Laboratory Exercise # 8: Other Staining Techniques

Purpose:

The purpose of this laboratory exercise is to acquaint the student with staining techniques other than the Gram stain that are routinely used to help identify microorganisms.

Introduction: The Capsule Stain

Many bacteria secrete a slimy, viscous covering called a capsule. Capsules are normally composed of complex polysaccharides forming a loose gel. However, the chemical composition of the capsule is unique to each genus of bacteria that secretes it. The presence of a capsule on an invading bacterium allows it to evade the host defense of white blood cell phagocytosis.

Materials:

- Skim milk culture of Enterobacter aerogenes
- Glass slides
- Inoculating loop
- Crystal violet stain
- 10% Copper sulfate solution

Procedure:

1. Stir the skim milk culture of Enterobacter aerogenes with your sterile cooled loop and place 2-3 loops of the culture on a glass slide.

2. Using your loop, spread the culture out over the entire slide to form a thin film.

3. Let the smear completely air dry. **DO NOT HEAT FIX!!** Capsules stick well to glass and heat may destroy the capsule.

4. Stain with crystal violet for **one minute**.

5. Wash off the excess with copper sulfate solution. **DO NOT USE WATER!!**

6. Let the smear air dry by placing it on an angle on a piece of paper towel.

7. Observe the smear using oil immersion. The organism and the milk dried on the slide retain the purple dye, while the capsule will remain colorless.
Data:

Sketch what you observe under oil immersion in the space provided below.

Questions:

1. What other function may a capsule have other than that of evading phagocytosis?

2. Name two microorganisms that can produce a capsule.
Introduction: Flagella Stain

What is the function of a flagellum? Movement (called *taxis*) of course, but for what purpose? Bacteria live in an ever changing environment and their need for nutrients is constant. If the bacteria have a flagellum then it has the ability to change its location. The concentration of different chemicals within its environment provides the stimulus for movement (called *chemotaxis*). Wastes of course should cause a bacterium to move away, while fresh nutrients should cause the bacterium to move towards its source.

In a clinical setting, the presence or absence of a flagellum is used to categorize (identify) a bacterium. Unfortunately, the observance of the flagellum is beyond the resolving power of the brightfield microscope. Therefore, staining of the flagellum is required. Since the flagella are very thin in structure, a series of multiple dyes and mordants are required in order to observe them. This is a very tedious and delicate process, which will not be undertaken in this exercise. But, the student will observe prepared flagella smears.

Materials:

Prepared slides of flagella stain

Data:

1. Sketch what you have observed on the demonstration slide using oil immersion objective in the space provided below.

2. There are four basic arrangements of flagella, using your textbook as a reference, sketch and label each type of arrangement.
Introduction: Endospore Stain

The genera *Clostridium* and *Bacillus* are able to produce endospores. The multi-layered structure of the spore is produced when environmental conditions are unfavorable. First the genetic material is replicated and then several layers called a coat surround the DNA, plus a small amount of cytoplasm. These coats protect the genetic material from harsh environmental conditions such as drought and heat. Further protection is afforded by the presence of dipicolinic acid and calcium ions found in high concentration within the spore. When environmental conditions again become favorable, the many layers surrounding the DNA dissolve and the spore then germinates into a vegetative cell.

Materials:

- Glass slides
- Inoculating loop
- Bunsen burner
- Tripod/Ring stand with wire gauze
- Small beaker
- Malachite green stain
- Safranin stain
- Trypticase soy agar plate of *Bacillus subtilis* - at least 72 hours old

Procedure:

1. Prepare the bacterial smear by removing some of the growth from the agar plate.

2. Mix the bacteria with water on a glass slide.

3. Allow the smear to air dry then heat fix the smear.

4. Set up the tripod/ring stand with a wire gauze. Light the Bunsen burner and slide it under the tripod.

5. Place the beaker, 2/3 full of water on the tripod/ring stand and carefully place the slide on the top of the beaker.

6. Cover the smear with Malachite green stain. As the smear starts to steam, add more stain so that the slide does not dry out.

7. Continue steaming the slide for 5 minutes. After staining remove the smear and let it cool.

8. Wash smear thoroughly with water to remove the excess stain.

9. Counterstain with Safranin for 1 minute.

10. Rinse the slide with water and gently blot dry.
11. Observe the smear under 100X objective.

**Data:**

Make a sketch of your smear, in the space provided below.

![Image of a circle for sketching]

**Questions:**

1. List two genera that form endospores.

2. How do these two genera differ with regards to their oxygen requirements?

3. When does endospore formation occur in bacteria?

4. Is the spore considered a reproductive structure?

5. List the three different locations that an endospore can have within a bacteria cell.

6. Why is the smear covered with malachite green and steamed for 5 minutes?

7. What color is the vegetative part of the cell?

8. What color is the endospore?
Introduction: Acid Fast Stain

The genera *Mycobacterium* and *Nocardia* are the only genera capable of producing a waxy outer covering made of mycolic acid. This makes *Mycobacterium* or *Nocardia* hard to treat with antibiotics, but a simultaneous treatment with rifampin and isoniazid is usually successful. The waxy coating also makes this bacterium exceedingly hard to stain with standard staining techniques, like the Gram stain procedure. These bacteria normally stain gram variable if at all. The **acid fast stain** is a differential staining process using carbol fuschin dye as the primary stain, an acid alcohol rinse and methylene blue as the secondary stain.

Materials:

TSA plates of:

- *Nocardia* or *Mycobacterium*
- *Bacillus subtilis*

<table>
<thead>
<tr>
<th>Glass slides</th>
<th>Small beaker</th>
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<tbody>
<tr>
<td>Inoculating loop</td>
<td>Acid alcohol</td>
</tr>
<tr>
<td>Carbol fuchsin</td>
<td>Bunsen burner</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Tripod/Ring stand with Wire gauze</td>
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Procedure:

1. Using sterile technique obtain bacteria from each TSA plate and place on opposite ends of a glass slide in a drop of water and mix.

2. Air dry the smear, then heat fix.

3. Set up the tripod/ring stand with a wire gauze. Light the Bunsen burner and slide it under the tripod.

4. Place a beaker that is 2/3 full of water on the tripod/ring stand and carefully place the slide on top of the beaker.

5. Cover the smear with Carbol fuchsin and allow to steam for 5 minutes. Add more stain as needed. **Do not let the smear dry out!**

6. After steaming remove the slide from the beaker and allow it to cool.

7. Rinse the smear thoroughly with acid alcohol. Then rinse with water.

8. Counterstain with methylene blue. For 1 minute.

9. Rinse the smear with water. Blot dry and **observe the smear under 100X.**
Data:

Make a sketch of your smear in the space provided below.

Questions:

1. Name the two genera that are identified by the use of the Acid fast staining procedure.

2. What is the composition of the outer waxy coating surrounding bacteria that are acid fast?

3. In the staining procedure what is the primary stain?

4. Why does the primary stain need to be steamed during the staining procedure?

5. How is acid alcohol different from Grams alcohol?

6. What is the secondary stain?

7. What color do acid fast bacteria stain? Non-acid fast?