

Bacterial Flagella Stain Protocol

Created: Monday, 08 September 2008

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Information **History**

Robert Koch published a stain for bacterial flagella in 1877 (16) and subsequently, several methodologies for staining these structures were developed (14). In 1930, Leifson published a simple flagella stain (6), and using this method he observed and concluded that mutations leading to nonflagellated from flagellated bacteria were common, as well as mutation leading to loss of motility without loss of the flagellum itself (7). A silver-plating stain for flagella was developed in 1958 (15) and simplified in 1977 (18). More recently a fluorescent protein stain, NanoOrange from Molecular Probes (Eugene, OR), has been used to screen for bacteria possessing flagella by light microscopy (3), as well as a commercially available staining procedure from Presque Isle Cultures (Erie, PA). However, staining and visualizing the flagellum in the classroom setting has always been somewhat difficult.

Purpose

The purpose of the protocol is to stain bacterial flagella, thus to reveal the presence or absence of flagella as well as their arrangement on the perimeter of the cell. These traits can be used to characterize bacteria phenotypically, as not all bacteria are flagellated and those that are will possess these structures in various locations extending from the cell membrane. Bacteria may possess a single polar flagellum (monotrichous), tufts of flagella at each pole (lophotrichous), one or more flagella at both poles (amphitrichous), or flagella surrounding the perimeter of the cell (peritrichous).

Bacteria may be motile by a variety of mechanisms (5), but the most common involves flagella. Procedures that reveal the presence of flagella will thus indicate bacterial motility. Powered by a proton motive force or in some instances a sodium gradient (12, 13), the flagellum can rotate up to an astounding 100,000 rpm in the case of some *Vibriosp.* (11) thus allowing, in this case, *V. alginolyticus* bacteria to swim at a maximal rate of 147 mm/sec (10). Motile bacteria can respond to chemical and light gradients via chemotaxis and phototaxis, respectively. The presence or absence of flagella is important to bacterial survival and growth, and visualization of these appendages by staining and subsequent light microscopy is an important tool for their characterization.

Theory

Bacterial flagella are composed of protein subunits called flagellin and are distinct in structure from flagella found on eukaryotic cells (17). Flagella are anchored to the bacterial cytoplasmic membrane and cell wall by basal bodies (1), and are assembled via flagellin subunits traveling through the basal bodies, then through the center of the flagellum itself, and are added to the distal end of the appendage (2, 4). The intrinsic structure of the flagellum is helical, making propulsion possible (8).

Flagella are of variable lengths that can extend several times the length of the bacterium itself, with widths generally between 10 and 50 nm (9). Due to their narrow width the best direct method of observing bacterial flagella is by the electron microscope, because the normal limit of resolution of light microscopy is ~200 nm. For those who do not have access to electron microscopy, bacterial flagella can be observed via the light microscope in combination with stains. All flagella stains use mordants, like tannic acid and potassium alum, to coat and thus thicken the flagellum in order to be within the limits of size observable by light microscopy.

The Leifson flagella stain method uses tannic acid (6, 7), while the Presque Isle method uses proprietary components. For the Leifson flagella stain, tannic acid and the dye form a colloidal precipitate that when absorbed by the flagellum causes it to increase in diameter and become colorized, thus amenable to viewing by light microscopy. The tannic acid-dye complex is more soluble in alcohol than water, and also more soluble with decreased pH. The alcohol concentration in the Leifson solution is sufficient to maintain solubility of the components. When the prepared sample is stained, the alcohol evaporates faster than the water, and the concentration of the tannic acid and dye increases to cause precipitation, leading to staining of the flagella. Salt concentration also affects the staining, presumably by altering charge of the tannic acid-dye complex and the flagellum itself. The flagella stain is finicky because many variables affect the outcome: age of the bacterial culture, thickness of the culture on the microscope slide, age of the staining solutions, pH, temperature, alcohol concentration, dye concentration, heat, and/or airflow.

PROTOCOL

The Leifson flagella staining procedure (7) herein was developed from that described in Murray et al. (14). As an alternative, commercially available reagents can be purchased from Presque Isle Cultures (Erie, PA) and flagella can be visualized by light microscopy using the manufacturer's reagents and staining procedure outlined below. Presque Isle Cultures recommends staining the flagella of *Pseudomonas fluorescens* as a positive control, but in theory the appendages of any flagellated bacterium can be viewed by this methodology or the Leifson stain.

Materials

- New microscope slides

- 95% ethanol and Kimwipes to clean slides
- Heat—provided by a Bunsen burner, alcohol lamp, ceramic heater, or other appropriate heat source
- Micropipettors to deliver 5 to 200 µl
- Sterile disposable tips for micropipettors
- Distilled water
- Bibulous paper

Solutions

Leifson flagella stain (14)

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

Mix equal volumes of solutions A and B; 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.

