7 Bacterial Structures

Bacteria are complex organisms with intricate structural details. Among the important bacterial structures are the endospore, capsule, and flagellum.

Endospores are formed by members of several 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 sporeformers are tetanus, botulism, gas gangrene, and anthrax.

The **capsule** is a layer of polysaccharides and proteins secreted by certain bacteria, including many pathogens. The layer adheres to the cell surface and serves as a buffer between the cell and its external environment. The capsule protects the bacterium against dehydration and traps nutrients from the surrounding environment. It contributes to the establishment of disease by lending resistance to phagocytosis. Various species of bacilli and cocci form capsules. When thin and flowing, the layer is called a **slime layer**. The term **glycocalyx** refers to both capsule and slime layer.

Flagella are protein appendages that facilitate motion (motility) of bacteria. Many species of bacterial rods and spirilla, and a few species of cocci possess flagella. Although flagella are often many times the length of the cell, they normally cannot be seen with the light microscope because they are extremely thin.

In this exercise, bacterial endospores and capsules will be visualized by special staining techniques, and the presence of flagella will be inferred by observing evidence of bacterial motion.

A. Spore Stain Technique

PURPOSE: to contrast vegetative cells from endospores.

Bacterial endospores 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 normal bacterial cells, or vegetative cells, are initially stained with the same stain as the spores, but they then are decolorized and stained with a different stain for contrast.

Special Materials

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

Procedure

I. The Standard Technique

1. Since dripping can occur, it is recommended that newspaper or paper towels covering the laboratory bench be used 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 7.1**A. Light the flame to begin heating the water while the slide is being prepared.
2. Prepare air-dried, heat-fixed smears of *Bacillus* and/or *Clostridium* species as outlined in Exercise 4A. The instructor will indicate which organisms are to be used. Normally, a smear will contain both spores and vegetative cells. A hay infusion may also be used as a source of spores. Such an infusion consists of grass clippings placed in a flask of water and incubated for two or three days.

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

3. Cut a piece of blotting (bibulous) paper just large enough to cover the smears. (Do not use lens tissue for this purpose.) When the water in the beaker has begun boiling, balance the slide over the steam, and cover the smears with the blotting paper. Do not allow the paper to hang over the edge of the slide since this will cause stain to drip.
4. Saturate the blotting paper with **malachite green** stain (Figure 7.1A). Allow the slide to remain over the steam for 3 minutes, continually adding stain during this period to keep the paper wet. The heat will force the stain into the spores and vegetative cells, and both will become green.
5. After 3 minutes, use forceps or a clothespin to remove the slide from the steam bath. Gently peel off the paper and wash the slide thoroughly with a gentle stream of water (Figure 7.1B). The vegetative cells will lose their color during this washing, but the spores will remain green. It is not necessary to blot the smear.

Quick Procedure
Spore Stain

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

6. Flood the smears with the red stain **safranin** for 1 minute (Figure 7.1C). This dye will stain the vegetative cells but have no effect on the spores. Wash the slide with water (Figure 7.1D) and blot it dry (Figure 7.1E).
7. Examine the smears under the low power lens, then high power and oil immersion. 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 smear of the hay infusion may also be examined for the presence of green, oval spores. If the slide is to be retained, label the slide with your name, the name of the organisms, the date, and "spore stain."

