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A Simple, Rapid Method for Demonstrating Bacterial Flagella

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We developed a simple, rapid method for demonstrating flagellation of bacteria using the fluorescent protein stain NanoOrange (Molecular Probes, Eugene, Oreg.). The NanoOrange reagent binds to hydrophobic regions of proteins, which results in substantial enhancement of fluorescence. Unbound reagent is essentially nonfluorescent. NanoOrange fluorescently stained bacterial cell bodies, as well as flagella and other appendages, which could be directly observed by epifluorescence microscopy. Detection of flagella was further improved by using a charge-coupled device camera for image capture and processing. The reliability of the method was tested by using 37 pure cultures of marine bacteria. Detection of flagella on the isolates by NanoOrange staining was compared to detection by transmission electron microscopy (TEM). For 36 of 37 cultures, the two methods yielded the same results. In one case, flagella were detected by TEM but not by NanoOrange, although the difference may be attributable to differences between the culture preparations. NanoOrange staining is rapid (10 to 15 min) and does not require fixation or dehydration, so live samples can be stained. Since NanoOrange is a general protein stain and works directly in seawater, it may also prove to be useful for staining other proteinaceous material that is of interest to aquatic microbial ecologists.

Flagellation is a useful parameter for identifying certain bacteria, such as nonfermentative and anaerobic gram-negative bacilli. Clinical studies, for example, have demonstrated the importance of examining bacterial flagella for characterization and identification of anaerobic bacteria (10). Flagellation also appears to be a common characteristic of bacteria in natural environments. For example, identification of flagellin gene homologues has been used as a biomarker in natural communities (21), and in one study, 45 to 70% of the bacteria in coastal seawater samples collected off Scripps Pier from August to October 1997 were motile (8). The motility exhibited by marine bacteria has been found to be markedly different from the run and tumble model motility derived from work on *Escherichia coli* (16). Visualization of flagella could prove to be useful in studies in which researchers examine the mechanisms of motility used by diverse bacteria isolated from aquatic environments.

Workers have described a number of methods for staining bacterial flagella, including the tannic acid-fuchsin method (10) and modifications of this method (3, 4, 7, 11–13), as well as silver staining methods (5, 10, 17, 20). These approaches are satisfactory but involve complicated protocols. Also, each method has limitations, such as unstable reagents and fixation-induced alterations. A simple, rapid method for demonstrating bacterial flagella would facilitate characterization of clinical and environmental isolates.

In this paper we describe a novel, rapid method for visualizing bacterial flagella with the fluorescent protein stain NanoOrange (Molecular Probes, Eugene, Oreg.) and epifluorescence microscopy. Flagella could be directly observed with a microscope, but visualization was greatly improved when a charge-coupled device camera was used for digital image capture and processing.

Bacterial isolates. Thirty-seven bacterial isolates were tested (Table 1). These strains had previously been isolated in the fall and winter of 1993 from seawater collected ~1 km off Scripps Pier (15). Stock preparations were kept frozen (−80°C) until they were grown on ZoBell 2216E medium plates (1.5% [wt/vol] agar [Difco], 5 g of peptone, and 1 g of yeast extract in 1 liter of prefiltered [pore size, 0.45 μm] seawater; autoclaved at 121°C for 30 min). To monitor flagellation during bacterial growth, 50-ml portions of autoclaved ZoBell 2216E medium were inoculated with bacteria grown on plates and incubated in 100-ml Erlenmeyer flasks for 26 h at 21°C on a shaker table. Aliquots were taken at 1- to 2-h intervals during exponential growth and after 23 and 26 h (early stationary phase). Growth of bacteria was monitored by determining optical density at 650 nm with a spectrophotometer (Lambda 4; Perkin-Elmer). All values were corrected with a blank consisting of autoclaved ZoBell 2216E medium.

Bacterial motility. Bacterial motility was observed by dark-field microscopy (16). The equipment used consisted of a plastic covered depression slide (thickness, 0.5 mm; Fisher) and a light microscope (Standard 16; Carl Zeiss, Oberkochen, Germany) equipped with a dry dark-field condenser (0.7/0.85) and a Plan 16/0.35. Aliquots (100 μl) of culture were transferred onto the depression slide and observed for 1 min at a magnification of ×320. Bacteria which exhibited directed movement were considered motile. An isolate was considered motile even if only a small portion of the cells displayed motility at the time of sampling.