Presque Isle Cultures flagella stain

Solutions are available from Presque Isle Cultures. The components of the flagella stain are proprietary.

Flagella mordant (Solution I)

Silver stain (Solution II)

Solutions I and II can be stored at room temperature for several weeks.

A. Preparation of Cultures

Background

Solid or liquid media cultures can be used for flagella staining (7). Cultures should incubate between 16 and 20 hours before staining, as older cultures tend to lose flagella. For example, newer cultures are particularly important for *Bacillus* spp. that undergo spore formation and lose flagella during this developmental process. If vortexing is necessary for suspension after centrifugation of liquid cultures, or because cultures clump, do so gently as flagella are easily sheared from the bacterium.

Method

From an agar plate or slant cultures, prepare a suspension by removing a small amount of growth, approximately one-fourth of the colony, with an inoculating loop using proper aseptic technique. Emulsify in 100 ml of distilled water in a microcentrifuge tube by gently vortexing. The emulsion should be only slightly cloudy. Using too much inoculum results in the inability to visualize the flagella.

For staining from liquid cultures, Leifson (7) recommends two rounds of centrifugation and final suspension in distilled water to remove any medium components. Place 100 ml of the liquid culture in a microcentrifuge tube, centrifuge, and remove spent medium. Resuspend in 100 ml of distilled water by gently vortexing, again centrifuge, and remove supernatant. Form a slightly cloudy emulsion by resuspending in ~200 ml of distilled water. Gently vortex. Again, emulsions should be only slightly cloudy prior to proceeding to staining. Optimization of the washing procedure will most likely be necessary to maximize quality of flagella stain.

B. Preparation of Slides

1. Wipe clean a new microscope slide with 95% ethanol and a Kimwipe. Flame to dry thoroughly. Use slides immediately.
2. When the slide is cool enough to handle, label it using tape with the name of the organism you will be staining.
3. Place 5 to 10 ml of the culture emulsion on one end of the slide using a micropipettor and spread the emulsion using the same pipette tip held parallel to the microscope slide.
4. Allow the sample to dry at room temperature. Do not heat fix as this will destroy the proteinaceous flagella structure.

C. Flagella Staining

Leifson flagella stain (14)

1. Take a prepared slide and using a wax pencil draw a rectangle around the dried sample. Place slide on staining rack.
2. Flood Leifson dye solution on the slide within the confines of the wax lines. Incubate at room temperature for 7 to 15 minutes. The best time for a particular preparation will require trial and error.

3. As soon as a golden film develops on the dye surface and a precipitate appears throughout the sample, as determined by illumination under the slide, remove the stain by floating off the film with gently flowing tap water. Air dry.

4. View using oil immersion, at 1,000x magnification, by bright-field microscopy. Bacterial bodies and flagella will stain red.

Presque Isle Cultures flagella stain

1. Prepare slide as described above. Place slide on staining rack.
2. Flood slide with Presque Isle Cultures Solution I, the mordant. Incubate at room temperature for 4 minutes.
3. Gently rinse with distilled water. Shake excess water from slide.
4. Flood with Presque Isle Cultures Solution II, the silver stain.
5. Heat over Bunsen burner by moving slide back and forth, just until steam is emitted. If a Bunsen burner is not available then an alternate heat source can be used, but optimization will be necessary. Be careful not to overheat sample, as excess heat will destroy the flagella. Incubate at room temperature for 4 minutes.
6. Rinse with distilled water. Carefully blot dry with bibulous paper.
7. View using oil immersion, at 1,000x magnification, by bright-field microscopy. Bacteria and flagella will appear golden brown. Excess stain is often observed on the slides and illustrates the necessity of beginning the procedure with thoroughly cleaned microscope slides.

