Staining Bacterial Flagella Easily

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A wet-mount technique for staining bacterial flagella is highly successful when a stable stain and regular slides and cover slips are used. Although not producing a permanent mount, the technique is simple for routine use when the number and arrangement of flagella are critical in identifying species of motile bacteria.

Staining bacterial flagella differs from staining other bacterial structures because it usually requires extraordinary care for the slides, stain, and cells. Our experience with the traditional flagellum-staining procedures (2, 4) and the newer variations (1, 3) has been unsatisfactory. There are usually few cells with stained flagella per slide, most cells are separated from their flagella, and cells are difficult to find within a heavily precipitated smear.

Other impediments include the need for acid-cleaned slides, freshly prepared stain, washed cells, Formalin-treated cells, and other handling that tends to remove flagella from cells. While these are not major obstacles, they discourage use of a flagellum stain, particularly when the success rate is irregular for observing cells with flagella intact. Clinical microbiologists may delay examining bacteria for the number and arrangement of flagella because of the time involved in making and scanning a preparation.

Recently, two techniques for staining flagella were published: a wet-mount procedure (5) and a more traditional dried-smear preparation (3). We discovered that a combination of the wet-mount technique of Mayfield and Innis (5) and the stain of Ryu (6), as suggested by Kodaka et al. (3), overcame most difficulties in staining flagella.

The Ryu stain has two components. Solution I, the mordant, contains 10 ml of 5% aqueous solution of phenol, 2 g of tannic acid, and 10 ml of saturated aqueous solution of

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FIG. 2. The precipitate in the background of the flagellum-stained *Alcaligenes faecalis* cells is typical of slides held overnight or of preparations from a rich liquid medium. Magnification, ca. ×1,500.

aluminum potassium sulfate–12 hydrate. Solution II, the stain, is a saturated ethanolic solution of crystal violet (12 g in 100 ml of 95% ethanol). The final stain was prepared by mixing 1 part solution II with 10 parts solution I and then filtering the mixture through filter paper to remove coarse precipitate. For use, the final stain was kept at room temperature in a syringe fitted with a 0.22-μm-pore-size porous membrane between syringe and needle. The needle was capped or stuck into a rubber stopper to prevent the stain from drying.

Bacterial cells grown in a noninhibitory medium for 16 to 24 h were used for flagellum staining; sheep blood agar plates

FIG. 3. Polarly flagellated cells of *Campylobacter jejuni*. Magnification, ca. ×1,500.
FIG. 4. Oligella ureolytica demonstrates a long polar flagellum with shorter lateral flagella. Magnification, ca. ×1,500.

and tryptic soy plates incubated overnight served well. Drops of motile cells were prepared in sterile water by using either of two nonstirring techniques: (i) touching the colony margin with an applicator stick (or wire) and then touching the stick into a drop of water on a clean slide; or (ii) touching a loopful of water to the colony margin, allowing cells to swim into this loopful of water, and then touching the loopful of motile cells to a drop of water on a slide. The faintly turbid drop was covered with a cover slip and examined for motile cells. After 5 to 10 min, when about half of the cells were attached to the glass of either slide or cover slip, two drops of Ryu stain were applied to the edge of the cover slip. The stain flowed under the cover slip by capillarity and mixed with the cell suspension. The cells were examined for flagella after 5 to 15 min at room temperature. To delay observation or to preserve the preparation overnight, the slide was held in a moist chamber.

Typical results of this staining procedure are presented in Fig. 1 to 4. Flagella on clinical isolates or control cultures of Bacillus cereus, Proteus mirabilis (Fig. 1), Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas cepacia, Alcaligenes faecalis (Fig. 2), Campylobacter jejuni (Fig. 3), Campylobacter fetus, Vibrio parahaemolyticus, Clostridium spp., and Oligella ureolytica (Fig. 4) were successfully and

FIG. 5. Caulobacter crescentus cells in liquid culture show stalked cells, polarly flagellated cells (swarmer cells), and stalked cells with flagellated daughter cells when stained with this technique. The flagella in the background were shed in the development of swarmer cells to stalked cells. Magnification, ×4,500. (Picture courtesy of J. S. Poindexter.)
dependably stained. Flagella on clostridia were stained by preparing drops of cells in an anaerobic chamber. The slides were removed from the chamber, and cover slips were applied as described above. Flagella on marine bacteria were stained in drops of sterile saline with good results.

For use with phase-contrast microscopy, the stain was prepared with 1/10 the amount of crystal violet described above; this dilute stain demonstrated both flagella and stalks on Caulobacter crescentus (Fig. 5). Additionally, the dilute stain greatly reduced precipitation so that 48 h later the preparation looked just as good as within the first 0.5 h (J. S. Poindexter, personal communication).

The stain was stable for weeks at room temperature. The staining reaction was temperature dependent; adequate staining required about 2 h at 10°C but as little as 2 min at 33°C. If cells older than 24 h exhibited flagellar motility in the wet mount, their flagella were stainable. Materials in rich nutrient media tended to cause background precipitation when cells were stained directly in drops of liquid cultures. Collecting motile cells in a loopful of water usually made a lighter suspension of cells than the applicator stick technique and ensured a higher proportion of flagellated cells. Agitating the loop or stick in the droplet on the slide sheared flagella from the cells. A proper wet mount had barely enough liquid to fill the space under the cover slip; small air pockets around the edge were useful in getting the stain to penetrate the mount. Cells with flagella were in a zone of optimum stain concentration usually halfway from the edge of the cover slip to the center of the mount. One subjective observation was that the blue-violet of the Ryu stain was easier to detect visually than the red of flagellar stains that use basic fuchsin.

This wet-mount technique was successfully employed by beginning students in microbiology; over 60% of the students stained flagella on their first attempt. Although the preparations are not permanent because the stain precipitates as the wet mount dries, there is little manipulation of cells for staining and the technique is easy to repeat. It is simple enough for routine use when the number and arrangement of flagella are critical in identifying species of motile bacteria. General advantages of this method are as follows: regular slides and cover slips can be used, the Ryu stain is stable at room temperature, cells can be stained directly from any medium which supports the development of flagella, and there is a high proportion of cells with flagella among the cells in the preparation.

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LITERATURE CITED