Exercise: 6

Bacterial Staining Procedures: Simple staining, Gram's staining and Endospore staining

Introduction

Bacteria are typically 0.2 to 2.0 µm on longest dimension, they are difficult to see with the light microscope.

Coloured bacteria like cyanobacteria and other photosynthetic bacteria can be viewed by wet mounts.

However, the constant movement of cells, either due to natural motility or Brownian movement, made accurate morphological studies very difficult.

The simplest way to increase contrast with the surrounding media was to use dyes which are taken up by the bacterial cells.

In 1875, Weigert, a German contemporary of Koch, found that the dye, methyl violet, would color or "stain" bacterial cells in some tissue preparations.

Prior to staining with dyes, cells must be "fixed."

Fixation is a process of killing, immobilizing and preserving the bacterial cell.

For bacteria, heat fixation is most common and chemicals such as formaldehyde, glutaraldehyde, acids and alcohols can be used.

Dyes used to stain bacterial cells are organic compounds which have affinity for specific cellular components.

Many dyes are positively charged (cationic) and combine strongly with negatively charged cellular materials such as nucleic acids and acidic polysaccharides.

Other dyes are anionic (negatively charged) and combine with positively charged constituents such as proteins.

A dye is composed of two components:

An auxochrome group which in itself does not produce color but gives the dye its acidic or basic properties,

Eg: NH_2; OH; OCH_3; I; Br; and Cl.

A chromophore group which imparts color to the dye molecule,

Eg: ñ N = N ñ (Azo); C = O (Carbonyl); ñ N = O (Nitroso); C = C (Ethylene); O N (Nitro); and C = S (Thiocarbon).

Types of staining:

Simple staining: Use of a simple dyes, mostly basic dyes.

Differential Staining: Involves the use of a combination of chemicals and dyes.

Gram staining, Acid-fast staining, Endospore staining etc.

Special Staining: Use special procedures for observing cell structures like nuclear material, capsule, flagella, storage granules etc.

SIMPLE STAINING

Simple staining is merely the use of a single dye such as Crystal violet, Malachite green Methyl blue etc. used to increase the contrast of cells for microscopy.

As an example, a simple stain would be used to detect the presence of bacteria in some natural material.

Positive stain: Dye sticks to the bacteria and colours it. Bacteria are normally heat fixed Eg. Crystal violet.

Negative stain: Dye settles outside of the bacteria. It is simple staining and there is no heat fixation. Eg: Nigrosin

Objective:

To illustrate the use of simple stains in the study of bacterial morphology.

Material:

1. Bacterial cultures 2. Crystal violet 3. Nigrosin 4. Glass slide 5. 100 X objective 6. Immersion Oil

Procedure

Positive staining:

•Clean microscope slides with soap and water, rinse, and then blot dry.

•Using an inoculating loop, put a drop of water on the slide.

•Flame sterilize, an inoculating loop and using aseptic technique, remove a small amount of bacteria from some solid medium and mix with the drop of water.

•The smear should be about the size of a dime and must be fairly dilute.

• If the smear is too dense, the morphology of individual cells will be impossible to determine.

•Allow the smear to air-dry.

•Heat-fix the slide by passing it through the flame of a Bunsen burner two or three times, or until the slide is slightly warm when touched to the back of the hand.

• Heat coagulates the protein of the cells so that the cells stick to the glass surface and are not washed off during the staining and rinsing procedures.

•Do not overheat the slide, however, as this may cause distortion of cell shape and uneven staining.

•Allow the slide to cool, then flood with a stain crystal violet for 1 min.

•At the end of the staining time, pour off the stain and wash the slide gently but thoroughly with tap water.

• Blot dry with a paper towel.

•Examine the dry smear using low-power, high-power and oil-immersion objectives.

• Describe the cellular morphology of each bacterial species.

Negative staining:

- Place a drop of nigrosin towards one end of the slide.

- Place a loopful of inoculums into the drop of stain and mix with a sterile inoculating loop.

- Place a slide vertically against a drop od suspension of organism and allow the drop to spread along the edge of the slide.

- Push the second slide away from the previously spread drop of organism to form a smear

- Observe under microscope.

- Bacteria are negatively charged and the dye is an acidic dye.
- The acidic stain with negative charge will not penetrate the bacterial cell due to the negative charge of the bacterial cell and hence the unstained cells are clearly discernible against a coloured background

DIFFERENTIAL STAINING

INTRODUCTION

Differential staining is a procedure that takes advantage of differences in the physical and chemical properties of different groups of bacteria.

It allows us to differentiate between different kinds of bacterial cells or different parts of a bacterial cell.

GRAM STAINING:

The most commonly used differential stain is the Gram stain, first described in 1884 by Christian Gram, a Danish physician.

The Gram reaction divides bacteria into two groups, those which are Gram-positive and those which are Gram-negative.

Those organisms which retain the primary stain (crystal violet) are stained purple and are designated Gram-positive;

those which lose the crystal violet and are subsequently stained by a safranin counter stain appear red and are designated Gram-negative.

1. **The crystal violet treatment must precede iodine treatment.**

   Iodine acts as a mordant, i.e., it increases the affinity of the cells for the crystal violet.