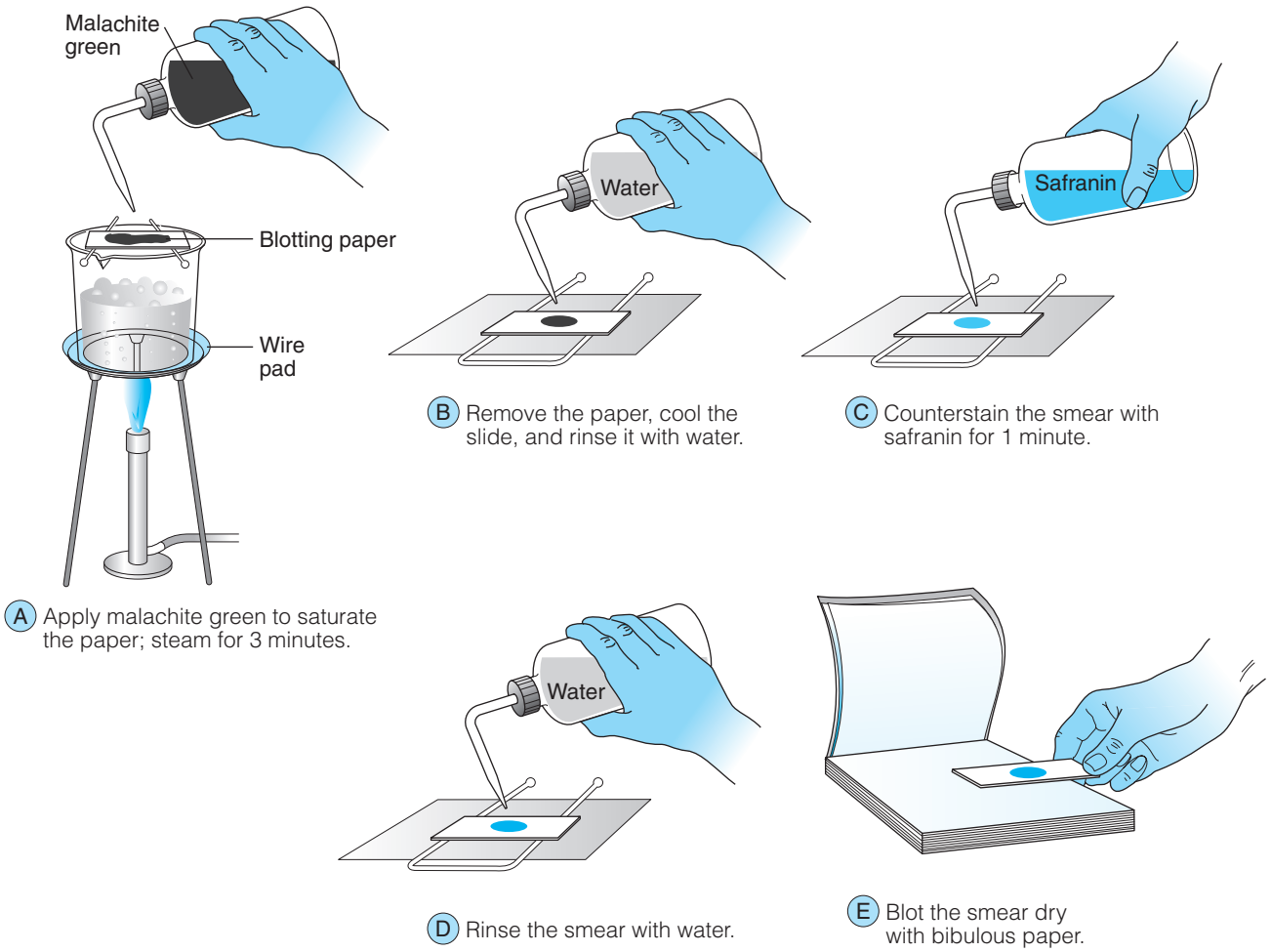


FIGURE 7.1
The spore stain technique.

II. An Alternative Technique

1. Spore staining without the use of steam may be performed as follows: Prepare air-dried bacterial smears as usual but heat-fix the slides by passing them through the Bunsen flame about 20 times. Then cool the slides briefly in air. 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). Wash the stain off the slide, and flood the smears with safranin for 1 minute as in step 6, above. Continue with step 7 to complete the procedure.

B. Capsule Stain Technique

PURPOSE: to detect the presence or absence of a bacterial capsule.

Visualization of the bacterial capsule by staining methods is a two-step procedure involving negative and simple staining. In the first step, the back-

ground area is stained to outline the capsule. The cells then are stained in the second step. Water and heat should not be used in either step because capsules are easily destroyed by both. Also, it is helpful to use milk cultures of organisms because media containing milk encourage capsule production.

