Examination of Bacterial Flagellation by Dark-Field Microscopy

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A method is described for visualizing unstained bacterial flagella by dark-field light microscopy. Since individual filaments can be seen, a genus such as Salmonella, which is peritrichously flagellated, can readily be distinguished from a polarly flagellated genus such as Pseudomonas. Polarily flagellated bacteria generally swim much faster than peritrichously flagellated bacteria, and turn by abrupt reversals. The differences in flagellation and motility provide diagnostic criteria that may be useful in clinical microbiology.

Among motile rod-shaped bacteria, the number of flagella per cell, and their points of origin on the cell (polar versus peritrichous), is a primary taxonomic criterion (9) of clinical significance (4). The type of flagellation can readily be ascertained by electron microscopy, but this technique is frequently not available to a clinical laboratory. Flagellar staining procedures for light microscopy (5) are available, but consistent results are not easily obtained.

In this paper, a method is described that allows the observation by dark-field microscopy of individual unstained flagella on whole cells. Various species of Pseudomonas have been examined as examples of polar flagellation, and Salmonella typhimurium, Bacillus subtilis, and Escherichia coli as examples of peritrichous flagellation. The substantial differences in velocity and turning pattern between the two classes are also demonstrated and discussed as a taxonomic criterion.

(It is commonly stated in microbiological texts that because bacterial flagella have a diameter [typically 15 nm] that is small compared with the wavelength of visible light [500 nm] they cannot be seen by light microscopy. Such statements are erroneous and are based on a misconception of the limit set by the wavelength of light. This limit is one of resolution, not of detection. Any particle immersed in a medium of different refractive index will, no matter how small it is, scatter some light and therefore in principle be detectable. Two particles separated by less than the wavelength of light will be seen as a single scattering source, as a result of the resolution limit.)

MATERIALS AND METHODS

Bacterial cultures. These should be grown in liquid medium to mid-log phase (optical density at 650 nm, ~0.6) and diluted to a suitable concentration for microscope observation. There should be enough cells in the field of view for a statistically significant observation to be made, but they should be sufficiently separated to prevent overlap of the flagella of adjacent cells. If the cells grow in a minimal medium they can be observed in it directly. Complex media such as nutrient broth give high background scattering, but may be required for growth. In this case, better contrast in the microscope will be obtained by sedimenting (e.g., in a clinical centrifuge for 10 min) and then gently resuspending the cells in a simple buffer such as 10 mM potassium phosphate, pH 7, plus 100 μM potassium ethylenediaminetetraacetic acid (1) or in Vogel-Bonner citrate medium (12).

Preparation of slides. Because of the very high light intensity used, particular attention should be paid to cleanliness of the sample. No cleaning technique can remove glass blemishes; if these seem excessive, the only recourse is to try a different source of slides. For cleaning, we have found a nonfoaming spray-on glass cleaner such as Bon-Ami, which is wiped off with tissue after a few seconds, to be as effective as acid washing procedures. The coverslip must be of the thickness (usually 0.17 mm [size 1½]) for which the microscope objective has been corrected. It can be satisfactorily cleaned by firm wiping with double layers of dry tissue. Sample size should be just sufficient to allow the liquid to spread to the area of the cover slip, since excessive liquid depth lowers image quality by providing a background of out-of-focus scattering sources. A loopful (3-mm loop diameter) of sample is about the right amount.

Light source. A high surface brightness is the important parameter, rather than overall flux. Short-arc xenon, mercury, or xenon-mercury lamps are suitable. Flagella can be seen using a 75-W arc, but a higher-wattage arc is preferred.

An adjustable collimating lens system and spherical rear mirror are needed and are usually an integral part of commercially available lamp housings. A tempered-glass heat filter is advisable to protect the microscope optics and the sample. Because of the
sensitivity of bacterial motility to high-intensity blue light (reference 7, and see Results section below) a high-pass yellow filter is also needed for observation of motility.

