EXPERIMENT 5

DIFFERENTIAL AND CYTOLOGICAL STAINS

In Experiment 3, simple and Gram stains were done. The Gram stain is a differential stain: it stains one type of cell in one color and other types of cells in a different color. In this experiment several differential stains are done: some reveal different cell types but others stain one part of the cell one color and other parts a different color. Most often this experiment is not done in total, but parts are done as separate experiments throughout the course.

THE SPORE STAIN

In Experiment 4, you may well have seen bacterial spores. They are refractile to most staining procedures and appear as clear areas in the Gram stain. Poly-beta hydroxybutyrate (bacterial fat) and other intracellular inclusions can also not stain in the Gram stain and appear as clear areas. The spore stain allows you to clearly demonstrate that the clear area in a Gram stained or simple stained cell is a spore. Since spores are refractile to stains, the Malachite green stain must be "driven" into the spore by heat. That is, during the spore stain, you will heat the slide being stained to steaming....this causes breaks in the spore such that the stain can penetrate the spore coat, cortex and spore wall. After cooling the slide is rinsed in water and counterstained with safranin. The cells stain red and the spores stain green.

MATERIALS

1. Two Nutrient Agar slant cultures of *Bacillus* species. One grown for 48 hours and another grown for 24 hours.
2. Shaeffer-Fulton spore stain: 0.5% Malachite Green.
3. Safranin.
4. Slides and slide holders.

PROCEDURE

1. Prepare two slides with the two cultures of *Bacillus* as heat fixed smears.

2. Simple stain one with Crystal Violet.

3. Spore Stain the other as follows:

a. Drop Spore stain onto the smear and cover with a piece of paper towel. The paper towel must be cut so that it does not extend beyond the sides of the slide (Figure 1.)

b. Heat the slide to steaming for 5 minutes. Do not let the stain dry on the slide, to prevent this add stain when it gets close to drying out.

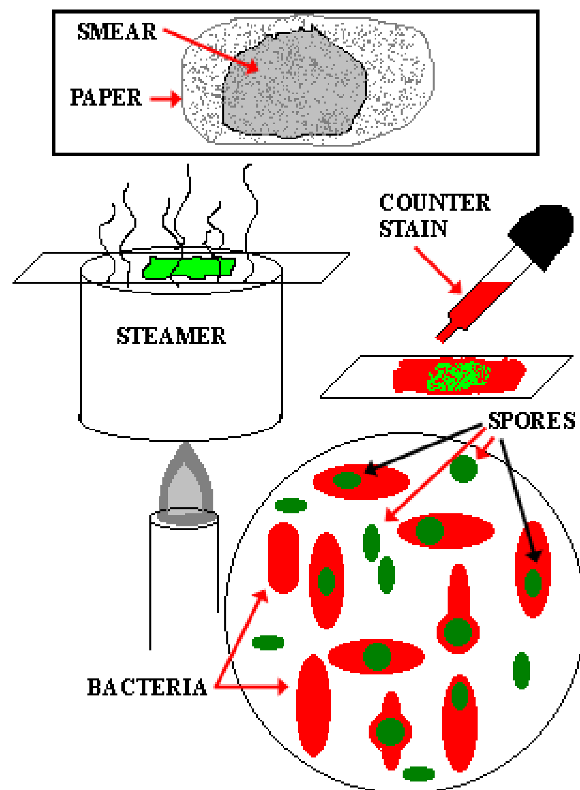

Figure 1. Spore stain procedure

c. Allow the slide to cool (place it on the cool bench). Remove the paper towel and put it in the waste bucket (**not the sink!**).

d. Rinse with water.

e. Counterstain with safranin for 1 minute.

f. Rinse with water, dry and observe with the oil immersion Lens (Figure 2.).

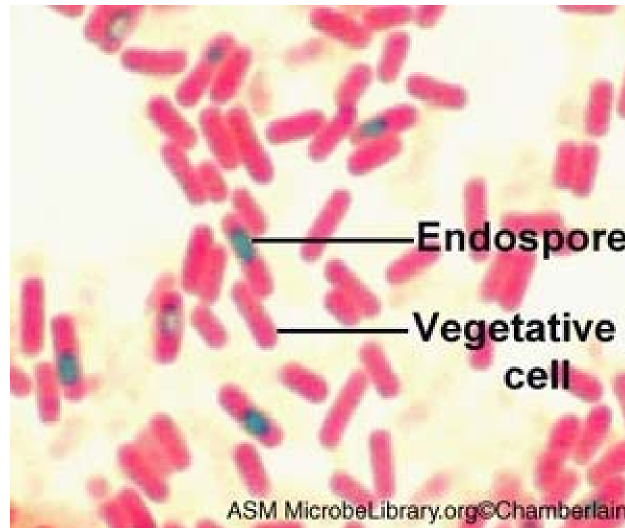

Figure 2. Spores of *Bacillus* sp. seen under a microscope

ACID FAST STAIN

Acid fast bacteria are Gram positive bacteria that possess waxes in their cell walls. They are generally difficult to stain, but the Acid Fast Stain uses heat to drive the stain through the wall. This stain also uses a challenge step: a rinse in acid alcohol (3% HCl in alcohol) which washes out the acid fast stain primary stain (carbol fuchsin) from cells that do not possess waxes in their walls. The acid fast stain is an important diagnostic tool in the detection of tuberculosis and leprosy.