Special Materials

- Selected encapsulated bacterial species
- Saline solution (0.85% NaCl)
- Nigrosin or India ink
- Crystal violet

Procedure

1. Prepare a **negative stain** of a selected bacterial organism using nigrosin or India ink and the technique described in Exercise 5. Allow the slide to air-dry thoroughly on the laboratory bench or warming tray. The acidic dye will outline the bacterial capsules.
2. Flood the slide with **crystal violet** for 1 minute. This will stain the bacterial cells.
3. Very carefully wash the excess stain from the slide using **saline solution** instead of water. Saline will help preserve the integrity of the capsule. Remember to be gentle because the slide has not been heat-fixed, and the bacteria may be lost with the stain if too much saline is applied. Blot the slide very gently.
4. Observe the slide under low power, then high power and oil immersion. Search for purple cells surrounded by capsules, which appear as white halos as illustrated in **Figure 7.2**. The background should be stained dark, and the cells and halos will appear as "motheaten" areas within the mat of stain. It may be necessary to repeat this technique several times before a successful capsule stain is observed.
5. Enter representations of encapsulated bacteria in the appropriate spaces in the Results section. Be sure to label the capsules to distinguish them from the cells. If the slide is to be retained, label the slide.

Quick Procedure
Capsule Stain

1. Prepare negative stain of bacterial smear with nigrosin as in Ex. 5.
2. Stain with crystal violet 1 min.
3. Wash gently with saline solution; dry; observe.

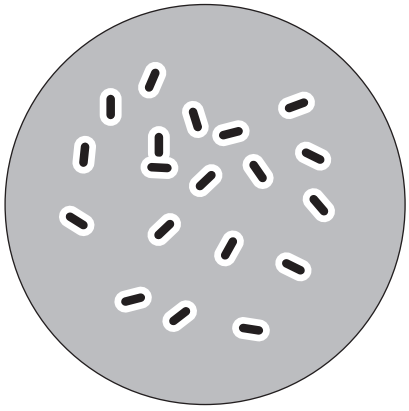


FIGURE 7.2
A schematic diagram of several bacterial rods stained to show their capsules.

C. Bacterial Motility

PURPOSE: to determine if a bacterial cell is motile (has one or more flagella).

Evidence for the presence of bacterial flagella is obtained by observing motility. Two methods are available. In the first method, live unstained bacteria are seen moving about in the hanging drop technique. In the second method, bacteria are inoculated into a semisolid medium. During the incubation, they migrate from the inoculation site and form a characteristic pattern of growth, which indicates motility.

Special Materials

- Selected motile bacterial species in broth cultures
- Selected nonmotile species in broth cultures
- Concave depression slides
- Coverslips
- Petroleum jelly
- Applicator sticks
- Tubes of motility test agar
- Inoculating needles

Procedure

I. The Hanging Drop Technique

1. Thoroughly clean a concave **depression slide** and a coverslip. Using an applicator stick, place a small amount of petroleum jelly at the corners of the coverslip. Position the coverslip face up on the desk.
2. Obtain a broth culture of a motile bacterial species, and aseptically place two or three loopfuls in the center of the coverslip. A hay infusion or sample of teeth scrapings may be used.
3. Invert the concave depression slide, and lower it onto the inoculated coverslip, pressing gently so that the petroleum jelly seals the corner of the coverslip to the slide. Quickly invert the slide so that the drop hangs into the concave depression, as shown in **Figure 7.3**.

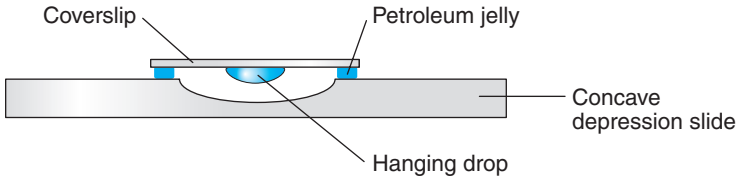


FIGURE 7.3
The hanging drop preparation.

Quick Procedure
Hanging Drop

1. Prepare coverslip with petroleum jelly adhesive.
2. Add two or three loopfuls of broth culture to coverslip.
3. Invert a depression slide onto the coverslip.
4. Turn the depression slide upright; observe.

