

Capsule Stain Protocols

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Information

History

Since the 1900s various methods have been devised to observe bacterial capsules (7). One very simple approach is mixing cells in a preparation of India ink. The large particles of ink will not penetrate the tight layers of the capsule or stain the bacterium. The particles of the ink will however provide a negative background that allows visualization of cells and capsules.

Capsules may be visualized by methods including electron microscopy, phase-contrast microscopy, and various staining techniques. Two staining techniques originally developed in the early 1900s will be described in this protocol section: Anthony's capsule stain was developed by E. E. Anthony in 1931 (1) and Maneval's staining method (8).

Note: encapsulated bacteria when growing on agar media take on a characteristic smooth appearance. Bacterial colonies of encapsulated bacteria have been described as "smooth" and colonies of nonencapsulated bacteria described as "rough" (5, 6). The classic experiment that revealed DNA as the transforming principle in bacterial DNA transformation involved the transformation of a rough strain of *Streptococcus pneumoniae* to a smooth strain (6).

Purpose

The purpose of the capsule stain is to reveal the presence of the bacterial capsule. The water-soluble capsule of some bacterial cells is often difficult to see by standard simple staining procedures or after the Gram stain. Capsule staining methods were developed to visualize capsules and yield consistent and reliable results.

Theory

Many bacteria, including both gram-positive and gram-negative, may be surrounded by an outer polysaccharide-containing layer termed the glycocalyx (Madigan, et al., 2000). When the composition of this layer is tightly bound and remains attached to cells, it is referred to as a capsule. More loosely bound layers that are difficult to see and do not exclude particles (India ink) are termed slime layers (Madigan et al., 2000). Capsules are usually composed of polysaccharides; however they may also contain polyalcohols and polyamines (Madigan et al., 2000). The

capsule of *Bacillus anthracis* is composed of polymers of amino acids (10).

In the case of human pathogens, a large number of different capsule serotypes have been identified. For example, over 80 different capsular polysaccharides or K antigens have been described for *Escherichia coli* (13, 15). For *Streptococcus pneumoniae*, over 90 different serotypes exist defined by unique antigenic components of capsular polysaccharides. The presence of the capsule is required for virulence in *Streptococcus pneumoniae* disease, and capsule antigenic variation provides significant challenge to vaccine efforts (14). Beta-hemolytic streptococci have been classified serologically on the basis of Lancefield group antigens found in the capsule.

Capsules are considered protective structures. Various functions have been attributed to capsules including protection from desiccation (11, 13) and adherence to surfaces and other bacteria contributing to biofilm formation (13). Capsules also often play a role in pathogenicity (3) acting as virulence factors to protect cells from phagocytosis and/or complement-mediated killing. Important plant pathogens such as strains of *Pseudomonas*, *Rhizobium*, and *Erwinia* require capsules for pathogenicity (13).

The size and constituency of the capsule varies with species and strains. Biosynthesis and assembly of capsules is a complex process (12, 15). The synthesis of the capsular material depends upon the environment, and for specific strains of bacteria, capsules are not required at all times. Organisms lacking capsules grow well at least under laboratory conditions, but it is important to note that these layers are essential for survival in certain natural environments and in some cases responsible for virulence of bacterial pathogens (e.g., *Streptococcus pneumoniae*). In the laboratory, it is common that special media such as milk broth or litmus milk broth are used to support the growth of encapsulated strains of bacteria.

Capsular polysaccharides are highly hydrated molecules containing over 95% water (13). As such, capsules are best demonstrated in preparations without heat fixation as heat will cause distortion and shrinking of the capsule.

Capsules are characterized by poor staining with standard dyes. Capsule staining methods thus depend upon revealing the presence of the capsule indirectly. Often capsule staining methods are accomplished using a combination of the following: (i) a basic dye that interacts with the negative ions of the bacterial cell, (ii) a mordant that causes the precipitation of the capsular material, e.g., metal ions, alcohol, and acetic acid (9), and (iii) an acidic stain used to color the background.