Detection of bacterial flagella. (i) TEM. Bacteria were preserved by adding formaldehyde (final concentration, 2%). Carbon-stabilized Formvar supports on 200-mesh copper transmission electron microscopy (TEM) grids (Ted Pella) were rendered hydrophilic by high-voltage glow discharge (9). In

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most cases, bacteria were adsorbed to TEM grids by floating the grids Formvar side down on an undiluted drop of culture for at least 5 min. In some cases, aliquots of fixed cultures were diluted to a volume of 10 ml with an artificial seawater medium and then pelleted onto TEM grids mounted on acrylic platforms in the bottoms of polyallomer ultracentrifuge tubes (19). The centrifugation conditions used ranged from 10,000 to 41,000 rpm for 0.5 to 4 h in a swinging bucket rotor (type SW41; Beckman). The bacteria on grids were stained by submerging the grids for 20 s in 0.5% (wt/vol) uranyl acetate and then were rinsed three times (10 s each) by submersion in aliquots of Milli-Q water. The grids were examined with a Hitachi model H-500 TEM by using an accelerating voltage of 80 to 100 kV.

(ii) NanoOrange staining. A 0.5-μl portion of a NanoOrange stock solution (Molecular Probes) was added to 10 μl of a live bacterial culture on a microscope slide. Samples on the slide were then mixed with 10 to 20 μl of 30% polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Mo.) and covered with a coverslip. Polyvinylpyrrolidone was used for mounting both to reduce convection and to serve as a cryoprotectant, which allowed storage of slides for 1 to 2 weeks in a freezer. After waiting 10 to 15 min for staining to occur, we examined the slide at a magnification of ×1,250 with an epifluorescence microscope (model BH2; Olympus, Tokyo, Japan) equipped with a blue filter set (excitation, wavelength, 490 nm; emission wavelength, 520 nm). The NanoOrange stock preparation was mainly used undiluted, but a dilution of 1:20 also worked fine. Higher dilutions (1:100 and 1:1,000) resulted in reduced image brightness, as judged qualitatively by eye. Staining times of 10 to 15 min in the dark assured adequate staining, whereas shorter staining times resulted in images that were qualitatively less bright. However, prolonged staining times (up to 6 h) did not change the quality of the image. Images were recorded with a video camera (model CCD-300 TIFG; Dage MTI) and were examined with an image-processing system (model IFG-300; Dage MTI) with output to a black and white video monitor. This system electronically increased image size by a factor of five and increased the brightness of the recorded image, which facilitated detection of flagella and other thin appendages. Images of *Escherichia coli* (Fig. 1 and 2) were recorded with a high-speed TEM camera (Mega View II; SIS, Münster, Germany) mounted on a Zeiss epifluorescence microscope (Axioskop 2) and were processed by using an image analysis system (analySIS 3.0; SIS).

Bacterial flagella were readily observed by epifluorescence microscopy after NanoOrange staining and by TEM, as shown for three isolates in Fig. 3. Scoring for the presence or absence of flagella by the NanoOrange and TEM methods yielded the same results for 36 of 37 isolates of marine bacteria grown in a rich medium (Table 1). For strain BBFL6, flagella were observed by TEM but not by the NanoOrange method. Since different cultures were used to detect flagella by the NanoOrange method and TEM, the results obtained for BBFL6 might reflect real differences in flagellation between the two different preparations rather than a difference between the techniques. Ideally, the same culture would have been used for both methods, but the NanoOrange procedure was developed several years after the more time-consuming TEM work had been completed. Despite the one equivocal result, our comparison of the two methods indicated that NanoOrange staining is a reliable method for visualizing flagella of cultured bacteria.

Detection of flagella with NanoOrange was found to vary with the growth phase for six cultures of the motile bacteria examined. Flagella were readily observed with the majority of bacteria during exponential growth. In contrast, few or no flagellated cells were observed in the initial lag and late stationary phases. This phenomenon is not unique to the NanoOrange method, since similar results were obtained with the tannic acid-fuchsin method when other bacteria were examined in a previous study (14). After 23 to 26 h of incubation, NanoOrange staining also revealed that the cell surface of most bacteria was covered with proteinaceous material, which consisted mostly of long fibers or fibrils which may have represented fimbria, pili, or perhaps some proteinaceous exudate.