   Iodine alone has no bacterial staining capabilities.

2. **Decolorization must be short and precise.**
   Too long an exposure to 95% alcohol will decolorize Gram-positive as well as Gram-negative cells.

   One explanation for the differential staining reaction emphasizes the higher lipid content of the cell walls in Gram-negative bacteria.

   During the decolorization step, alcohol may extract the lipids, increasing the porosity or permeability of the cell walls. Thus, the crystal violet-iodine complex is easily lost.

The Gram-positive bacteria, however, do not have lipid-rich cell walls.

Their cell walls become dehydrated during the alcohol treatment, decreasing the porosity so that the crystal violet-iodine complex is retained.

It is important to note that Gram-positive organisms are not always constant in their staining reaction.

Older cultures of some Gram-positive bacteria are subject to autolysis

Enzymatic breakdown of the cell wall causes older cells to become Gram-variable (both red and purple cells present) or Gram-negative.

The pH of the culture medium will also influence the staining of Gram-positive cells.
Cultures for Gram staining should be grown in media low in sugars to avoid the formation of acidic end products during cell growth.

Materials:

1. 24-48 hour broth cultures
2. Gram stain reagents

a. crystal violet
b. Gram's (or Lugol's) iodine solution
c. 95% alcohol
d. Safranin

Procedure:

•Prepare smears of the organisms listed above and heat-fix smear in the usual way.

• Allow the slide to cool, then flood with crystal violet.

•After one minute, pour off the stain and wash the slide gently but thoroughly with tap water.

•Flood the smear with Gram's (or Lugol's) iodine.

After one minute, pour off the iodine solution and rinse with tap water.

At this point, try to remove most of the excess water.

•Decolorize with 95% alcohol.

•Hold the slide at an angle and "drip" the alcohol over the smear. Observe these drips.

•As soon as the drips lose the faint touch of blue, rinse the slide with tap water immediately.

•The decolorization step should not have taken more than 15-20 seconds.

•Counter stain with safranin for 1 minute and wash gently with tap water.

•Blot dry and examine the smear under oil immersion.

•Record results including sizes of each morphotype in microns.

ENDOSPORE STAINING

Introduction

Endospores are hard dry structure which form inside certain cocci and rod genera.

When the vegetative (dividing) cell dies, the endospore remains and preserves life.

They contain may hydrophobic amino acids and exist in what is known as a cryptobiotic state, this is a state with no measurable metabolic activity.

These tiny 'containers' have large amounts of dipiolinic acid and calcium that are not normally present in vegetative cells.

Most endospores have an outer spore coat known as exosporia.

Due to their metabolically inert life-form, they are resistant to drying, ultraviolet radiation and can survive for a time in strong bases, acids, and disinfectants.

They tend to resist staining with the normal aniline dyes used and when unstained are highly refractile (shiny, light reflective).

Endospores can be located terminally, subterminally, or centrally.

If the endospore diameter is larger than the vegetative cell that holds (sporangium), the vegetative cell is swollen.

The endospore characteristics are stable for each species and therefore are identification characteristics.

There are several genera that are capable of forming endospores in the bacterial kingdom.

There are four common rod genera that form endospores are *Bacillus, Sporolactobacillus, Clostridium* and *Desulfotomaculum*.

***Sporosarcina* and *Oscillospira* are two common cocci genera that form endospores.**

Materials

Three days old culture of *Bacillus*,
Microscope slides,
Schaeffer-Fulton endospore staining reagents:
Malachite green 5% aqueous (aq.),
Safranin, 5% aq.,
Paper towel,
Acid-alcohol,
Bunsen burner,
Striker,
Inoculating needle,
Clothes pin,
Water bottle,
Bibulous paper
Lens paper.

Objective:

Learn to stain endospore-forming rods using the Schaeffer-Fulton endospore staining technique and be able to stain non-endospore-forming cocci using the same technique and learn to interpret the procedure accurately.

To identify the bacterial genera that commonly form endospores.

Schaeffer-Fulton Endospore Stain

This technique requires that heat be applied to drive the primary dye (malachite green) into the endospore.

Once an endospore is dyed it is difficult to decolorize it.

Vegetative cells that are present are stripped of the primary dye by a water rinse.

The counter stain of safranin then dyes all non-endospore-containing cells pink.

Procedure:

I. Schaefer/Fulton Staining of *Clostridium* or *Bacillus*:

1.Make a smear of *Clostridium* or *Bacillus*.

2.Allow to air dry.

3.Heat-fix the organisms.

4.Place a piece of paper towel on the slide, securing with a clothes pin.

5. Apply malachite green to the slide and begin heating.

6. Heat 5 minutes.

DO NOT ALLOW STAIN TO BOIL. Add extra stain if it begins to dry out!

1.Cool briefly, remove the paper towel and rinse with deionized water for about 15- 30 seconds or until the water runs clear with no green present.

2.Apply safranin and allow to set for 1 minute. Rinse briefly with water.

3.Blot dry with bibulous paper. Remove any burned on dye with acid-alcohol.

Observe each slide through the microscope on oil immersion (100x).