At the completion of the preparation, the capsule is revealed as a clear halo between the colored background and the stained cell. In some capsule staining preparations, cells are exposed to antibody against capsular antigens to enlarge the capsule for easier visualization (9).



FIG. 1. Encapsulated *Bacillus* sp. stained using Maneval's capsule staining method. Note the pointer indicates the clear halo.

Various protocols for revealing capsules through staining methods are described in the literature. Two methods are outlined here: Anthony's capsule stain and Maneval's capsule staining method.

In Anthony's capsule stain, crystal violet is used as the primary stain, interacting with the protein material in the culture broth or added during the staining, and copper sulfate serves as the mordant. There is no additional negative stain. At the completion of the stain, the bacterial cells and the background will be stained by the crystal violet while the unstained capsule will appear white.

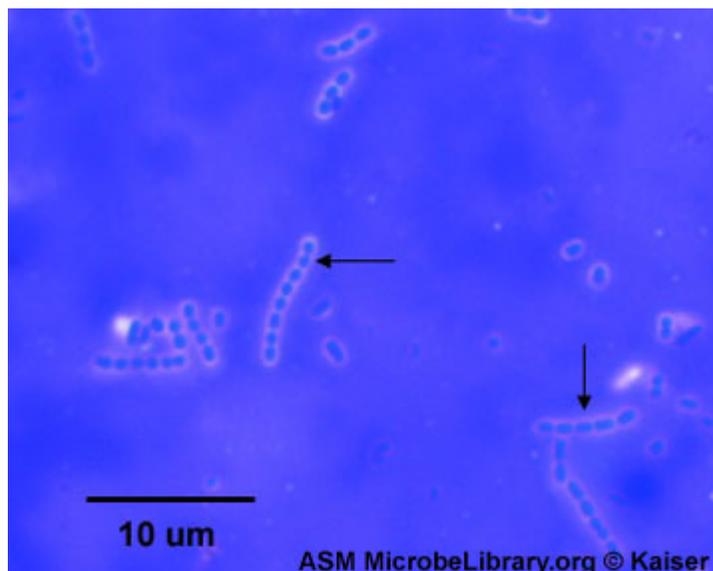


FIG. 2. Encapsulated *Streptococcus lactis* stained using Anthony's capsule stain.

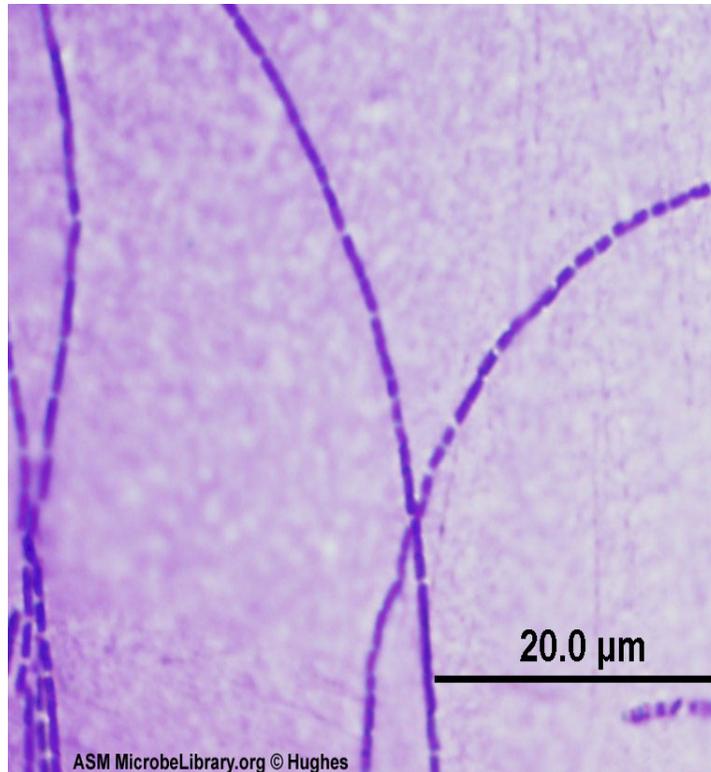


FIG. 3. Nonencapsulated *Bacillus megaterium* stained using Anthony's capsule stain.

