Lab 7: Endospore Stain and Bacterial Motility

A. ENDOSPORE STAIN

DISCUSSION

A few genera of bacteria, such as *Bacillus* and *Clostridium* have the ability to produce resistant survival forms termed endospores. Unlike the reproductive spores of fungi and plants, these endospores are resistant to heat, drying, radiation, and various chemical disinfectants (see Labs 19 and 20).

Endospore formation (sporulation) occurs through a complex series of events. One is produced within each vegetative bacterium. Once the endospore is formed, the vegetative portion of the bacterium is degraded and the dormant endospore is released.

First the DNA replicates and a cytoplasmic membrane septum forms at one end of the cell. A second layer of cytoplasmic membrane then forms around one of the DNA molecules (the one that will become part of the endospore) to form a forespore. Both of these membrane layers then synthesize peptidoglycan in the space between them to form the first protective coat, the cortex. Calcium dipicolinate is also incorporated into the forming endospore. A spore coat composed of a keratin-like protein then forms around the cortex. Sometimes an outer membrane composed of lipid and protein and called an exosporium is also seen (Fig. 1M).

Finally, the remainder of the bacterium is degraded and the endospore is released. Sporulation generally takes around 15 hours (see Fig. 1K). The process is summarized in Fig.1.

The endospore is able to survive for long periods of time until environmental conditions again become favorable for growth. The endospore then germinates, producing a single vegetative bacterium (see Fig. 1N).
Bacterial endospores are resistant to antibiotics, most disinfectants, and physical agents such as radiation, boiling, and drying. The impermeability of the spore coat is thought to be responsible for the endospore's resistance to chemicals.

The heat resistance of endospores is due to a variety of factors:

- Calcium-dipicolinate, abundant within the endospore, may stabilize and protect the endospore's DNA.
- Specialized DNA-binding proteins saturate the endospore's DNA and protect it from heat, drying, chemicals, and radiation.
- The cortex may osmotically remove water from the interior of the endospore and the dehydration that results is thought to be very important in the endospore's resistance to heat and radiation.
- Finally, DNA repair enzymes contained within the endospore are able to repair damaged DNA during germination.

To view an electron micrograph of an endospore of *Bacillus stearothermophilus*, see the Microbe Zoo web page of Michigan State University.

Scanning electron micrograph of *Clostridium botulinum* with endospore; courtesy of Dennis Kunkel's Microscopy.

Scanning electron micrograph of *Bacillus anthracis* endospores; courtesy of CDC.

For more information on bacterial endospores, see Unit 1, Section B3e in your Lecture Guide.

Due to the resistant nature of the endospore coats, endospores are difficult to stain. Strong dyes and vigorous staining conditions such as heat are needed. Once stained, however, endospores are equally hard to decolorize. Since few bacterial genera produce endospores, the endospore stain is a good diagnostic test for species of *Bacillus* and *Clostridium*.

ORGANISMS

Trypticase Soy agar plate cultures of *Bacillus megaterium*.

PROCEDURE (to be done individually)

1. Heat-fix a smear of *Bacillus megaterium* as follows:

   a. Using the dropper bottle of deionized water found in your staining rack, place 1/2 of a normal sized drop of
water on a clean slide by touching the dropper to the slide (see Fig. 13). Alternatively, use your sterilized inoculating loop to place a drop of deionized water on the slide.

b. Using your sterile inoculating loop, aseptically remove a small amount of the culture from the edge of the growth on the agar surface (see Fig. 14) and generously mix it with the drop of water until the water becomes visibly cloudy (see Fig. 15).

c. Incinerate the remaining bacteria on the inoculating loop.

d. After the inoculating loop cools, spread the suspension over approximately half of the slide to form a thin film (see Fig. 16).

e. Allow this thin suspension to completely air dry (see Fig. 17).

f. To heat-fix the bacteria to the slide, pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear near the opening of the microincinerator for 10 seconds (see Fig. 18) as demonstrated by your instructor. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

2. Place a piece of blotting paper over the smear and saturate with malachite green (see Fig. 19).

3. Let the malachite green sit on the slide for one minute and proceed to the next step.

4. Fill a glass beaker approximately one-fourth full with tap water, place it on a hot plate, and bring the water to a boil. Reduce the heat so the water simmers and place your slide on top of the beaker (see Fig. 20). Your slide will get hot so be sure to handle the slide with a test tube holder. Steam the slide for 5 minutes. As the malachite green evaporates, continually add more. Do not let the paper dry out!

5. After five minutes of steaming, wash the excess stain and blotting paper off the slide with water. Don't forget to wash of any dye that got onto the bottom of the slide.

6. Blot the slide dry.

7. Now flood the smear with safranin and stain for one minute (see Fig. 21).

8. Wash off the excess safranin with water (see Fig. 22), blot dry (see Fig. 23), and observe using oil immersion microscopy. With this endospore staining procedure, endospores will stain green while vegetative bacteria will stain red (see Fig. 2).

9. Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink.

10. Observe the demonstration slide of Bacillus anthracis (see Fig. 3). With this staining procedure, the vegetative bacteria stain blue and the endospores are colorless. Note the long chains of rod-shaped, endospore-containing...
bacteria.

11. Observe the demonstration slide of *Clostridium tetani* (see Fig. 4). With this staining procedure, the vegetative bacteria stain blue and the endospores are colorless. Note the "tennis racquet" appearance of the endospore-containing *Clostridium*.

12. Endospore stain of *Clostridium botulinum* (see Fig. 13). Endospores stain green while vegetative bacteria stain red.

### B. BACTERIAL MOTILITY

#### DISCUSSION

Many bacteria are capable of motility (the ability to move under their own power). Most motile bacteria propel themselves by special organelles termed flagella.

A bacterial flagellum has 3 basic parts: a filament, a hook, and a basal body.

1) The **filament** is the rigid, helical structure that extends from the cell surface. It is composed of the protein flagellin arranged in helical chains so as to form a hollow core. During synthesis of the flagellar filament, flagellin molecules coming off of the ribosomes are transported through the hollow core of the filament where they attach to the growing tip of the filament causing it to lengthen. With the exception of a few bacteria, such as *Bdellovibrio* and *Vibrio cholerae*, the flagellar filament is not surrounded by a sheath (see Fig. 5).

2) The **hook** is a flexible coupling between the filament and the basal body (see Fig. 5).

3) The **basal body** consists of a rod and a series of rings that anchor the flagellum to the cell wall and the cytoplasmic membrane (see Fig. 5). Unlike eukaryotic flagella, the bacterial flagellum has no internal fibrils and does not flex. Instead, the basal body acts as a rotary molecular motor, enabling the flagellum to rotate and propell the bacterium through the surrounding fluid. In fact, the flagellar motor rotates very rapidly.

The **MotA and MotB proteins** form the stator of the flagellar motor and function to generate torque for rotation of the flagellum. The **MS and C rings** function as the rotor (see Fig. 5). Energy for rotation comes from the **proton motive force** (def) provided by protons moving through the Mot proteins along a concentration gradient from the peptidoglycan and periplasm towards the cytoplasm.


Bacterial motility constitutes unicellular behavior. In other words, motile bacteria are capable of a behavior called *taxis*. Taxis is a motile response to an environmental stimulus and functions to keep bacteria in an optimum environment.

The arrangement of the flagella about the bacterium is of use in classification and identification. The following flagellar arrangements may be found (see Fig. 6):
1. **monotrichous** - a single flagellum at one pole (see Fig. 7A and Fig. 7B).
   - Scanning electron micrograph showing monotrichous flagellum of **Vibrio**; courtesy of CDC.

2. **amphitrichous** - a single flagellum at both poles. (see Fig. 8A)

3. **lophotrichous** - two or more flagella at one or both poles of the cell (see Fig. 8).
   - Scanning electron micrograph of **Helicobacter pylori** showing lophotrichous arrangement of flagella; from Science Photolab.com

4. **peritrichous** - completely surrounded by flagella (see Fig. 9).
   - Transmission electron micrograph of **Proteus mirabilis** showing peritrichous arrangement of flagella; from Microbe World

One group of bacteria, the **spirochetes**, has internally-located axial filaments (see Fig. 10) or endoflagella. Axial filaments wrap around the spirochete towards the middle from both ends. They are located above the peptidoglycan cell wall but underneath the outer membrane or sheath.

   - Axial filaments of the spirochete **Leptospira**; Midlands Technical College, Bio 255 course site

For more information on bacterial flagella, see Unit 1, Section B4b in your Lecture Guide.

To detect bacterial motility, we can use any of the following three methods: 1) direct observation by means of special-purpose microscopes (phase-contrast and dark-field), 2) motility media, and, indirectly, 3) flagella staining.

1. **Direct observation of motility using special-purpose microscopes.**

   a. **Phase-contrast microscopy**

   A phase-contrast microscope uses special phase-contrast objectives and a condenser assembly to control illumination and give an optical effect of direct staining. The special optics will convert slight variations in specimen thickness into corresponding visible variation in brightness. Thus, the bacterium and its structures appear darker than the background.

   Phase contrast microscopy of motile **Pseudomonas** from YouTube.

   b. **Dark-field microscopy**

   A dark-field microscope uses a special condenser to direct light away from the objective lens. However, bacteria (or other objects) lying in the transparent medium will scatter light so that it enters the objective. This gives the optical effect of an indirect stain. The organism will appear bright against the dark background. Dark field microscopy is especially valuable in observing the very thin spirochetes (see Fig. 11 and Fig. 14).

