NOTES

Practical Procedure for Demonstrating Bacterial Flagella

H. KODAKA,† A. Y. ARMFIELD, G. L. LOMBARD, AND V. R. DOWELL, JR.*

Anaerobic Bacterial Branch, Hospital Infections Program, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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We developed a simple, rapid method for demonstrating bacterial flagella with Ryu staining solution that gave satisfactory results for numerous motile and nonmotile bacteria. Two major advantages of this method are that the staining solution, ready for use, is stable at ambient temperature indefinitely and that microscopic examination of bacteria in the stained drop preparations can be performed rapidly.

Flagellum length, shape, curvature, number per cell, and arrangement on the cell are important phenotypic characteristics for taxonomic classification of bacteria (5, 10). These characteristics are also useful in identifying motile bacteria, especially nonfermentative aerobic, gram-negative bacilli (5, 6), encountered in clinical microbiology laboratories. In recent years, as isolates of Anaerobiospirillum, Campylobacter, Selenomonas, and Wolinella and other motile, gram-negative anaerobes from human clinical material are received more frequently by the Centers for Disease Control (CDC) Anaerobe Reference Laboratory, we have become increasingly aware of the differential value of examining bacterial flagella in the characterization and identification of anaerobic bacteria.

Numerous methods for staining bacterial flagella have been described by others. Some of the more commonly used methods include the original Leifson tannic acid-fuchsin method (7) and its modifications (1, 2, 4, 8, 9) and silver plating methods (3, 12). Although satisfactory results can be obtained with any of these methods if used correctly, each has certain drawbacks, such as unstable reagents, a requirement for scrupulously cleaned slides, or a need for heat treatment of slides during staining.

In this paper, we describe a simple, rapid procedure for staining bacterial flagella with a stable staining solution developed by E. Ryu, National Taiwan University, Taipei, Taiwan (11). The procedure is as convenient to perform as the Gram stain procedure.

Staining solution. (i) Solution 1. Mix 10 ml of 5% carboxylic acid solution (Mallinckrodt Chemicals, Inc., St. Louis, Mo.), 2 g of tannic acid (powdered; J. T. Baker Chemical Co., Phillipsburg, N.J.), and 10 ml of saturated aluminum potassium sulfate 12-hydrate (crystal, J. T. Baker Chemical Co.).

(ii) Solution 2. Prepare a saturated solution of crystal violet in ethanol (12 g per 100 ml).

(iii) Final stain. Mix 10 parts of solution 1 (mordant) with 1 part of solution 2 (stain) and store the mixture at ambient temperature. Ryu (11) recommends storing solutions 1 and 2 separately at ambient temperature and mixing them just before use. The practice of storing the staining solution in ready-for-use form was introduced to H. K. by S. Okuma, Nihon Universi-

† Present address: Department of Microbiology, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga setagaya-ku, Tokyo, Japan 138.

FIG. 1. Sketch illustrating how drop preparations of bacteria are prepared for demonstration of flagella. Colonies on anaerobe blood agar are carefully picked with an inoculating needle and transferred to 2 drops of water on a microscope slide by touching the surface of the water drops as shown. To avoid excessive loss of flagella, the bacteria and water are not mixed with the needle.
FIG. 2. Photomicrographs (×1,347) of flagellated bacteria grown on anaerobe blood agar and stained with Ryu stain as described in text. (a) 18-h culture of Proteus mirabilis with peritrichous flagella; (b) 48-h culture of Campylobacter jejuni with polar monotrichous and amphitrichous flagella; (c) 48-h culture of Campylobacter sputorum subsp. bubulus with polar monotrichous flagella; (d) 24-h culture of Campylobacter sputorum subsp. mucosalis with polar monotrichous and amphitrichous flagella; (e) 48-h culture of Wolinella succinogenes with polar monotrichous and amphitrichous flagella; (f) 72-h culture of Anaerobiospirillum sp. with polar multitrichous flagella.

Procedure for demonstrating bacterial flagella.

(i) Step 1. Streak a pure-culture isolate on CDC anaerobe blood agar (6) to obtain isolated colonies and incubate in an appropriate gaseous environment (candle extinction jar for facultative anaerobes; chamber containing 5% O₂-10% CO₂-85% N₂ for microaerophiles; anaerobic system containing 5% CO₂-10% H₂-85% N₂ for obligate anaerobes) at 35°C for 18 to 72 h.

