

Bacterial Structures: Spore Stain Technique

Materials and Equipment

- Cultures of *Bacillus* and/or *Clostridium* species
- Steaming apparatus
- Forceps or clothespin
- 5% malachite green
- Safranin
- Newspaper or paper towels to cover laboratory desk
- Blotting paper
- Brightfield microscope



Watch This Video

- Preparing an Endospore Stain

Endospores are formed by members of a few gram-positive bacterial genera, such as *Bacillus* and *Clostridium*. The spores are extremely resistant structures; resistant to boiling water temperatures for two hours or more. They contain little water and exhibit very few chemical reactions. When the external environment is favorable, the spore's protective layers break down and the vegetative cell emerges to grow and reproduce. Among the notable diseases caused by different species of endospore-forming bacteria are tetanus, botulism, gas gangrene, and anthrax (**FIGURE 16.1**).

In this exercise, bacterial endospores will be visualized by special staining techniques.

SPORE STAIN TECHNIQUE

PURPOSE: to view and contrast vegetative cells from endospores with the microscope.

Identifying endospore-forming pathogens is important in food and medical microbiology. However, the spores contain numerous protective layers, which cannot be penetrated easily by stain using the simple or Gram stain techniques. It therefore is necessary to

apply heat to assist stain penetration. The heat will force the stain into the spores and vegetative cells, and both will become green. By rinsing the slide, the vegetative cells will lose their color but the spores will remain green. Counterstaining with safranin will stain the vegetative cells pink, but have no effect on the spores.



FIGURE 16.1 Colonies of *Bacillus anthracis* growing on blood agar.

Courtesy of CDC/ Megan Mathias and J. Todd Parker.

PROCEDURE

I. The Schaeffer-Fulton Method

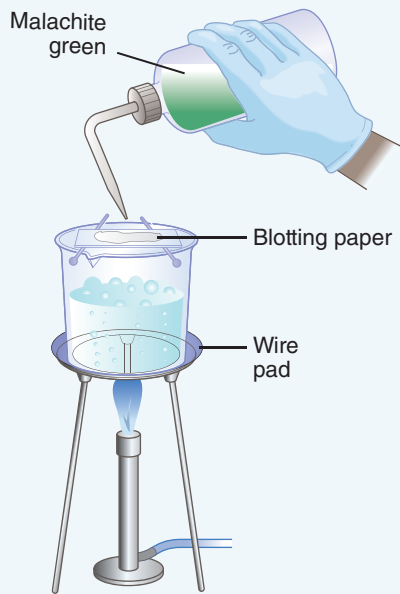
1. Prepare air-dried, heat-fixed smears of *Bacillus* and/or *Clostridium* species as outlined in Exercise 12. The instructor will indicate which organisms are to be used. Normally, a smear will contain both spores and vegetative cells.
2. While the smear is air drying, set up the staining apparatus as follows:
 - Since dripping can occur, newspaper or paper towels should be used to cover the laboratory hood for this procedure.
 - Set up a steaming apparatus consisting of a Bunsen burner, tripod, wire pad, beaker of water, and slide rack of glass rods, as illustrated in **FIGURE 16.2A**.
3. Light the flame to begin heating the water.
4. Cut a piece of blotting (bibulous) paper just large enough to cover the smear. (Do not use lens paper for this purpose.)
 - When the water in the beaker has begun boiling, balance the slide over the steam, and cover the smear with the blotting paper. Do not allow the paper to hang over the edge of the slide since this will cause stain to drip.
5. Saturate the blotting paper with **malachite green** stain (**FIGURE 16.2A**).
 - Allow the slide to remain over the steam for 3 minutes, continually adding stain during this period to keep the paper wet.
6. After 3 minutes, use forceps or a clothespin to carefully remove the slide from the steam bath.
 - Gently peel off the paper and wash the slide thoroughly with a gentle stream of water (**FIGURE 16.2B**).
 - It is not necessary to blot the smear.
7. Flood the smears with **safranin** for 1 minute (**FIGURE 16.2C** and **FIGURE 16.3**).
 - Rinse the slide with water (**FIGURE 16.2D**) and blot it dry (**FIGURE 16.2E**).
8. Using the microscope, examine the smears under the low-power (10×) lens, then high power (40×) and oil immersion (100×).
 - Scan the slide and note the oval spores stained green and the long vegetative cells stained red-orange. Look carefully to determine whether any spores are still within vegetative cells, and note the spores' position (central, subterminal, or terminal). Young cultures often contain spores within the vegetative cells, while older cultures contain more free spores and fewer vegetative cells.
 - Draw representative spores and vegetative cells in the Results section.



The fumes from malachite green are toxic. Always do the staining in the fume hood.



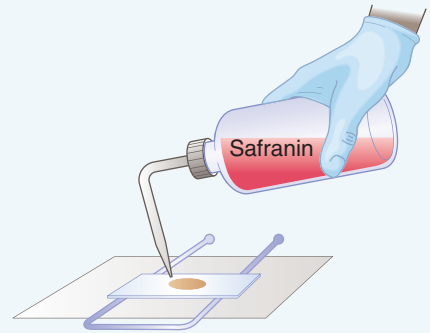
The slide will become rather hot when placed over the boiling water. Be sure to use the forceps (or clothespin) to handle the slide.