Microscope. Any good-quality microscope can be used. The light beam is brought in directly without diffusing filters.

The condenser should be an oil immersion dark-field condenser of high numerical aperture (inner 1.2, outer 1.4).

Choice of objective is critical for optimum image quality. Considerable experimentation indicated that a medium power (40×), dry fluorite objective, of moderately high numerical aperture (0.75), gave the best combination of image brightness, contrast, and freedom from flare. Specifically, the use of higher power oil immersion objectives gave inferior images because of flare from bacterial cell bodies.

Standard eyepieces are used; we usually use 16×, but 10× are satisfactory.

Adjustment of optics. Because of the low scattering power of the flagella, careful adjustments of all elements of the system are necessary to maximize intensity; most of these will be necessary only during initial setup. A check list follows: (i) All optical surfaces must be kept scrupulously clean. (ii) The arc must be centered on the collimating lens axis, and the secondary image of the arc from the spherical rear mirror (if the housing has one) superposed on the primary image. (Personnel should acquaint themselves with precautions against ultraviolet light and other hazards before making these adjustments.) (iii) The microscope condenser should be centered on the objective axis, using a precentered built-in light source if available. A suitable "sample" is a microscope slide, heavily scratched with emery paper; a loopful of water is first added and then the appropriate thickness cover slip. The oil connection between the condenser and slide must be free of bubbles and be copious enough to ensure that the entire beam is transmitted. Insufficient oil will manifest itself as an off-center connection. (iv) The beam axis should be centered on the microscope optical axis. If a built-in 45° mirror is used, it of course requires that the incident beam be horizontal. More flexibility in layout is obtained by the use of a universal mirror (e.g., Zeiss 46 51 07). (v) The beam from the housing should be adjusted until it is approximately collimated, and then the microscope condenser should be adjusted to give critical illumination of the sample. This adjustment will be recognized by a minimum in the central illuminated area, with a corresponding maximum in intensity. No attempt should be made to achieve uniform intensity over the whole field. (vi) Different degrees of collimation (slightly convergent, slightly divergent) and, if possible, different distances of the lamp from the microscope should be tried; in each case, the condenser should be adjusted for critical illumination of the central area. When the overall optimum beam arrangement has been ascertained, it can be used from then on, the only adjustment needed on a routine basis being the vertical position of the microscope condenser, which must be made with each new slide.

Motility measurements. These were made photographically by measuring the contour lengths of the tracks generated during a 0.5-s exposure time (11), or less accurately by use of a stopwatch to estimate the time required for a cell to cover a defined distance on an eyepiece reticle. Calibration of the reticle is accomplished with a stage micrometer.

Summary of optical equipment used in our laboratory. The setup comprised the following: light source: Hanovia 500-W xenon arc, 959C-98; light housing: Oriel 6140, 48-mm f.0 collimating lens; power supply: Oriel 6242; heat filter: Optical Industries 03 FHA 023; yellow filter: Optical Industries GG495 or Corning 3-69; microscope: Zeiss standard RA; condenser: Zeiss oil immersion dark-field Ultracondenser, numerical aperture 1.2/1.4; objective: Zeiss Neofluar 40×, 0.75; eyepiece: Zeiss wide-angle Kpl 16×; beam geometry: lamp to collimating lens = 50 mm; lens to condenser = 0.5 m (lens to image plane in absence of condenser = 1.2 m), i.e., beam slightly convergent. However, note that geometry should be optimized empirically in any given apparatus to give maximum image brightness.

RESULTS

By use of the methods described above, individual flagella can readily be seen. Before going on to describe the observations in more detail, it is necessary to make some comments about the difficulty of photographic documentation in this context. No photographic film can match the capabilities of the human eye in terms of sensitivity, dynamic range, and perception of the form of a moving object. All three of these capabilities are in demand here: (i) sensitivity, because the flagella are weak scattering sources; (ii) dynamic range, because the cell body and flagellum differ in brightness by about three orders of magnitude; (iii) perception of moving forms, because active movement and even Brownian movement interfere with the formation of a sharp photographic image. Exposure time is therefore a compromise between factors (i) and (iii). For these reasons, a clinical laboratory will probably not wish to attempt photographic documentation, nor is there any need for it to do so. Photographs are given here for illustrative purposes, but it must be strongly emphasized that the quality of the direct visual image is far higher.