Some interesting differences between motility and flagellation were observed. For example, six isolates (SWAT5, SWAT7, LHFL5, LHAT3, BBFL4, and BB2AT2) were motile as determined by dark-field microscopy, but no flagella were detected by NanoOrange staining or TEM (Table 1). It is possible that these spiral-shaped bacteria move by means of periplasmic flagella, as described previously for spirochetes (2). Internal flagella are unlikely to have been detected by either NanoOrange staining or TEM of unsectioned cells. It is also possible that motility of these strains is achieved by means

TABLE 1. Motility and flagellation of bacterial isolates[a]

Isolate	Motility	Flagellation detected by:	
		TEM	NanoOrange staining
SWFL1	No	Yes	Yes[b]
SWFL2	Yes	Yes	Yes
SWAT1	No	No	No[c]
SWAT2	Yes	Yes	Yes
SWAT3	No	Yes	Yes
SWAT4	No	No	No[c]
SWAT5	Yes	No	No[c]
SWAT7	Yes	No	No
SWAT8	Yes	Yes[b]	Yes
SWAT9	Yes	Yes[b]	Yes[b]
LHFL1	Yes	Yes[b]	Yes[b]
LHFL3	Yes	Yes	Yes[b]
LHFL5	Yes	No	No[c]
LHAT1	Yes	Yes	Yes[b]
LHAT2	Yes	Yes	Yes[b]
LHAT3	Yes	No	No[c]
LHAT4	Yes	Yes	Yes
LHAT5	Yes	Yes	Yes
LHAT7	Yes	Yes	Yes
LHAT9	Yes	Yes	Yes
BBAT1	No	No	No[c]
BBAT4	Yes	Yes[b]	Yes
BBFL1	Yes	Yes[b]	Yes[b]
BBFL2	Yes	Yes	Yes
BBFL3	Yes	Yes	Yes[b]
BBFL4	Yes	No	No[c]
BBFL5	No	No	No[c]
BBFL6	No	Yes	No
BBFL7	No	No	No
BBFL8	Yes	Yes	Yes[b]
BBFL9	Yes	Yes	Yes
BB2FL1	Yes	Yes	Yes[b]
BB2FL2	Yes	Yes	Yes[b]
BB2FL3	No	No	No[c]
BB2AT1	No	No	No
BB2AT2	Yes	No	No
BB2AT3	Yes	Yes[b]	Yes[b]

[a] Motility was determined by dark-field microscopy. Flagella were detected by TEM and NanoOrange staining.
[b] Very thin flagella are hard to see.
[c] Proteinaceous appendages are present on the cell surface.