**Movies of bacterial motility:**

   - Movie of motile **Escherichia coli** with fluorescent labelled-flagella #1 Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.
• Movie of motile *Escherichia coli* with fluorescent labelled-flagella #2 Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of motile *Escherichia coli* with fluorescent labelled-flagella #3 Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of motile *Escherichia coli* with fluorescent labelled-flagella #4 Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of swimming *Escherichia coli* as seen with phase contrast microscopy Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of tethered *Escherichia coli* showing that the bacterial flagella rotate Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of swarming motility of *Escherichia coli* Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of motile *Pseudomonas* from YouTube.

• Movie of motile *Rhodobacter spheroides* with fluorescent labelled-flagella Courtesy of Dr. Howard C. Berg from the Roland Institute at Harvard.

• Movie of motile *Borrelia burgdorferi*, the spirochete that causes Lyme disease. From You Tube, courtesy of CytoVivo.

• Movie of motile *Borrelia burgdorferi*, the spirochete that causes Lyme disease.

2. **Motility Test medium**

Semi-solid Motility Test medium may also be used to detect motility. The agar concentration (0.3%) is sufficient to form a soft gel without hindering motility. When a **non-motile organism** is stabbed into Motility Test medium, **growth occurs only along the line of inoculation**. Growth along the stab line is very **sharp and defined** (see Fig. 12A). When **motile organisms** are stabbed into the soft agar, they swim away from the stab line. Growth occurs **throughout the tube** rather than being concentrated along the line of inoculation. Growth along the stab line appears much more **cloud-like as it moves away from the stab** (see Fig. 12B). A tetrazolium salt (TTC) is incorporated into the medium. Bacterial metabolism reduces the TTC producing formazan which is red in color. **The more bacteria present at any location, the darker red the growth appears**.

3. **Flagella staining**

If we assume that bacterial flagella confer motility, flagella staining can then be used indirectly to denote bacterial motility. Since flagella are very thin (20-28 nm in diameter), they are below the resolution limits of a normal light microscope and cannot be seen unless one first treats them with special dyes and mordants that build up as layers of precipitate along the length of the flagella, making them microscopically visible. This is a delicate staining procedure and will not be attempted here. We will, however look at several demonstration flagella stains.

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**ORGANISMS**

Trypticase Soy broth cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. **Caution: handle these organisms as pathogens.**
Motility Test medium (2 tubes)

**PROCEDURE** (to be done individually and in pairs)

1. Observe the phase-contrast microscopy demonstration of motile *Pseudomonas aeruginosa*.
   
   Movie of motile *Pseudomonas* from YouTube.

2. Observe the dark-field microscopy demonstration of motile *Pseudomonas aeruginosa*.

3. Take 2 tubes of Motility Test medium per pair. Stab one with *Pseudomonas aeruginosa* and the other with *Staphylococcus aureus*. **Stab the bacterium about 1/2 - 3/4 of an inch into the agar, taking care not to tilt or twist the loop so that the loop comes up through the same cut as it went down.** Incubate the tubes in your test tube rack at 37°C until the next lab period.

4. Observe the flagella stain demonstrations of a *Vibrio* species (*monotrichous*), *Proteus vulgaris* (*peritrichous*) and *Spirillum undula* (*amphitrichous*) as well as the dark-field photomicrograph of the spirochete *Leptospira*. When observing flagella stain slides, keep in mind that flagella often break off during the staining procedure so you have to look carefully to observe the true flagellar arrangement.

**RESULTS**

A. Endospore Stain

Make drawings of the various endospore stain preparations.

Endospore stain of *Bacillus megaterium*

Endospore stain of *Bacillus anthracis*

Endospore stain of *Clostridium tetani*

B. Bacterial Motility

1. Observe the phase contrast and dark-field microscopy demonstrations of bacterial motility.

2. Observe the two tubes of Motility Test medium.
PERFORMANCE OBJECTIVES FOR LAB 7

After completing this lab, the student will be able to perform the following objectives:

A. ENDOSPORE STAIN

DISCUSSION
1. Name two endospore-producing genera of bacteria.

2. State the function of bacterial endospores.

RESULTS

1. Recognize endospores as the "structures" observed in an endospore stain preparation.

2. Identify a bacterium as an endospore-containing *Clostridium* by its "tennis racquet" appearance.

B. BACTERIAL MOTILITY

DISCUSSION

1. Define the following flagellar arrangements: monotrichous, lophotrichous, amphitrichous, peritrichous, and axial filaments.

2. State the function of bacterial flagella.

3. Describe three methods of testing for bacterial motility and indicate how to interpret the results.

RESULTS

1. Recognize bacterial motility when using phase-contrast or dark-field microscopy.

2. Interpret the results of Motility Test Medium.

3. Recognize monotrichous, lophotrichous, amphitrichous, and peritrichous flagellar arrangements.

SELF-QUIZ

Self-quiz

Answers

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