4. Observe the slide with the low power objective, and lower the light considerably. Then locate the edge of the drop. Now switch to high power, and locate the edge of the drop again. Careful focusing and reduction of the light to achieve contrast are essential to success. Locate organisms within the fluid, and note that the motion of certain cells has direction, with a consistent waving pattern. This is evidence of true motility. Do not use oil immersion except at the suggestion of the instructor. Note in the Results section the patterns of motion displayed by the organisms, where they accumulate, their relative sizes and shapes, any configurations they display, and the speed of their movement. When your observations are complete, enter representations of the cells, showing the direction of motion. Since the slides contain live organisms, they should be placed into a beaker of disinfectant after use. Rinse the slide with disinfectant before reuse.
5. Prepare a second hanging drop preparation with loopfuls of a nonmotile species. Note the erratic vibrations of the cells in place and the lack of directed movement. This is **Brownian motion,** a phenomenon caused by molecules striking the organisms and displacing them briefly. It should be compared with true motility. Representations of these organisms and their pattern of movement may be entered in the Results section. Conclusions may be drawn on the type of motion observed as evidence of the presence of flagella.
6. Though technically not a hanging drop, a useful alternative can be prepared as follows: Smear a clean glass slide with a drop or two of immersion oil. Then add two or three loopfuls of a broth culture of bacteria to a coverslip. Now invert the slide onto the coverslip, turn it upright, and examine the slide microscopically. Broth droplets containing bacteria will be trapped within the field of oil, and live bacteria may be observed moving about within the droplets.

II. Motility Test Agar Technique Using Unknowns

1. Obtain broth cultures of the two unknown bacteria to be used. **Motility test agar** is a semisolid growth medium containing a reduced amount of agar. The semisolid state allows bacteria to move freely through the medium. Obtain two tubes of motility test agar, and label them with your name, the date, the codes of the two unknown organisms to be used, and the name of the medium "motility test agar." One tube will be inoculated with a motile species, the other with a nonmotile species.
2. Obtain an inoculating needle and hold it as you would hold an inoculating loop. Sterilize the needle in the Bunsen burner flame. Aseptically obtain a sample of one of the unknown cultures. Inoculate the appropriate tube of motility test agar by inserting the needle into the medium at least halfway down. Carefully withdraw the needle along the same line. Re-sterilize the needle.
3. Inoculate the second tube of motility test agar with the other unknown species.
4. Incubate the tubes at 37° C for 24 to 48 hours, or as directed by the instructor. An uninoculated control tube may be included for comparison purposes. At the end of the incubation period, the tubes may be refrigerated to preserve them until observed.

5. Observe the tubes and determine which unknown was motile. Motile organisms will spread out from the line of inoculation and establish a broad zone of growth in various patterns. The motility test agar may become cloudy with growth. Nonmotile species, however, will grow only along the line of inoculation, and a white line may be seen in the medium. The remainder of the medium will be clear. Enter representations of the tubes in the Results section, showing the evidence for motility and, by inference, the presence of flagella. Carefully placed labels should be used to guide the reader, and a word or two of explanation can be included. A "talking picture" should result.
6. Prepared slides may be available for microscopic observation of bacterial flagella.

Questions

1. Explain how the extremely high resistance of bacterial endospores has an influence on sterilization practices, food microbiology, and disease processes.
2. Why is heat necessary for the successful performance of the spore stain technique?
3. What mistakes might contribute to the inability to locate any cells on the slide at the conclusion of the capsule stain technique? How can these mistakes be corrected?
4. In what ways can Brownian motion be distinguished from true bacterial motility in the hanging drop preparation?
5. Explain why a tube with a nonmotile species is necessary for reliable determination of bacterial motility by the motility test agar technique.