MATERIALS

1. Cultures of *Mycobacterium* species and *Staphylococcus aureus*.
2. Zielhl's Carbol Fuchsin.
3. Acid Alcohol.
4. Methylene Blue.

PROCEDURE

1. Prepare on one slide a smear of *S. aureus* and a smear of one of the *Mycobacterium* species provided. Allow the smears to air dry and then heat fix.

2. Cover the smear with Carbol Fuchsin and a paper towel as was done in the spore stain. Heat to steaming for 5 minutes making sure to not let the slide dry out....add more stain if the slide appears to becoming dry (Figure 3.).

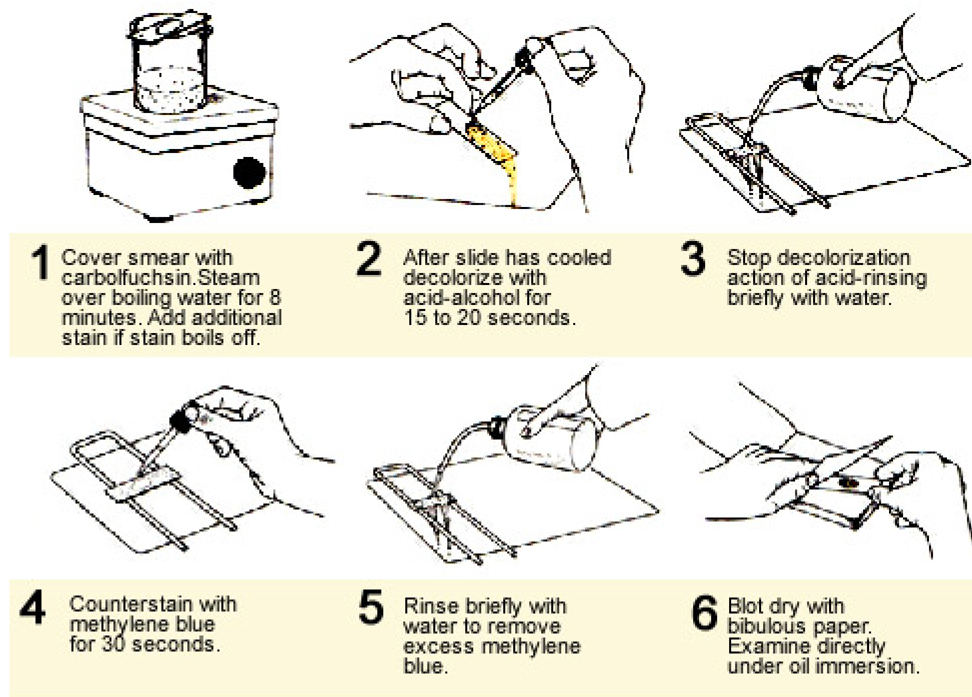

Figure 3. Acid fast staining procedure

3. Cool and then rinse the slide with tap water.
4. Rinse with acid alcohol until color no longer runs out of the smears. Avoid over rinsing.
5. Rinse with water.
6. Counterstain with Methylene Blue for 1 minute.
7. Rinse with water, dry, and observe with the oil immersion lens (Figure 4.).

