Endospore Stain Protocol

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Information

History

Although endospores were observed and reported by numerous scientists (including Perty, 1852; Pasteur, 1869; Koch, 1876; and Cohn, 1872), these structures were primarily described as refractile bodies because they could not be stained using the typical compounds (carmine, methylene blue, carbol fuchsin, safranin, etc.) applied in early staining protocols.

Once the endospores were understood to be a dormant form of certain bacteria that demonstrated a unique resistance to treatment with heat, research efforts emphasized control of the endospores to improve sterilization, prevent infection, and limit contamination of foods.

In 1922, Dorner published a method for staining endospores. It employed a lengthy heating step but resulted in differential staining of endospores and vegetative cells in the same sample. Endospores and free spores appeared green or blue in contrast to red dye taken up by the vegetative cells.

Shaeffer and Fulton modified Dorner's method in 1933 to make the process faster, but heating with a Bunsen burner was still messy. As researchers identified what was required to make spores stainable, additional modifications to the Dorner and Shaeffer-Fulton methods were made to make staining and viewing spores occur quickly, easily, with less mess, and with sharp contrast (Bartholomew and Mittwer, 1950; Bartholomew, Roberts, and Evans, 1950; Robinson, 1951; Bartholomew and Mittwer, 1952; Fitz-James, 1953; Bradley and Williams, 1957; Hashimoto, Black, and Gerhardt, 1959; and Foster, 1960).

For many years, just identifying the presence of endospores (and/or free spores) was sufficient. Often, the spores' refractility was evident with simple staining or Gram staining. Characterization or identification of the organism could proceed using other criteria. Even today, spore stains are applied in the early steps of bacterial identification. Since relatively few genera of bacteria form endospores, it is a significant finding to learn of the presence of endospores in an unknown bacterial sample.

Beyond identification of unknown organisms, there is a line of research

devoted to understanding the nature of the endospore and its composition. In 1941, Georges Knaysi proposed a differential staining process that would allow individual layers within a cell to be recognizable. Work that followed in the 1950s and 1960s determined that endospores contained higher levels of calcium than did the vegetative cells, dipicolinic acid (not found in vegetative cells), and very low water content. These advances and significant developments using electron microscopy have dramatically improved the body of knowledge about the endospores of bacteria. (1, 6, 8).

Purpose

Gram staining and simple staining techniques may or may not reveal the presence of endospores in a bacterial sample. Since confirming the presence or absence of the endospores establishes the identification pathway to pursue, methods for visualizing the endospores and the free spores in a sample were developed. Relatively few species of bacteria produce endospores, so a positive result from endopore staining methods is an important clue in bacterial identification. In a clinical setting, identification of pathogens includes describing the shape and position of the endospores present. During microscopic examinations, the endospore stain can yield this information. While staining is probably the most common approach, laboratories that use phase-contrast microscopy can obtain the same information without the staining step (9; http://www.hpa-standardmethods.org.uk/documents/bsopid/pdf/bsopid8.pdf).

Theory

Differential stains are useful when it is important to visualize structurally different organisms. Like the Gram stain and the acid-fast stain, the endospore stain is a differential stain. Structurally, endospores are different from vegetative cells, so the endospore stain is used to demonstrate endospores forming within vegetative cells and make free spores easy to detect (8, 9, 14).

Bacterial endospores are differentiated cells formed within the vegetative cells. In bacteria, the endospores serve as a protective structure for survival of the organisms; they do not have a role in reproduction (9, 14). Some endospores are visible within vegetative cells; others appear outside of vegetative cells when the cellular content that surrounds an endospore disintegrates (lyses). The endospores located outside of cells are called free spores (2).