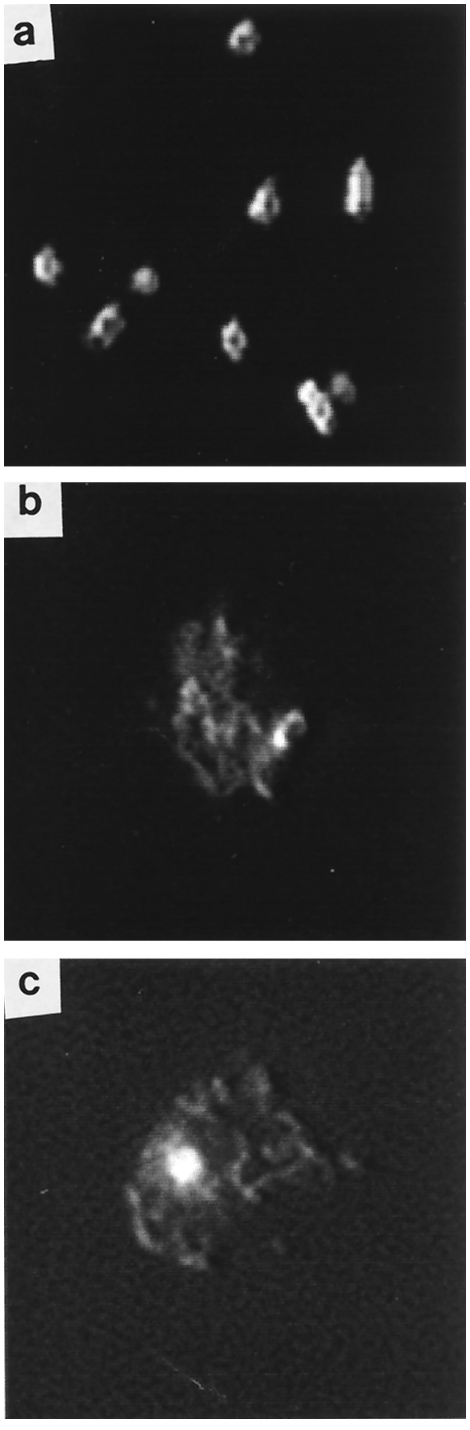

FIG. 1. Staining of nonmotile, nonflagellated *E. coli* cells (a) and motile, flagellated *E. coli* cells (b and c).

other than flagellar rotation. One alternative mechanism has been proposed for the marine cyanobacterium *Synechococcus* sp. (18), which may move by generating surface waves (6). In another study, Brahamsha (1) showed that an abundant cell surface polypeptide is required for swimming by this nanoflagellated cyanobacterium. Proteinaceous structures other than flagella were observed on the cell surfaces of SWAT5, LHFL5, LHAT3, and BBFL4, but whether these structures are involved in motility was not studied. In addition, two isolates (SWFL1 and SWAT3) were flagellated but not motile under our culture conditions. However, one of these isolates (SWAT3) was motile when it was grown on glucose minimal medium (R. Long, personal communication), suggesting that motility depended on the type and/or concentration of substrate.

To prove the validity of our method, we stained the well-characterized gram-negative bacterium *E. coli* in its nonmotile, nonflagellated form as well as in its motile, flagellated form (Fig. 1). Nonmotile cells of *E. coli* did not exhibit any flagellation (Fig. 1a), whereas motile cells were surrounded by numerous flagella (Fig. 1b and c). Unfortunately, our image analysis system was not capable of resolving single flagella very well, which made it more difficult to clearly distinguish different types of flagellation. Since NanoOrange is a general protein stain, it should also be useful in numerous other applications. For instance, preliminary results have shown that flagella of protozoans are also readily stained and visualized (Grossart and Azam, unpublished data). The fine fibrils observed on bacteria in some stationary-phase cultures suggest that other bacterial appendages, such as the appendages on stalked bacteria, can be visualized by NanoOrange staining (Fig. 2). The ability to quickly and easily visualize these appendages could be useful in studies of bacterial attachment to solid surfaces, conjugation, and/or pathogenicity. The fluorescent stain might also be used in aquatic microbial ecology studies to visualize protein release by exudation and cell lysis of phytoplankton and bacteria.

In summary, we describe a novel use of the fluorescent protein stain NanoOrange for rapid visualization of bacterial flagella. Flagella were discernible by epifluorescence microscopy in exponentially growing cultures of marine bacteria. Detection of flagella was greatly facilitated by digital image acquisition with a charge-coupled device camera and a simple image processor. The NanoOrange method is simpler and faster than TEM, but it was equally reliable for demonstrating flagellation of a variety of marine bacterial isolates. Some significant advantages of the NanoOrange method are that it does not require fixation of the cells, it involves very little sample manipulation, and staining can be carried out directly in a growth medium; thus, artifacts due to fixation, dehydration, and excessive sample manipulation can be avoided.

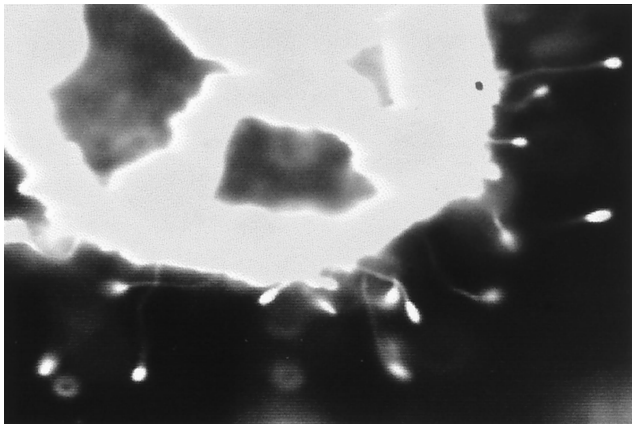

FIG. 2. Stalked bacteria on a marine diatom (*Thalassiosira rotula*) after NanoOrange staining. Note that the diatom is strongly stained by the dye, indicating that large amounts of proteinaceous material were present on the algal surface.