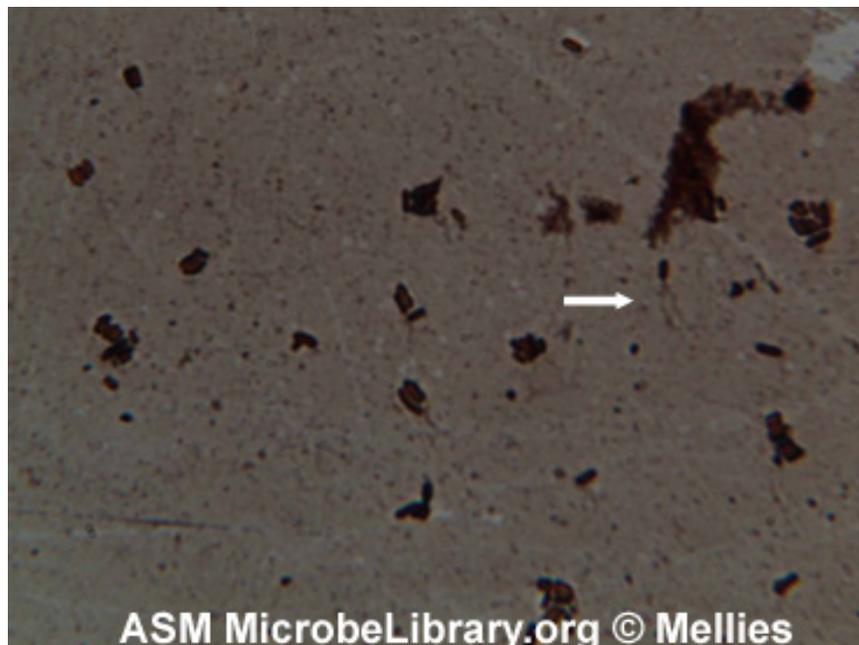


FIG. 1. Arrow points to lophotrichous flagella of *Pseudomonas fluorescens* cultured on nutrient agar, stained using the Presque Isle flagella stain, and visualized under bright-field microscopy using oil immersion at 1,000x magnification.

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](#).

TIPS AND COMMENTS

Chance of success increases with the number of slides prepared. In his modification of the Loeffler's flagella stain, Shunk (15) states that, "As a usual rule, flagella staining has been a more or less hit and miss process." I believe this still to be true, but a welcome challenge to a class of eager students (Jay Mellies, Reed College).

REFERENCES

1. **Cohen-Bazire, G., and J. London.**1967. Basal organelles of bacterial flagella. *J. Bacteriol.* **94**:458–465.
2. **Emerson, S. U., K. Tokuyasu, and M. I. Simon.** 1970. Bacterial flagella: polarity of elongation. *Science* **169**:190–192.
3. **Grossart, H. P., G. F. Steward, J. Martinez, and F. Azam.**2000. A simple, rapid method for demonstrating bacterial flagella. *Appl. Environ. Microbiol.* **66**:3632–3636.
4. **Iino, T.**1969. Polarity of flagellar growth in *Salmonella*. *J. Gen. Microbiol.* **56**:227–239.
5. **Jarrell, K. F., and M. J. McBride.**2008. The surprisingly diverse ways that prokaryotes move. *Nature reviews. Microbiology* **6**:466–476.
6. **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.
7. **Leifson, E.**1951. Staining, shape and arrangement of bacterial flagella. *J. Bacteriol.* **62**:377–389.
8. **Macnab, R. M.**1977. Bacterial flagella rotating in bundles: a study in helical geometry. *Proc. Natl. Acad. Sci. USA* **74**:221–225.
9. **Macnab, R. M.**1999. The bacterial flagellum: reversible rotary propellor and type III export apparatus. *J. Bacteriol.* **181**:7149–7153.
10. **Magariyama, Y., S. Sugiyama, K. Muramoto, I. Kawagishi, Y. Imae, and S. Kudo.**1995. Simultaneous measurement of bacterial flagellar rotation rate and swimming speed. *Biophys. J.* **69**:2154–2162.
11. **Magariyama, Y., S. Sugiyama, K. Muramoto, Y. Maekawa, I. Kawagishi, Y. Imae, and S. Kudo.** 1994. Very fast flagellar rotation. *Nature* **371**:752.
12. **Manson, M. D., P. Tedesco, H. C. Berg, F. M. Harold, and C. Van der Drift.**1977. A protonmotive force drives bacterial flagella. *Proc.*

Natl.

Acad. Sci. USA **74**:3060–3064.

13. **Muramoto, K., I. Kawagishi, S. Kudo, Y. Magariyama, Y. Imae, and M. Homma.** 1995. High-speed rotation and speed stability of the sodium-driven flagellar motor in *Vibrio alginolyticus*. *J. Mol. Biol.* **251**:50–58.

14. **Murray, R. G. E., R. N. Doetsch, and C. F. Robinow.** 1994. Light microscopy. *In* P. Gephardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general molecular bacteriology*. American Society for Microbiology, Washington, DC.

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

16. **Shunk, I. V.** 1920. A modification of Loeffler's flagella stain. *J. Bacteriol.* **5**:181–187.

17. **Silflow, C. D., and P. A. Lefebvre.** 2001. Assembly and motility of eukaryotic cilia and flagella. Lessons from *Chlamydomonas reinhardtii*. *Plant Phys.* **127**:1500–1507.

18. **West, M., N. M. Burdash, and F. Freimuth.** 1977. Simplified silver-plating stain for flagella. *J. Clin. Microbiol.* **6**:414–419.

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