Most vegetative cells are stainable with standard aqueous dye solutions, but endospores possess a permeability barrier (Bartholomew and Mittwer, 1950) that prevents dyes from entering unless the barrier is destroyed by heat, chemicals (acid), ultraviolet light, or mechanical rupture (8). The barrier develops as the spore coats are formed to protect the cell's DNA and vital proteins from adverse environmental conditions (excessive heating, drying, radiation, low nutrient content, etc.). An endospore is structurally and chemically more complex than

the vegetative cell it comes from. Externally, there are more layers, and as the layers form increases in calcium ions and the synthesis of dipicolinic acid impart traits that make the endospore chemically unique. Endospores are often called metabolically inert. The low level of endospore activity is associated, at least in part, with the low water content. Researchers studying the resistance to staining exhibited by endospores eventually recognized the dehydrated state as the critical feature that both protects the cell and makes staining the spores especially difficult (1).

The cortex of the endospore is located below the spore coat and contains peptidoglycan. Once the protective layers have been penetrated, the typical dyes applied in staining can interact with the petidoglycan to produce the desired staining effect (8, 9).

The Schaeffer-Fulton method (or a modification) is the most common method used to perform endospore staining in undergraduate microbiology courses. A suspension of the culture is applied to a slide and allowed to air dry. The sample is heat fixed and fitted with a piece of absorbent paper. The slide is flooded with malachite green solution and a heat source is used to steam the stain into the cells and the spores. This requires approximately 5 minutes and must be done carefully to achieve steaming and not charring of the sample. This heating step stains the vegetative cells and the endospores. Once the heating period is complete, the slide is washed gently with tap water. The water acts as a decolorizer for the vegetative cells, but the stain is not released by the endospores and free spores. The safranin counterstain is used on the slide to give color to the vegetative cells (4, 7). The endospores will have retained the malachite green, appearing green (sometimes a little bluish), and the vegetative cells will be brownish-red or pinkish.

Many people find the steaming steps required in the endospore procedure and the acid-fast staining procedure to be messy, time-consuming, and variable in result. Consequently, since the 1930s, others tried to simplify the procedure by using increased dye concentrations, longer staining times, UV light, and other mechanisms to achieve results (Bartholomew,1950; Knaysi, 1948; Lechtman, 1965). Many of the methods are quite satisfactory, while others have their limitations in applying the method widely. As improvements in microscope technology occurred, the use of phase-contrast microscopy replaced the staining step in some applications. In larger laboratories, phase-contrast microscopes are often employed for faster more detailed results.

Sometimes determining the presence of spores is not the sole goal of performing an endospore test. Examining a sample for spore placement as well as the proportion of spores to vegetative cells may be useful in studying particular cultures or making diagnostic decisions in clinical samples. The environmental conditions of a culture or its habitat will determine what may be viewed. Young cultures of spore-forming microbes may not demonstrate any endospores because the vegetative cells may not have been subjected to sufficient stress to stimulate sporulation. To improve the odds that spore formers can be detected,

most methods suggest using cultures that are 18 to 36 hours old. Even with some standardization, there will be variation in the proportions of endopores to vegetative cells among species (Bartholomew and Mitwer, 1950; and Bartholomew, Roberts, and Evans, 1950).
Endospore staining is one tool in the arsenal of bacterial identification. With modifications, it is also useful in detecting spores produced by certain eukaryotes (e.g., *Cryptosporidium*). Advances in microscopy have lead to alternative and/or parallel testing for endospores, and continuing research to explain their structure and chemistry is testimony to their importance in the life cycle of the bacteria which form them.

RECIPES (4, 5)

A. Dorner method for staining endospores

Carbolfuchsin stain

0.3 g of basic fuchsin
10 ml of ethanol, 95% (vol/vol)
5 ml of phenol, heat-melted crystals
95 ml of distilled water

Dissolve the basic fuchsin in the ethanol; then add the phenol dissolved in the water.
Mix and let stand for several days. Filter before use.

Decolorizing solvent (acid-alcohol)

97 ml of ethanol, 95% (vol/vol)
3 ml of hydrochloric acid (concentrated)

Counterstain (Nigrosin solution)

10 g of nigrosin
100 ml of distilled water

Immerse the solution in boiling water for 30 minutes. Cool. Add 0.5 ml of formalin to preserve. Filter before use.

In a less messy variation of the Dorner method, the decolorizer is omitted and a 7.0% (wt/vol) aqueous solution of the nigrosin is used. See the protocol for details.