Flagellation. The distinction between peritrichous and polar flagellation should not be based on the appearance of moving cells, since the helical bundle of peritrichous cells tends to form at the pole (Fig. 1). Once it has been established that motile cells are present in the culture, they can be rendered stationary within 1 s or so by removal of the protective yellow filter. Under these conditions, peritrichous flagellation is recognized as a pattern of helical filaments radiating from the entire cell, as is seen in the photograph of S. typhimurium in
Fig. 1. (A) S. typhimurium during translational movement. The peritrichous flagella form a polar bundle (see cartoon, Fig. 1B) which could be mistaken for polar flagellation. Classifications should therefore be based on the appearance of flagella on stationary cells. Dark-field photomicrograph taken with high-intensity pulsed xenon arc. Bar equals 5 μm.

Fig. 2. The overall impression of a field of such cells leaves the observer in no doubt that flagellation is peritrichous.

Polar flagellation is seen as one or more flagella radiating from the polar regions only, as can be seen in the photographs of Pseudomonas stutzeri and Pseudomonas aeruginosa in Fig. 3. Although the point of origin of each flagellum
cannot, because of scattering from the cell, be determined exactly, it can be located much more accurately than the photographs suggest. In practice, one has little difficulty in determining whether or not an individual cell is polarly flagellated, and even less difficulty in making a decision based on a field of many cells. Further classification of cells may be made on the basis of the number of flagella per pole. Thus, in agreement with established classification (3), P. stutzeri and P. aeruginosa are observed to be predominantly monotrichous, although cells with two polar flagella were quite frequently observed (as in Fig. 3D).

These statements about observation of polar flagellation need to be qualified in one regard: Pseudomonas species sometimes possess very short flagella (ca. one complete helical turn), and under these circumstances scattering from the cell body makes flagellar observation very difficult even in cultures that have high motility.

Motility. The motility of Pseudomonas species differs markedly from that of peritrichously flagellated cells in two respects, velocity and turning pattern. The differences, described below, are being presented here because they are sufficient to constitute a useful diagnostic criterion that is not presently utilized in clinical microbiology.

For unknown reasons, the swimming velocity of vigorously motile cultures of Pseudomonas species is much higher than that of peritrichously flagellated cells (8). Velocity measurements of P. stutzeri and P. aeruginosa made photographically in the present study are shown in Table 1, along with data obtained in a similar manner for S. typhimurium in a previous study (6). Velocities comparable to, or lower than, these for S. typhimurium have been noted for other peritrichously flagellated species, such as E. coli (2), Serratia marcescens (8), and B. subtilis (R. Macnab, unpublished work).

Semi quantitative data were obtained with an eyepiece reticle and stopwatch. Measurements made in this way on 10 cells each of S. typhimurium and P. stutzeri yielded mean velocities with standard deviations of the mean of 25.4 (1.5) and 51.5 (3.4) μm per s, respectively, in fair agreement with the more accurate photographic data given in Table 1.

Peritrichous organisms such as E. coli or S. typhimurium terminate a period of translational movement by chaotic tumbling (2, 6) associated with dispersal of the coordinated bundle of flagella (7). Pseudomonas species, on the other hand, usually undergo abrupt double reversals (10), as can be seen in Fig. 4. This abrupt reversal does not appear with peritrichously flagellated cells although some may, after tumbling, fortuitously travel back along their path.