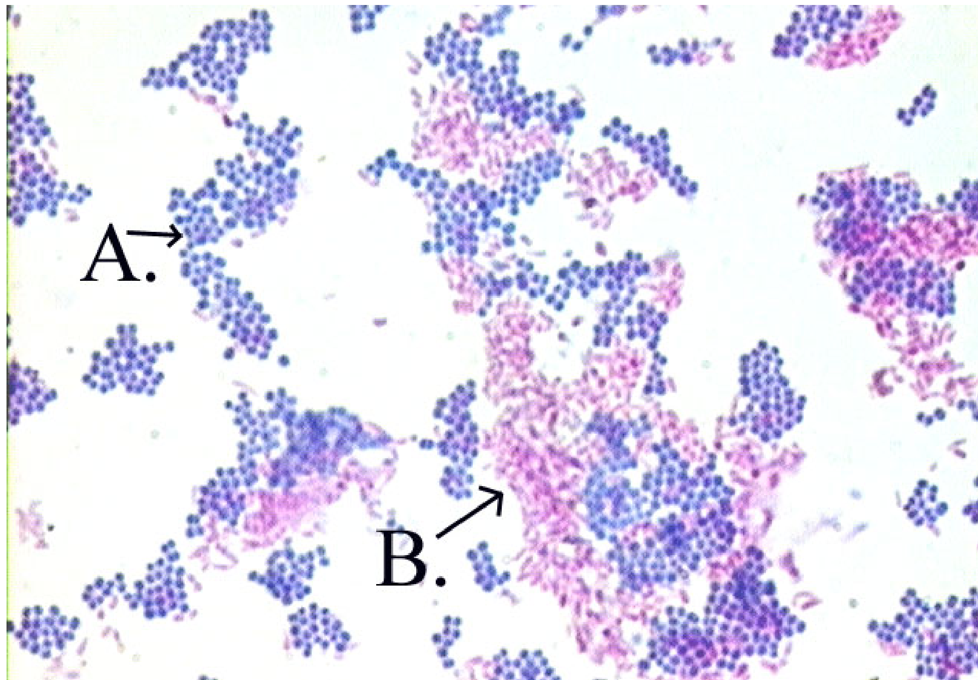

Figure 4. Difference between non-acid fast bacteria (A) and acid fast bacteria (B)

NEGATIVE STAIN for CAPSULE

Negative stain stains the surrounding glass but not the cells. The general negative staining reagents are india ink or nigrosin: both are jet black and added to a cell suspension. Note that a heat fixed smear is not done. The stain and cell suspension is then "pulled" across a glass slide thereby spreading out the suspension so that a one cell thick smear is obtained.

MATERIALS

1. Culture of *Flavobacterium capsulatum* or other capsulated bacteria growing in BHI broth with 1% glucose.

2. Nigrosin stain and Methylene Blue stain.

PROCEDURE

1. To the end of a slide add one loopful of culture.

2. Add a very small drop (loopful) of Methylene Blue.

3. With another slide, touch the suspension with its short side as diagramed below (Figure 5.) and then draw the suspension across the slide in one clean motion. Note that this **does not push** the suspension but rather **pulls** it along.

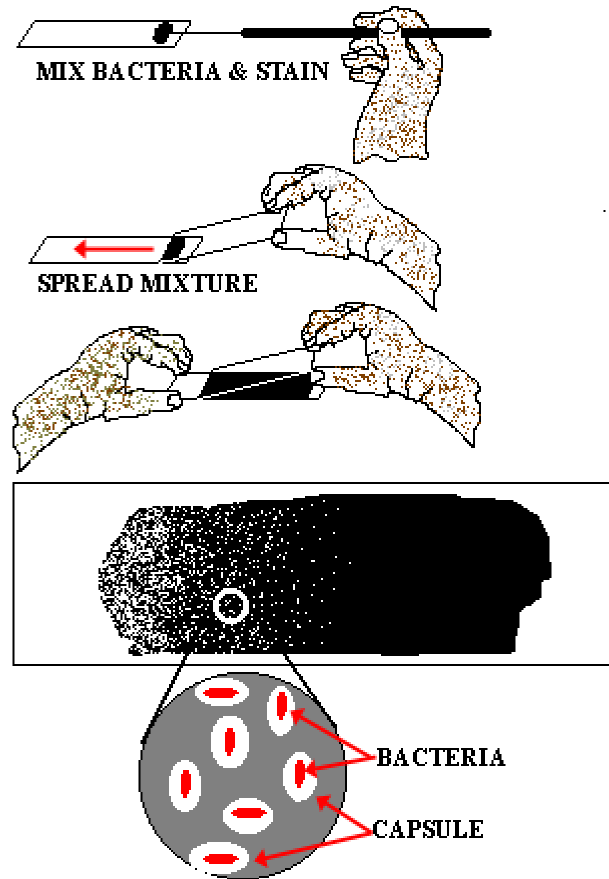

Figure 5. Negative stain procedure

4. Allow the slide to air dry and observe first with low power, then high dry to locate a good area before going on to the oil immersion lens (Figure 6.).