B. 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

Decolorizing agent

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

PROTOCOL (4, 5)

Note: the protocols that follow are those reported by Gerhardt et al. in the 1981 and 1994 editions of the textbooks noted here. Many common modifications of these protocols are used by professionals around the world. Some of the modifications are addressed in the Tips and Comments section as a result of valuable input from the reviewers.

A1. Dorner method for staining endospores

1. Air dry or heat fix the organism on a glass slide and cover with a square of blotting paper or toweling cut to fit the slide.

2. Saturate the blotting paper with carbolfuchsin and steam for 5 to 10 minutes, keeping the paper moist and adding more dye as required. Alternatively, the slides may be steamed over a container of boiling water.

3. Remove the blotting paper and decolorize the film with acid-alcohol for 1 minute; rinse with tap water and blot dry.

4. Dry a thin even film of saturated aqueous nigrosin on the slide.

5. Examine the slide under the oil immersion lens (1,000X) for the presence of endospores. Vegetative cells are colorless, endospores are red, and the background is black.

A2. Variation on the Dorner method for staining endospores

1. Mix an aqueous suspension of bacteria with an equal volume of carbolfuchsin in a test tube.

2. Immerse the tube in a boiling water bath for 10 minutes.

3. Mix a loopful of 7% nigrosin on a glass slide with one loopful of the boiled carbolfuchsin-organism suspension and air dry to a thin film.

4. Examine the slide under the oil immersion lens (1,000X) for the presence of endospores. Vegetative cells are colorless, endospores are red, and the background is black.

B. Schaeffer-Fulton method for staining endospores

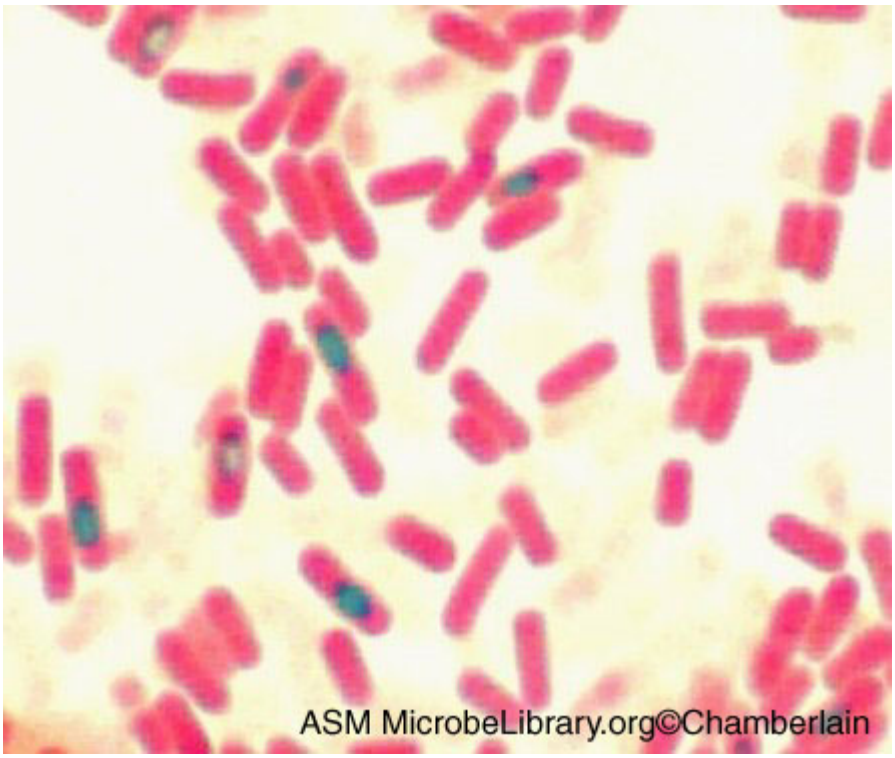
1. Air dry and heat fix the organism on a glass slide and cover with a square of blotting paper or toweling cut to fit the slide.