A In the lab hood, apply malachite green to saturate the paper; steam for 3 minutes. Do not let the malachite green stain dry, so add additional stain if it evaporates.



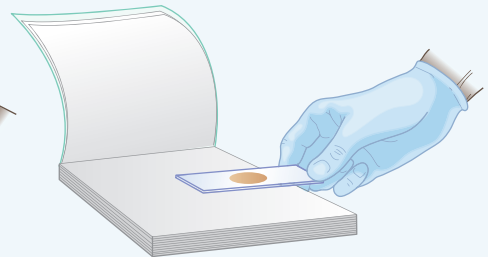
B Remove the paper with forceps and dispose of the paper in the biohazard container. Allow the slide to cool and then rinse it with water to remove the excess malachite green.



C Flood the smear with safranin for 1 minute.



D Rinse the smear with water to remove the excess safranin.



E Blot the smear dry with bibulous paper.

FIGURE 16.2 The spore stain technique.

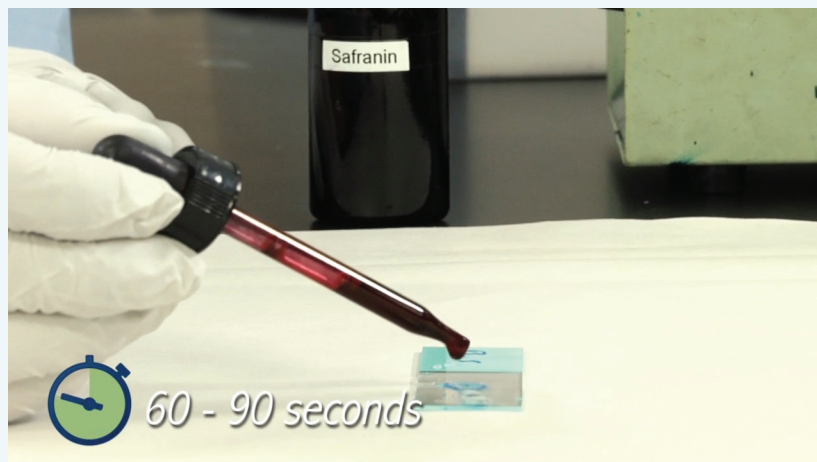


FIGURE 16.3 Counterstain with safranin for approximately 1 minute.

- If the slide is to be retained, label the slide with your name, the name of the organisms, the date, and “spore stain.” If it will not be retained, clean the slide well with soap and water, and then dry the slide.

II. An Alternative Technique

1. Spore staining without the use of steam may be performed as follows (watch **Microbiology Video: Preparing an Endospore Stain** to familiarize yourself with the technique):



Preparing an Endospore Stain

2. Prepare air-dried bacterial smears as usual but heat-fix the slides by passing them through the Bunsen flame about 20 times.
3. Cool the slides briefly in air.
4. Stain the slides by covering the smears with 7.5% malachite green, and allow the stain to remain for 10 minutes. This is a more concentrated stain than that used in the steaming method (if used with steam, the concentrated malachite green would precipitate rapidly and staining could not take place).
5. Wash the stain off the slide, and flood the smears with safranin for 1 minute as in step 7, above.
6. Continue with step 8 to complete the procedure.

Quick Procedure

Spore Stain

1. Stain with malachite green over boiling water for 3 min.
2. Rinse with water.
3. Stain with safranin for 1 min; rinse; dry; observe.

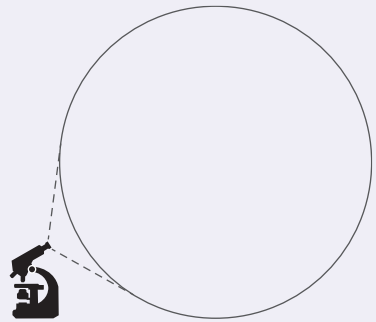
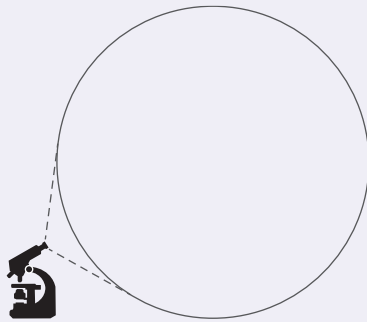
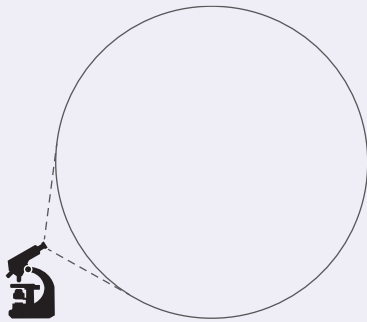
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EXERCISE
RESULTS

16

RESULTS

SPORE STAIN TECHNIQUE



Organism: _____

Total magnification: _____

Spore size: _____

Observations and Conclusions

Questions

1. Why is heat necessary for the successful performance of the spore stain technique using the Schaeffer-Fulton Method?

2. Explain how the extremely high resistance of bacterial endospores has an influence on sterilization practices, food microbiology, and disease processes.
