

SPECIAL STAINING

(Endospore / Capsule / Flagella / Metachromatic Granules)

Spore Staining

If spore bearing organisms are stained with ordinary dyes, or by Gram's stain, the body of the bacillus is deeply coloured, whereas the spore remains unstained. The vegetative bacterial cells are stainable with aqueous dyes, but endospores possess permeability barrier that prevents stain/dyes from entering the spore coat unless the barrier is destroyed by heating, UV light, mechanical rupture or by treatment of acid. The tough spore coat is formed to protect the bacterial cells DNA and important proteins from adverse environmental conditions (excessive heating, short of nutrients, drying etc.) Spore coat is a complex multilayered structure containing high calcium ions and dipicolinic acid which makes the structure more tough .

Below spore coat, lies the peptidoglycan. Once the protective tough spore coat is penetrated, the stain/dye interacts with peptidoglycan to produce the desired effect of staining.

There are other staining methods to introduce dye into the substance of the spore. When thus stained, the spore tends to retain the dye and resist decolourization. Several methods such as Acid Fast Staining Method for Spores (Spores stained bright and protoplasm of the bacillus stains blue), Hansen's Method, Dorner's method and Schaeffer and Fulton's Method are widely applied methods for staining spores in proper.

Commonly used staining methods for endospores includes:

1. The Schaeffer-Fulton method - Most common method used to stain endospores.
2. Dorner method
3. Hansens method

1. Schaeffer-Fulton method for staining endospores

Malachite green stain (0.5% (wt/vol) aqueous solution)

0.5 g of malachite green

100 ml of distilled water

Decolorizer

Tap water,

Safranin counterstain

Stock solution (2.5% (wt/vol) alcoholic solution)

2.5 g of safranin O

100 ml of 95% ethanol

Working solution

10 ml of stock solution

90 ml of distilled water

Procedure:

Schaeffer-Fulton method:

1. Fix the air dried smear by passing over the flame 2-3 times.
2. Flood smear with malachite green and heat for 5 min. Do not boil.
3. Allow to cool and wash with water.
4. Counter stain with dilute safranin (working solution) for 1 min.
5. Wash the smear and air dry it.

Spore stains: Endospore takes bright green and bacterial cells are brownish red to pink.

Dorner method for staining endospores –

Spore stains: Bacterial cells are colorless, endospores are red, and the background is black.
Carbol Fuchsin – primary stain and counterstain Nigrosin .

Hansen's Method

Material: Bacterial smear, Cocn. carbol fuchsin, 5% Acetic acid, Loeffler's methylene blue.

Procedure:

1. Fix the air dried smear by passing over the flame 2-3 times.
2. Stain the smear as follows,
3. Flood smear with Conc. carbol fuchsin and heat for 5 min. Do not boil.
4. Allow to cool and wash with water.
5. Decolorize with 5% Acetic acid for 1 min. and wash with water.
6. Counter stain with Loeffler's methylene blue for 3 min.
7. Wash the smear and air dry the smear and observe under microscope.

Spore stains: spores stain red and the vegetative cells blue.

Capsular Staining

The best way to demonstrate capsules of bacterial cells is to stain them by some procedure, which differentiates them from the bacterial cell itself. Anthony's method (with Tyler's modification) to stain capsule is the simplest method.

Anthony's method (With Tyler's modification)

Staining solution:

Acetic crystal violet	
Crystal violet (35% dye content)-----	0.1 g
Glacial acetic acid -----	0.25 ml
Distilled water -----	100 ml

Procedure:

Anthony's method (With Tyler's modification)

1. Prepare a smear of bacterial culture on the slide.
2. Dry it in the air.
3. Stain for 4-7 minutes in the 'acetic crystal-violet' solution.
4. Wash with 20% aqueous copper sulphate ($\text{CuSO}_4, 5\text{H}_2\text{O}$)
5. Dry with blotting paper and examine.

Capsules stains blue violet; Bacterial cell stains dark blue.

Flagellar Staining

Robert Koch published staining procedure for bacterial flagella in 1877. Subsequently several modifications and methods were developed for staining flagella were developed. In 1930, Leifson published a simple flagella stain. Many modifications or alternative methods includes a wet-mount procedure of Mayfield and Innis and a more traditional dried-smear preparation ,combination of the wet-mount technique of Mayfield and Innis and the stain of Ryu suggested by Kodaka et al. ,overcame most difficulties in staining flagella. **Presque Isle Cultures flagella stain – ready made staining method available commercially.**

A silver-plating stain for flagella was developed in 1958 and simplified in 1977. Recently a fluorescent protein stain, NanoOrange from Molecular Probes (Eugene, OR), is being applied to screen for bacteria possessing flagella by light microscopy .