2. Saturate the blotting paper with malachite green stain solution and steam for 5 minutes, keeping the paper moist and adding more dye as required. Alternatively, the slide may be steamed over a container of boiling water.

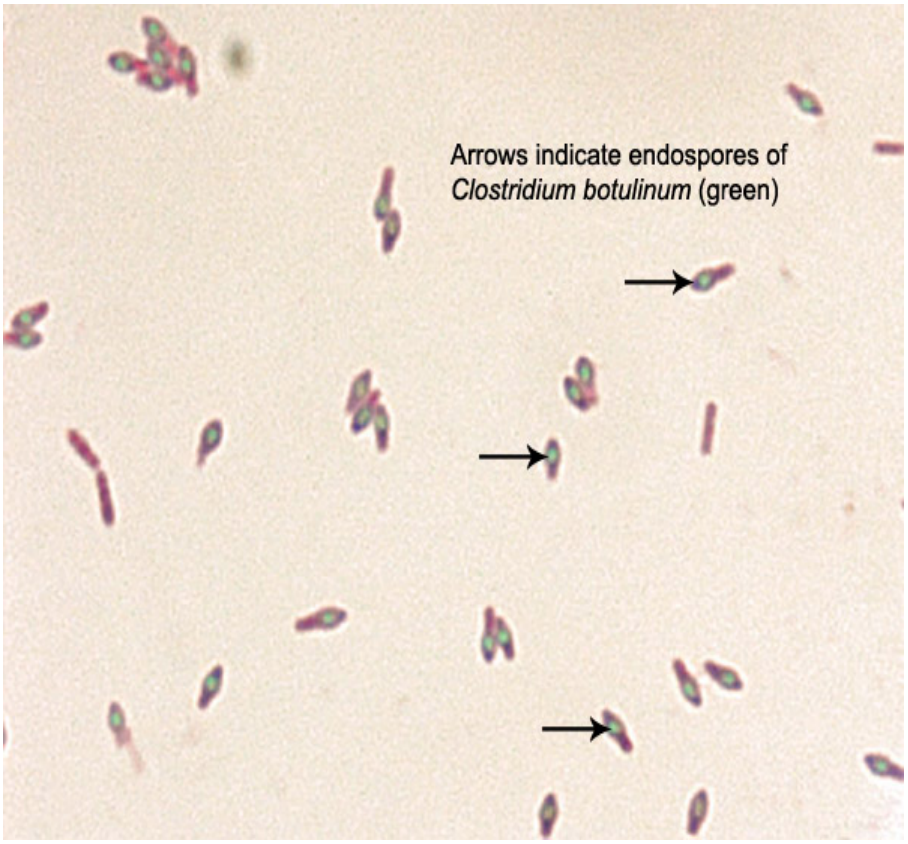
3. Wash the slide in tap water.

4. Counterstain with safranin for 30 seconds. Wash with tap water; blot dry.

5. Examine the slide under the oil immersion lens (1,000X) for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink.



Bacillus subtilis (Chamberlain, before 2002).



Clostridium botulinum (Public Health Image Library, before 2002).

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.

One safety concern with the endospore staining procedure involves the use of absorbent paper during staining. Please see the Comments and Tips section to review the suggestions that make the procedure safer for students to perform.

Material safety data sheet links

Basic fuchsin: http://www.jtbaker.com/msds/englishhtml/B0514.htm

Ethanol: www.emsdiasum.com/microscopy/technical/msds/15050.pdf

Phenol: www.jtbaker.com/msds/englishhtml/P1949.htm

Hydrochloric acid: www.jtbaker.com/msds/englishhtml/H3880.htm

Nigrosin: www.sciencelab.com/page/S/PVAR/23023/SLN1033

Malachite green: www.jtbaker.com/msds/englishhtml/M0286.htm

Safranin O: www.jtbaker.com/msds/englishhtml/S0240.htm

Potassium permanganate: www.jtbaker.com/msds/englishhtml/P6005.htm

Nitric acid: www.jtbaker.com/msds/englishhtml/N3660.htm

COMMENTS AND TIPS

Comments and tips came from discussions at the ASM Conference for Undergraduate Educators 2007.