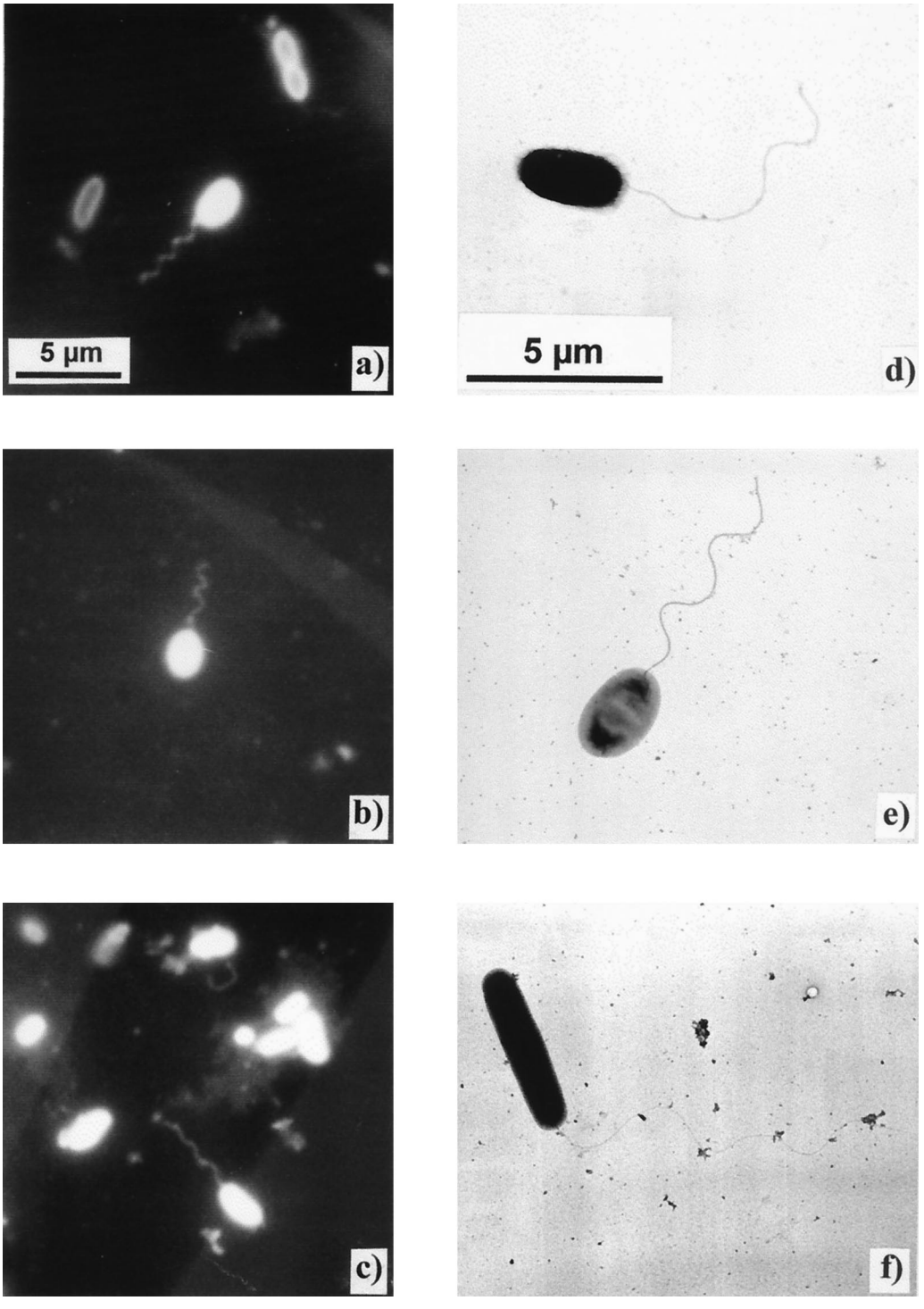

FIG. 3. Comparison of flagellar staining by NanoOrange and epifluorescence microscopy (a through c) and by TEM (d through f). The marine bacterial isolates used were SWAT8 (a and d), LHAT4 (b and e), and LHAT9 (c and f). The organisms in panels a and c are surrounded by proteinaceous material which is also stained by NanoOrange.

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