Materials

12-16 Hrs incubated bacterial culture, Microscope slides, 95% ethanol
Micropipette with sterile disposable tips, Distilled water.

Leifson flagella stain

Solution A:

Sodium chloride	1.5 g
Distilled water	100 ml

Solution B:

Tannic acid	3.0 g
Distilled water	100 ml

Solution C:

Pararosaniline acetate	0.9 g
Paraosaniline hydrochloride	0.3 g
Ethanol, 95% (vol/vol)	100 ml

Take equal volumes of solutions A and B and then add 2 volumes of the mixture to 1 volume of solution C. The resulting solution may be kept refrigerated for 1 to 2 months. (Ryu method - reagent stable at room temperature)

Preparation:

Bacterial cultures incubated for 12-16 Hrs can be used for flagellar staining. Collect small quantity of growth from agar medium and emulsify in 100 ml of distilled water. Take in a micro-centrifuge tube mix by gentle vortexing. Avoid too much of inoculums.

If culture is used from incubated broth, centrifuge the culture, remove spent medium.

Resuspend in 100 ml of distilled water by gently vortexing, again centrifuge, and remove supernatant. Finally, resuspend in 200 ml of distilled water and prepare slightly cloudy emulsion to be used for staining.

Preparation of smear:

1. Take ethanol treated clean new microscope slide and flame to dry before use.
2. Cool the slide, place 5 to 10 ml of the culture emulsion on one end of the slide and spread it with the help of pipette.
3. Dry at room temperature. Do not heat fix.(Heating destroys the flagella)

Staining procedure: Leifson flagella staining method

1. Take a prepared slide and mark an area of 1x1.5 inch² with grease pencil.
2. Flood Leifson dye solution on the slide within the marked area.
3. Incubate at room temperature for 7 to 15 minutes or allow to act till formation of fine precipitate.(Golden film develops on the dye surface).
4. Remove the stain by gentle wash with water steam and air dry.
5. Observe under oil immersion.

Bacterial body and flagella will stain red

Staining of Metachromatic Granules - Albert's Method

Metachromatic granules:

Special stains (Albert, Neisser) pick out the volutin granules and give the bacilli a beaded or barred appearance; the granules are polar in short bacilli. Volutin staining reactions are best seen in young cultures .

Albert staining :

Albert's stain

Toluidine blue	1.5 g
Malachite green	2.0 g
Glacial acetic acid	10 ml
Alcohol (95% ethanol)	20ml
Distilled water	1000ml

Dissolve the dyes in the alcohol and add to the water and acetic acid. Allow to stand for one day and then filter.

Albert's Iodine

Iodine	6 g
Potassium iodide	9 g
Distilled water	900ml.

Procedure:**Albert staining:**

1. Make the film dry and fix by heat.
2. Cover slide with Albert's stain and allow to act for 3-5 min.
3. Wash in water and blot dry.
4. Cover slide with Albert's iodine and allow to act for 1 minute.
5. Wash and blot dry.

Metachromatic granules stain bluish black; the protoplasm green and other organisms mostly light green.

Exercise:

1. Explain why spore coat is impermeable for routine dyes.
2. What is the role of tannic acid in staining flagella..
3. Difference between capsule & slime layer

Reference:

Clark W. A., 1976 A Simplified Leifson Flagella Stain, *J. Clin. Microbiol.*, p. 632-634 Vol. 3, No. 6.

Heimbrook, W L Wang and G Campbell, 1989. **Staining bacterial flagella easily** *J. Clin. Microbiol.*, 27(11):2612.

Kodaka, H., A. Y. Armfield, G. L. Lombard, and V. R. Dowell, Jr. 1982. Practical procedure for demonstrating bacterial flagella. *J. Clin. Microbiol.* 16:948-952.

Leifson, E.1930. A method of staining bacterial flagella and capsules together with a study of the origin of flagella. *J. Bacteriol.* **20**:203–211.

Leifson, E.1951. Staining, shape and arrangement of bacterial flagella. *J. Bacteriol.* **62**:377–389.

Rhodes, M. E. 1958. The cytology of *Pseudomonas* spp. as revealed by a silver-plating staining method. *J. Gen. Microbiol.* **18**:639–648.

Ryu, E. 1937. A simple method of staining bacterial flagella. *Kitasato Arch. Exp. Med.* 14:218-219.

Schaeffer, A. B., and M. Fulton. 1933. A simplified method of staining endospores. *Science* **77**:194.

www.microbelibrary.org