1. Larger laboratories use phase-contrast microscopy instead of staining to demonstrate the presence of spores. The increased density of mature spores makes them very bright during observation with bright-field microscopy, but it makes the sporagia in a sample difficult to view. This problem is eliminated with phase-contrast microscopes, because these instruments capitalize on the differences in density among various components in a sample (2).

2. **Term.** Free spores: free spores are those structures that demonstrate no evidence of the cellular matter (sporangium) that surrounds the endospore during its formation (2).

3. Some labs do not use an absorbent paper as described in the Schaefer-Fulton procedure. When steaming over a water bath or using a Bunsen burner to heat the slide, it is possible for a poorly-fitted piece of paper to burn or drip. This hazard is avoided if the paper is cut to fit only over the area occupied by the specimen or in a shape that is smaller than the area of the slide where the stain will be applied.

4. Known spore formers are grouped into relatively few genera. Most are rods. Phylogenetically, the known spore formers reported in Table 12.25 of *Brock Biology of Microorganisms* (9) are all members of the low GC subdivision of the gram-positive bacteria.

In most introductory courses, students are asked only to recognize *Bacillus* spp. and *Clostridium* spp. as spore formers. Some instructors include *Sporosarcina* to make the point with students that a few cocci also have the capability of forming endospores. Demonstration of endospores is an important early step in identification protocols.

It is useful to know that there are many other genera that are included as endospore-forming bacteria. These genera are (9, p. 404): *Paenibacillus, Sporolactobacillus, Desulfotomaculum, Thermoanaerobacter, Sporomusa, Sporohalobacter, Anaerobacter, Alicyclobacillus, Amphibacillus, Heliobacterium, Heliophilum, Syntrophospora,* and *Desulfitobacterium*. All are rod shaped.

5. In clinical microbiology, there are many differentiating physical and chemical characteristics used in identifying pathogens. Among the

characteristics commonly included on microscopy reports are the shape and position of endospores. Endospores are either round or oval in shape and will be seen in terminal, subterminal, or central locations within the cells. (Terminal spores are at the ends of the cells, central spores are seen in the middle of the cell, and those organisms that produce subterminal spores will create the spore in between the very end of the cell and its middle (central) location.)

6. Safer ways to perform the endospore stain

A. One reviewer reports using no heating. The malachite green and the carbolfuchsin are left on the slide for 10 minutes. The reviewer reports that the procedure works well for *Bacillus subtilis* and*Bacillus cereus* but not for soil microbes.

B. There are many variations reported for cold staining. Some use the Schaefer-Fulton reagents, some use Wright's stain. All use longer exposure times than if heating was applied. (Author's note: the cold methods do not appear to be standardized and would primarily be useful for demonstrating the presence of spores not for describing the amount of sporulation seen in a sample. Additionally, some microbes may not respond adequately to these methods. If readers want to pursue the cold methods, the next edition of this protocol could compare techniques and provide more thorough descriptions of the reagents and steps in the protocol.)

7. This protocol was developed to emphasize a common staining method in an undergraduate microbiology course. In addition to the endospore staining methods, endospores can be seen in many Gram stain preparations, using negative staining, or with phase-contrast microscopy. However, researchers have used many laboratory techniques to study the characteristics of spores. One technique that does not usually include staining is called the "popping test." It is reported by Gerhardt, et al. (5) as a direct test that produces a visible phenomenon (12) occurring in dormant spores. The spores are exposed to an oxidizer such as 0.1% $KMnO_4$ in 0.3N HNO_3. This results in rupture of the spore cortex and the release of the spore protoplast. The event occurs after about 5 minutes in the solution, with some drama. The "popping" is visible under an oil immersion lens without staining. To use this technique, mount a dried film of spores on a coverslip and place it in the oxidizing reagent for 10 to 20 minutes. Apply the cover slip to a slide and examine using the oil immersion objective. Once ruptured, the contents of the spore are stainable with basic dyes, so adding stain may make it easier to see the popping in samples with a small number of spores (5).

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