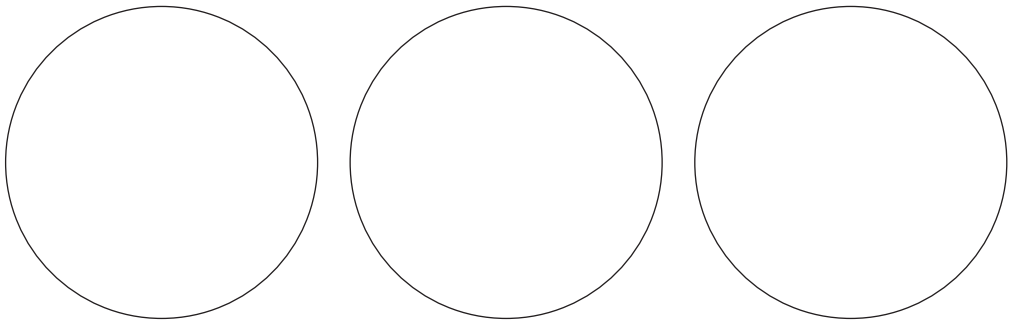
Name _____

Date _____ Section _____

Exercise 7 Results

Bacterial Structures

A. Spore Stain Technique



Organism: _____ _____ _____

Magnif.: _____ _____ _____

Observations and Conclusions: _____

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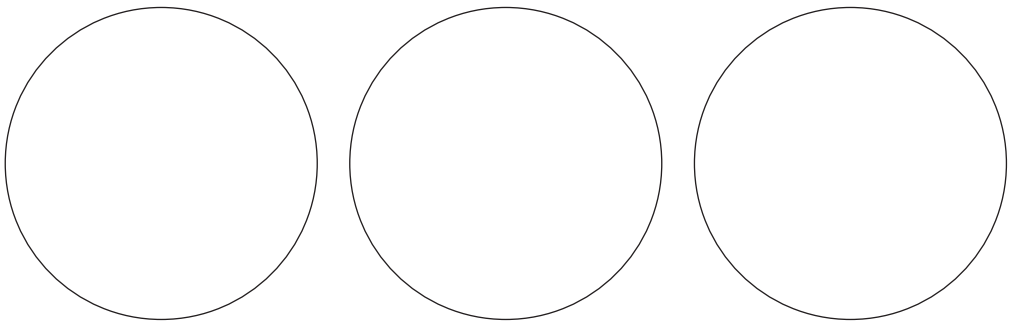
B. Capsule Stain Technique



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Observations and Conclusions: _____

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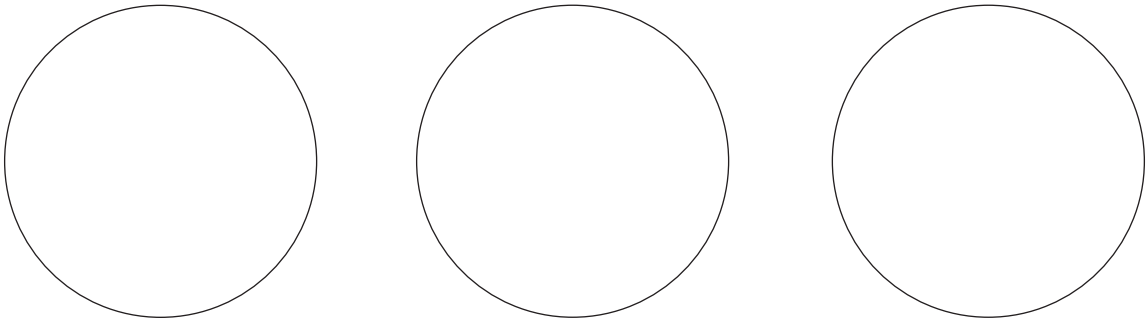
C. Bacterial Motility

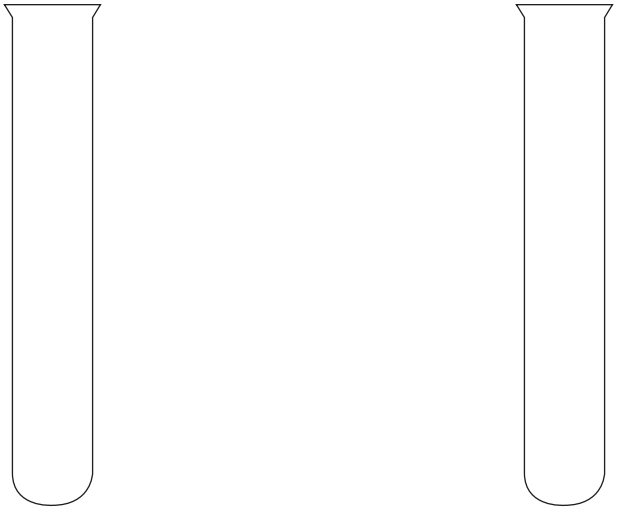
I. The Hanging Drop Technique



Organism: _____ _____ _____

Magnif.: _____ _____ _____

II. Motility Test Agar Technique Using Unknowns



Organism: _____ _____

Observations and Conclusions: _____

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