(ii) Step 2. As soon as adequate growth is evident, pick a colony with an inoculating needle, taking care not to pick up agar, and lightly touch the needle to the center of each of 2 drops of distilled water on a new microscope slide (precleaned by the manufacturer) (Fig. 1).

(iii) Step 3. Let the drop preparations dry in air at ambient temperature.

(iv) Step 4. After the preparations are dry, flood the slide with the staining solution, stain...
FIG. 3. Photomicrographs (×1,347) of flagellated bacteria that were grown on anaerobe blood agar and stained with Ryu stain as described in the text. (a and b) 72-h culture of an unidentified microorganism with lophotrichous flagella isolated from human blood; (c) 72-h culture of an unidentified microorganism, source unknown; (d) 72-h culture of a gram-negative microorganism with polar multitrichous flagella, isolated from a human rectal abscess; (e) 72-h culture of a gram-negative microorganism with peritrichous flagella, isolated from human blood; (f) 48-h culture of an atypical strain of *Clostridium difficile* with subpolar monotrichous flagella.

for 5 min, and then thoroughly wash the slide (front and back) in running tap water for 2 to 3 min.

(v) **Step 5.** When the slides are dry, examine the stained preparations microscopically under an oil immersion objective (×97), beginning at the periphery and moving toward the center.

**Points relative to specific steps.** (i) **Step 1.** Leifson (10) and others (6) have pointed out how a low pH can have a deleterious effect on flagella and have recommended omission of fermentable carbohydrates from the culture medium if possible. For this reason, we used CDC anaerobe blood agar, which contains no added carbohydrates, for cultivation of bacteria to be examined for flagella. We made no attempt in our study to compare the flagellation of the bacterial strains at an incubation temperature other than 35°C. However, we are aware that some microorganisms may require a lower or a higher temperature of incubation for maximum flagellation (6, 10).

(ii) **Step 2.** There is general agreement in the literature that the age of the culture can have a dramatic effect on flagellation. In general, the best flagellation occurs in the logarithmic and
FIG. 4. Photomicrographs (×1,347) of flagellated bacteria that were grown on anaerobe blood agar and stained with Ryu stain as described in the text. (a and b) 24-h cultures of *Clostridium tertium* with peritrichous and subpolar monotrichous flagella; (c) *C. tertium* cell in the process of sporulation; (d) 24-h culture of *Clostridium sporogenes* with peritrichous flagella; (e and f) 24-h culture of *Clostridium septicum* with peritrichous flagella.

Notes:

Early stationary phases of growth (10). It is very important to take care in transferring bacteria from a colony to the droplets of water on the slide so that flagella are not dislodged from the bacterial cells. We have found that the special cleaning of slides recommended by others (2, 5, 6, 10) to demonstrate bacterial flagella is not necessary.

(iii) Step 3. It is important to dry the drop preparations at ambient temperature (do not heat to hasten drying) before proceeding to the staining step to prevent the flagella and bacterial cells from being distorted, dislodged, or both.

(iv) Step 4. Thorough washing of the front and backs of slides is required to prevent formation of artifacts which can hinder microscopic examination. Wiping the back of the slide with a cloth is not necessary if the slides have been properly washed.

(v) Step 5. Beginning the microscopic examination at the periphery of the preparation saves time because characteristic flagellated cells are usually found in greater abundance in the outer portion of the preparation than in the center. In our experience, the cells in drop preparations could be examined microscopically much faster.
than those in preparations prepared by allowing cell suspensions to run down the slide, which are the kind of preparations we have used in the past (2).

During the last 18 months, we have used the staining procedure to study more than 100 strains of motile and nonmotile bacteria with satisfactory results. These included members of the genera *Anaerobiospirillum, Bacillus, Bacteroides, Campylobacter, Clostridium, Escherichia, Fusobacterium, Listeria, Proteus, Pseudomonas, Serratia,* and *Wolinella.* Photomicrographs of representative flagellated bacteria of various species are shown in Fig. 2 through 4 to illustrate the quality of the results. On the basis of these results, we now routinely use the procedure, instead of a modification of the Leifson method (2), for demonstrating bacterial flagella in the CDC Anaerobe Reference Laboratory. We keep the Ryu staining solution at ambient temperature in a plastic washing bottle near the reagents for the Gram stain.

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**LITERATURE CITED**