In Maneval's capsule staining method, the basic stain that interacts with the bacterial cell is acid fuchsin. Acid fuchsin is present in the formulation of Maneval's solution. The counterstaining is provided by Congo red. In the procedure for Maneval's capsule staining method, the bacterial cells are first introduced into a drop of Congo red solution on a slide. Congo red is a pH indicator that is blue at or below pH 3.0 and red above pH 5.2. At this step, Congo red dries to form a red background on the slide. Maneval's solution (a mixture of acetic acid and acid fuchsin) is added to the slide. The acetic acid lowers the pH in the sample and causes the Congo red to change from a red color to blue. The acid fuchsin (a basic dye) interacts with the bacterial cell, staining the cell bright red. The acetic acid serves as a mordant to stabilize the capsule structure. The capsule remains unstained, and, using the light microscope, it is clearly seen as white in this red, white, and blue preparation. (4, 8).



FIG. 4. Encapsulated *Bacillus* sp. stained using Maneval's capsule staining method. Note that the capsule is seen as a clear halo around the rod-shaped bacterium.

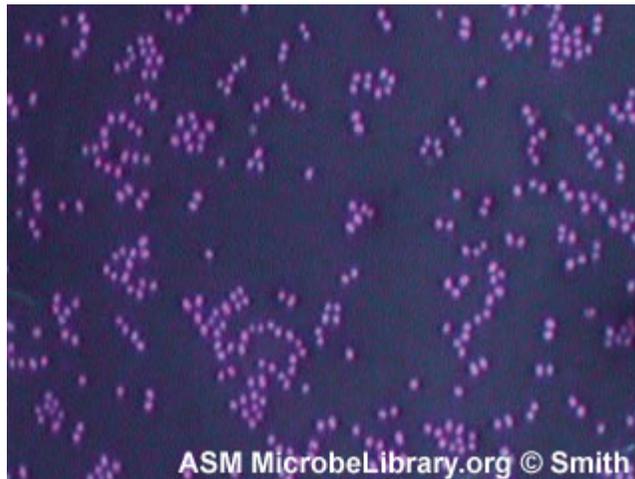


FIG. 5. *Staphylococcus epidermidis* stained using Maneval's capsule staining method. Note that there is no halo surrounding the cocci-shaped cells.

RECIPES

Milk broth culture recipe

Modified from *Handbook of Microbiological Media* (2)

Composition per liter:
9.5 g of skim powder milk
Autoclave 15 minutes at 15 psi.

Litmus milk recipe

Handbook of Microbiological Media (2)

Composition per liter:
100.0 g of skim powder milk

0.5 g of Azolitmin
0.5 g of Na₂SO₃
pH 6.5

Autoclave for 20 minutes at 10 psi and 115°C.

PROTOCOL FOR ANTHONY'S CAPSULE STAIN

A. General materials

- Staining tray
- Staining rack
- Slide holder
- Disposable gloves

B. Staining reagents

- Crystal violet 1% solution (primary stain)
- Copper sulfate 20% (decolorizer agent)

C. Procedure

1. Prepare a smear from a 12- to 18-hour culture grown in milk broth or litmus milk. (Serum protein may be used to prepare the smear if the organism was not grown in milk broth or litmus milk.) This is to provide a proteinaceous background for contrast.
2. Allow the smear to air dry. DO NOT HEAT FIX (to avoid destroying or distorting the capsule or causing shrinkage).
3. Cover the slide with 1% crystal violet for 2 minutes.
4. Rinse gently with a 20% solution of copper sulfate.
5. Air dry the slide. DO NOT BLOT. (Blotting will remove the un-heat-fixed bacteria from the slide and/or cause disruption of the capsule.)
6. Examine the slide under an oil immersion lens. Bacterial cells and the proteinaceous background will appear purplish while the capsules will appear transparent.