DISCUSSION

The procedures described allow identification of the type of flagellation possessed by motile bacterial cultures, without resort to the use of stains or electron microscopy. The example chosen in this paper is the differentiation between S. typhimurium, which is peritrichously flagellated, and Pseudomonas species, which are polarly flagellated, but the method is applicable whenever knowledge of morphology of flagellation (both the origin of the flagella on the cell body, and the number, length, and shape of flagella) would be of assistance in identifying a culture.

The ability to see individual, unstained flagella (7) is a significant advance in the power of dark-field light microscopy, yet it resists relatively standard microscopic equipment. For example, I have tested existing fluorescence microscopic equipment (Leitz Dialux microscope, oil immersion dark-field condenser, 200-W mercury arc lamp) in a clinical laboratory with satisfactory results.

The operating skills can be picked up quickly by anyone familiar with the use of the light microscope. No unusual visual acuity is needed for the recognition of peritrichous flagellation; many individuals with any prior experience or skills have done so when visiting my laboratory. Polar monotrichous flagellation is more demanding for two reasons. (i) The probability of one flagellum being suitably oriented is lower than the probability of a representative selection of flagella being suitably oriented in the peritrichous case. (ii) The flagella tend to be much shorter, and therefore more easily obscured by flare from the cell body. In spite of these difficulties, an observer with average visual acuity can, with a little practice, recognize polar flagellation in most cases. In cases where the observer fails to note flagella in motile cultures, this failure is in itself an indication of polar flagellation (provided control cultures have established that the optical equipment is adequate) since peritrichous bacteria with short flagella are poorly motile (3), presumably because the flagella cannot extend past the cell and form the bundle.

As can be seen from Table 1, the velocity of pseudomonads is approximately twice that of peritrichously flagellated species. This difference is sufficiently marked to be obvious on
FIG. 3. Polar flagellation in pseudomonads. (A) Stationary cell of P. stutzeri. (B) Same negative [overexposed during printing] to show cell outline. (C) Cartoon combining information from (A) and (B). (D–F) As for (A–C) but with P. aeruginosa (note presence of two flagella at pole, although species is predominantly monotrichous). Bar equals 5 μm.

FIG. 2. Peritrichous flagellation, exemplified by S. typhimurium. Dark-field photomicrograph of stationary unstained cells taken with continuous xenon arc. (A) Exposure chosen to show flagella. (B) Shorter exposure to show outlines of cell bodies. (C) Cartoon combining information found in (A) and (B). Arrow indicates much brighter image resulting from two flagella lying together, in phase. Bar equals 5 μm.
inspection of a slide. The simplest method is a qualitative side-by-side comparison with control cultures, but as was shown in the Results section, semiquantitative data may readily be obtained with an eyepiece reticle and a stopwatch. Although mean velocities in the range of 40 to 70 μm per s are strong evidence for polar flagellation, measurements in the range of 20 to 30 μm per s are of course not strong evidence against this type of flagellation, since any of a number of adverse conditions could reduce motility.
As far as turning behavior is concerned, translational movements, followed by tumbling in the case of peritrichously flagellated cells and by abrupt reversal in the case of polarly flagellated cells, are easily distinguished with little practice, and would seem to be a simple criterion which has been underutilized, but which is of course restricted to cultures that are highly motile.

ACKNOWLEDGMENTS
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LITERATURE CITED

Table 1. Swimming velocity of polarly and peritrichously flagellated bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of flagellation</th>
<th>No. of cells in sample</th>
<th>Mean velocitya (μm per s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total sample</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>Polar</td>
<td>90</td>
<td>48.5 (1.5)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Polar</td>
<td>92</td>
<td>58.3 (1.7)</td>
</tr>
<tr>
<td>S. typhimuriuma</td>
<td>Peritrichous</td>
<td>45</td>
<td>28.8 (0.8)</td>
</tr>
</tbody>
</table>

a Calculated from photographically recorded motility tracks taken at 24°C. Data from several photographs are included in each sample. Nonmotile cells are excluded from the calculations. Results are given with the standard deviation of the mean given in parentheses.

b Data of Macnab and Koshland (6).