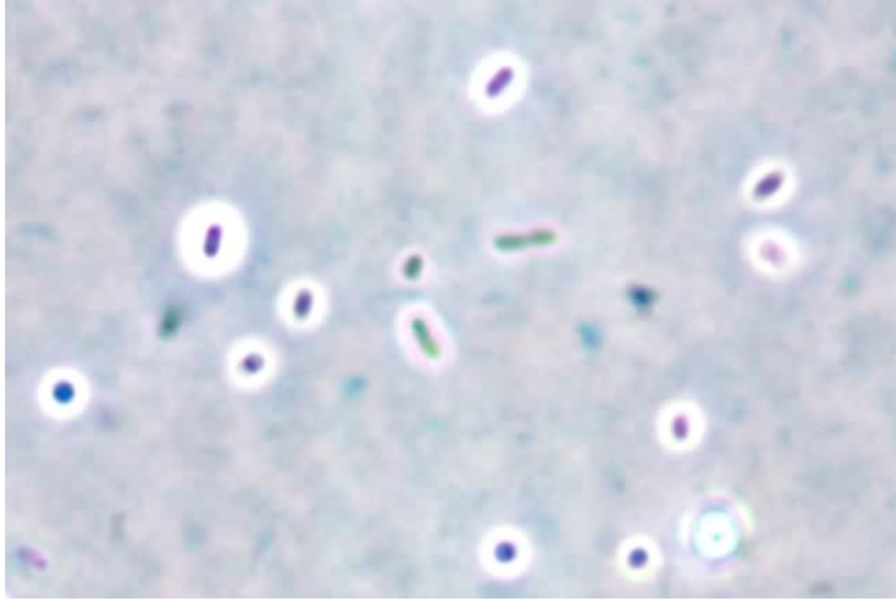

Figure 5. Capsule

FLAGELLA STAIN

Since the width of the bacterial flagella are smaller than the resolving distance of the light microscope, they can only be seen by light microscopy by using staining procedures which add bulk material to them thereby enlarging them. The bulk material (mordant) either can then be stained or already has the stain attached. The most common mordant used in flagella stains is tannic acid. Successful flagella stains require patience and a good preparation of cells. Generally young, log phase cultures are used as flagella (Figure 6.) often fall of cells in older cultures. Also, scrupulously clean slides are required. The mordant is not specific for flagella but will stick to any extraneous organic material and thereby stain just about anything on the slide.

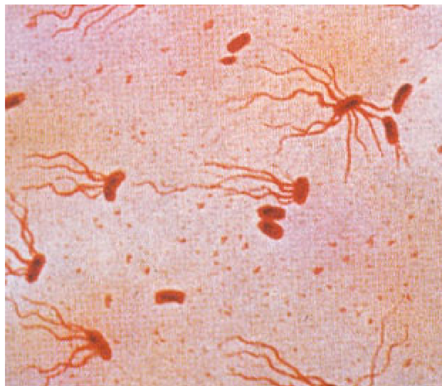

Figure 6. Flagella

In this experiment two flagella staining procedures will be attempted: the Liefson Flagella stain and the Ryu Flagella stain. The principles are the same, the procedure by which they are done are different.

MATERIALS

1. Plate streak cultures of *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Vibrio harveyi*, *Salmonella typhimurium* or other flagellated bacteria on BHI or Nutrient Agar.
2. Acid cleaned glass slides and coverslips.
3. Liefson and Ryu Flagella stains.

PROCEDURE

1. Add a few drops of distilled water to the side of young growth of the culture assigned. Allow the cells to suspend themselves to just barely form visible turbidity. Do this on two different organisms: a polarly and a peritrichously flagellated bacterium.

Ryu Flagella Stain Procedure

2. Carefully place a couple of loopfuls of one organism on a clean glass slide and cover with a coverslip. Repeat with the other organism.

3. Observe each at first with low power and then with the high dry lens. The bacteria must be clearly motile and not just moving by Brownian motion.

4. Allow the slides to sit for 5 to 15 minutes. Add a few drops of the Ryu Flagella Stain to the edge of the coverslip and allow it to slowly diffuse under the coverslip...5 to 15 minutes.

5. Observe with the high power, oil immersion lens. The slides should be examined in several areas. The cells that have attached to the glass slide surface or the undersurface of the coverslip are the best places to observe flagella. Remember that flagella are thin and even when coated with mordant and stain are still small.

Liefson Flagella Stain Procedure

6. Flame an acid cleaned slide and mark both the long and one side edge of the slide.

7. Place a loopful of one cell suspension on the top to one side and a loopful of the other cell suspension on the other side. Then allow each to run down an inclined slide.

8. Allow to dry, then flood the slide with Liefson's Flagella Stain.

9. Allow the stain to stand until a fine rust colored precipitate forms throughout the stain on the slide. This usually starts at one side then rapidly spreads over the slide.

10. Gently pour off the stain and rinse by placing the slide into a Petri plate filled with water. Rinse the slide with running water in the Petri plate without allowing the water to directly hit the slide.

11. Air dry and observe first under low power. Try to locate areas where there are not copious amounts of stain and areas where the cells are clearly visible. Remember the slide can not be heated and must be completely dry before using the oil immersion lens.

LAB REPORTS : Different Parts of this Experiment are done each semester, student must make up their own RESULTS pages.

HINTS

1. Make sure not to boil the stain. Always steam gently.
2. After steaming the slide, cool it before rinsing with cold water. If the slide is not cooled, it may crack when rinsed with cold water.