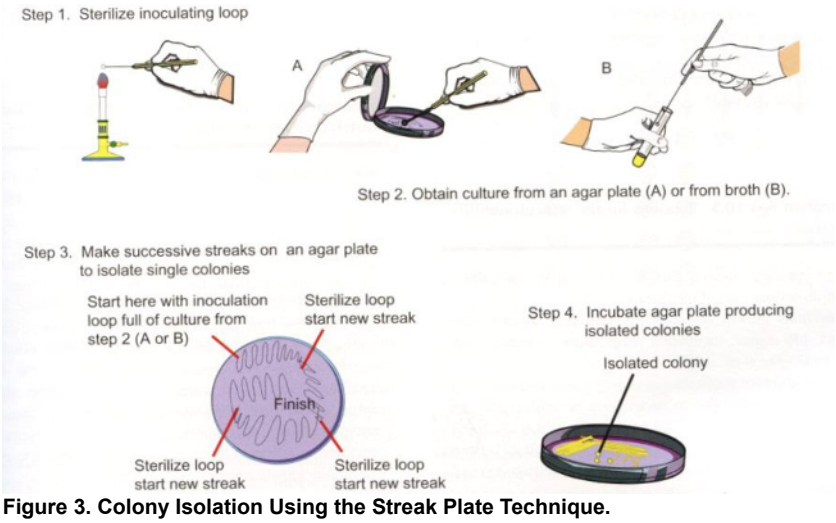

Figure 3. Colony Isolation Using the Streak Plate Technique.

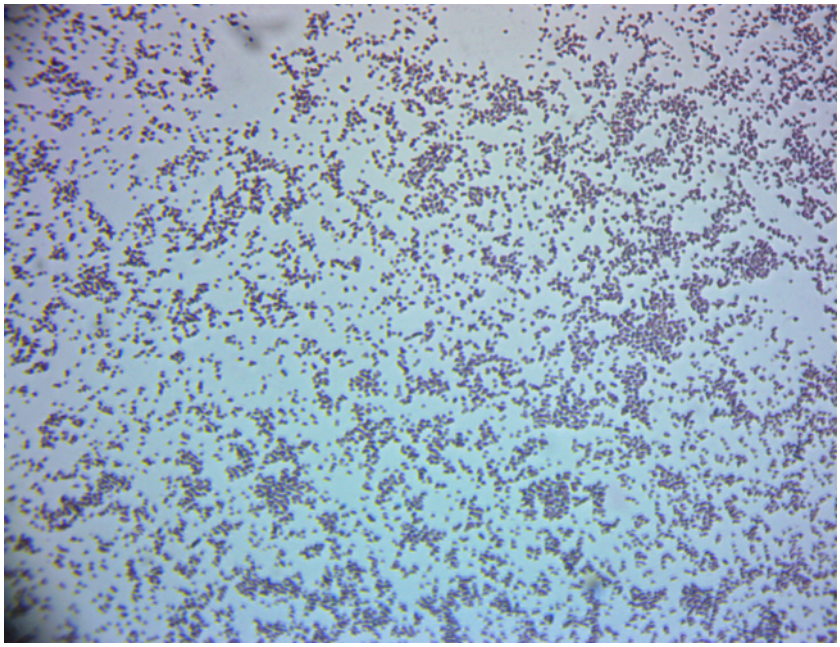

Figure 4. Gram-positive soil bacterium *Staphylococcus aureus*.

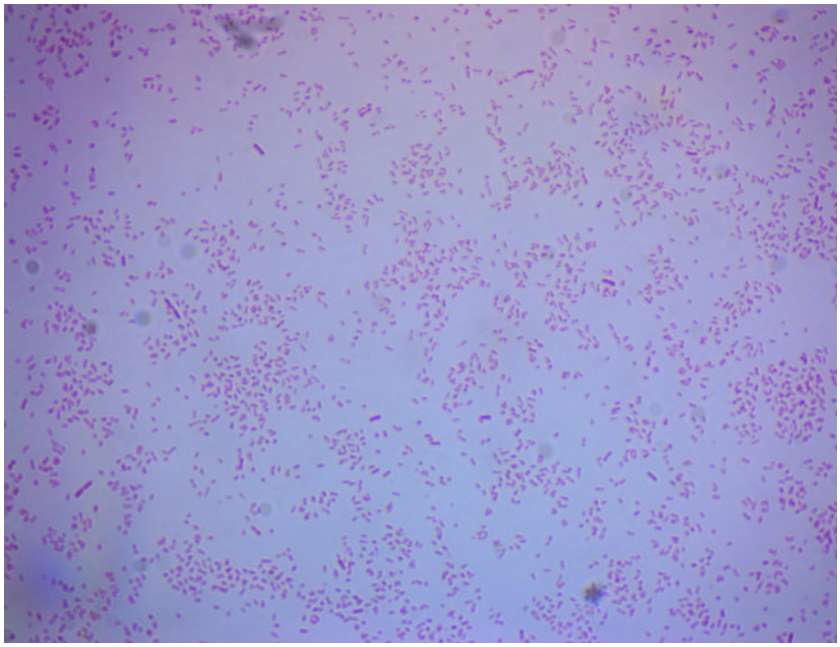

Figure 5. Gram-negative soil bacterium *Escherichia coli*.

Applications and Summary

The Gram stain is used in the many sub-fields of both environmental and clinical microbiology. Water quality scientists may use the Gram stain as a confirmatory tool for the detection of fecal bacteria in water samples. Bacterial isolates from soils are Gram stained in order to further characterize culturable soil communities. For environmental microbiologists, Gram stain aids in the categorization of bacterial populations according to cell wall structure. This, in turn, provides information about the general ability of a given microbial community to withstand desiccation and other environmental stressors. Knowledge of Gram stain designation is also of importance in the research and development of disinfectants and other antimicrobials, as Gram-positive bacteria tend to be more resistant to inactivation by particular chemistries than Gram-negative bacteria.

For clinical microbiology applications, the Gram stain is used to confirm the identity of bacteriological disease agents along with traditional diagnostic methods. It is also of great assistance when culturing has failed, or is not an option. Gram staining of clinical specimens can reveal the presence of etiologic agents that may not have been observed otherwise.

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