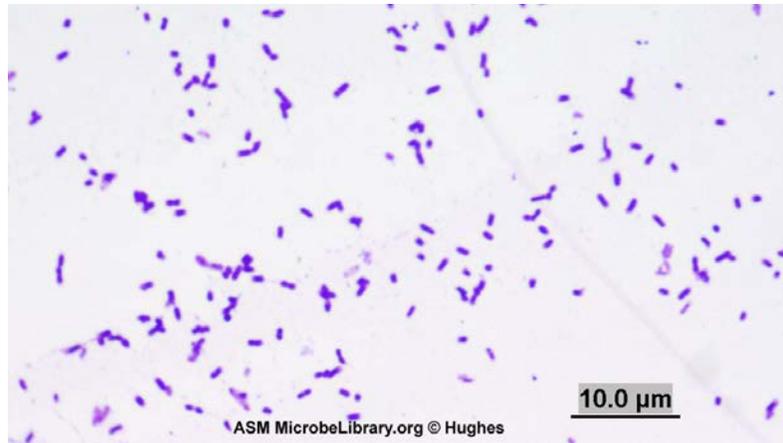


FIG. 6. Encapsulated *Klebsiella pneumoniae* grown in nutrient broth and stained using the Anthony's capsule stain. Note that no capsule is visible because no milk or other proteinaceous material was present or added to take up the crystal violet stain and provide a background.



FIG. 7. Encapsulated *Serratia marcescens* grown in skim milk broth and stained using the Anthony's capsule stain.

PROTOCOL FOR MANEVAL'S CAPSULE STAINING METHOD

A. General materials

- Staining tray
- Staining rack
- Slide holder
- Disposable gloves

B. Staining Reagents

- Congo red (1% aqueous solution)
- Maneval solution (see recipe or available from Carolina Biological Supply Company, NC)
- 0.05 g of fuchsin
- 3.0 g of ferric chloride
- 5 ml of acetic acid (glacial)
- 3.9 ml of phenol (liquified)
- 95 ml of distilled water

Prepare in a fumehood.

C. Procedure

1. Place a few drops of Congo red on a clean slide. Do NOT use a water drop in this sample preparation.
2. Mix in a small amount of culture.
3. AIR DRY. DO NOT HEAT FIX. (Heat fixing destroys protein capsules; it could also dehydrate the cells which would result in shrinking of the capsule)
4. Place the slide on a rack of the staining tray. Gently flood the smear with the Maneval solution and wait 5 minutes.
5. Lift the slide and gently pour off excess stain.

Rinsing with water is the next step. Rinsing must be done with great care. As the sample was not heat fixed to the slide, the sample preparation is fragile. Suggestions for careful rinsing:

- Place slide on bottom of staining tray.
- Add water to tray in the opposite corner from the placement of the slide. Add the water SLOWLY until a large puddle forms.
- Tilt staining tray and allow water to rinse the slide. Remove slide from tray.

6. Place slide on absorbent paper. Allow sample to AIR DRY. Do not blot.
7. Examine the slide with an oil immersion lens at 1,000X magnification.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

COMMENTS AND TIPS

1. Be very careful when preparing the smear or capsule or slime will be lost. Do not shake the media; slime layers can be shaken off with vigorous shaking of the tube.
2. Capsules are seen better at the edges of the slide, so scan the slide for a “good bacterial smear” (hard to keep capsule intact).
3. One of the organisms used in the Capsule Stain Atlas is the soil organism *Paenibacillus velaei*. This gram-positive bacillus (rod) has an extremely large, transparent capsule. *P. velaei* is a Biosafety Level 1 organism, and thus suitable for any lower-level microbiology laboratory. Burk’s medium is used for growing *Paenibacillus* due to the enhanced production of the polysaccharide capsule in this medium resulting in an extremely large capsule.

This section is to evolve as feedback on the protocol is discussed at ASMCUE. Please contact the project manager for further information.

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