Scanning Electron Microscope Observation of the Swarming Phenomenon of Vibrio parahaemolyticus

M. ROBERT BELAS† AND R. R. COLWELL*
Department of Microbiology, University of Maryland, College Park, Maryland 20742

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Scanning electron microscopy was used to study the production of lateral flagella and the swarming phenomenon in Vibrio parahaemolyticus. Differences in the size and diameter of the sheathed, polar flagellum and lateral flagella were apparent in these preparations. Swarming of V. parahaemolyticus was found to be similar to the swarming of Proteus spp. in that swarm cells which are heavily flagellated and elongated are formed.

Mixed flagella distribution as an unusual character (14), i.e., the presence of lateral (or peritrichous) flagella together with a slightly thicker polar flagellum, was observed by Houwink and van Iterson in 1950 (7). This phenomenon has also been reported to occur in certain strains of Aeromonas (9), Chromobacterium (16), and Pseudomonas (12, 13) and more recently in Vibrio (Beneckea) spp. (1–3, 15, 17–20).

Certain species of the genus Vibrio, notably V. parahaemolyticus and V. alginolyticus, exhibit mixed flagellation. Organisms of these species typically possess a single, sheathed, polar flagellum when grown in a liquid medium (1–3, 4, 17, 20). When these same bacteria are streaked onto a solid agar medium, the bacteria develop numerous lateral flagella in addition to the sheathed polar flagellum. The polar flagellum has a diameter of 24 to 30 nm and consists of a core (14 to 16 nm) surrounded by a sheath (4). The lateral flagella are unsheathed and have a diameter of 14 to 15 nm (1). The wavelength of the polar flagellum has been reported to be 1.5 μm, whereas the wavelength of the lateral flagellum is 0.9 μm (3).

Concomitant with the production of lateral flagella, the growing bacterial mass swarms over the surface of the agar medium. Lateral flagella have been implicated in the motility of these bacteria over the agar surface (15). Swarming of V. alginolyticus has been shown to involve a complex set of interactions between the temperature, pH, agar concentration, and NaCl concentration of the medium (2, 3, 17, 18). Swarming events can be induced on minimal medium by the addition of selected volatile acids, and it has been suggested that swarming results in the movement of the bacteria away from toxic metabolic by-products which develop during growth (18).

The swarming of V. parahaemolyticus and V. alginolyticus has been observed by light microscopic methods (12, 18). These studies have suggested that the swarming of V. parahaemolyticus resembles the swarming of members of the genus Proteus (12, 22). However, light microscopy of living bacteria on agar is not able to resolve individual flagella, nor can it allow us to associate the development of these flagella with swarming. Transmission electron microscopy is capable of distinguishing individual flagella but cannot conveniently be used to examine intact bacteria on an agar surface. Scanning electron microscopy is also able to distinguish individual bacterial flagella and, in addition, allows examination of bacteria on agar surfaces. The relationship of lateral flagella to the swarming of V. parahaemolyticus was examined by scanning electron microscopy and is reported in this paper.

V. parahaemolyticus strain H-926 was isolated from Chesapeake Bay plankton collected in the spring of 1979. The culture was frozen at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented by the addition of 10 g of NaCl per liter (1.5% NaCl BHI) and 20% (vol/vol) glycerol. When a fresh culture was required, a small amount of frozen suspension was scraped from the top of the vial of suspended bacteria and placed in 5 ml of fresh 1.5% NaCl BHI broth. Incubation at 25°C for 6 to 8 h resulted in a turbid suspension which was streaked as a thin line along the midsection of the 1.5% NaCl BHI (1.5% NaCl BHI and 15 g of agar per liter) plate, and the organism was incubated overnight (i.e., 8 to 12 h) at 25°C. Agar plates were dried at 25°C for a minimum of 24 h before being used for the study of bacterial swarming.

† Present address: Department of Biology, University of California at San Diego, La Jolla, CA 92039.
The bacteria were fixed by the technique recommended by Matsuguchi et al. (10). The fixative contained 1.0% glutaraldehyde (vol/vol) (Polysciences, Inc., Warrington, Pa.), 1.5% NaCl (wt/vol), and 0.1 M cacodylic acid (Sigma Chemical Co., St. Louis, Mo.), pH 7.60. To fix the bacteria after swarming had occurred, the fixative was gently floated onto the plate, replaced at 30-min intervals, and allowed to act for 2 h at room temperature. After fixation, remaining fixative was removed from the agar surface, and the surface was gently floated with 0.1 M cacodylic acid buffer, pH 7.60. The cacodylic acid buffer was removed by vacuum aspiration, and the process was repeated three times to remove traces of the glutaraldehyde fixative. The agar plates were then flooded with the cacodylic acid buffer and stored overnight at 4°C.

Blocks of the agar still covered with the cacodylic acid buffer were removed with a surgical blade. The agar blocks were placed in glass scintillation vials, and each block was dehydrated through either a graded ethanol or acetone series of final concentrations of 30, 50, 70, 95, and 100% (vol/vol) solvent. Three separate changes of the final 100% solvent were used to ensure that the specimen was dried properly. Each block was removed from the final dehydrating solution and critical point dried, with liquid carbon dioxide as the transition fluid. Once dried, the agar blocks were removed and mounted on aluminum stubs, with a small amount of conductive silver paint as an adhesive. Agar blocks were coated with gold-palladium alloy for 3 to 6 min with a sputter coat plating device. Specimens were examined with an ISI 60 (International Scientific Instruments) scanning electron microscope at 20 kV.

Broth- and agar-grown bacteria were also examined for flagellar morphology and arrangement by staining the flagella by the Mayfield and Inniss (11) modification of the Leifson flagella stain (8).

*V. parahaemolyticus* H-926 possessed a single polar flagellum when grown in a liquid medium and produced lateral flagella when streaked onto a solid agar medium, as observed by light microscopy. When grown on agar medium, the bacteria swarmed over the surface, eventually covering it entirely. Swarming of strain H-926 was not accompanied by the formation of zones or halos, an observation in agreement with that of Ulitzur (17), although these concentric rings have been reported to occur in other laterally flagellated *Vibrio (Benekea)* spp. (1) and do occur in the swarming of *Proteus mirabilis* (22).

Conditions of fixation and dehydration greatly influenced the morphology of the lateral flagella, although the bacterial soma appeared to retain their morphology. Ethanol was inappropriate for dehydration because it destroyed the morphology of the lateral flagella, an observation in agreement with that of Matsuguchi et al. (10). When the organisms were dehydrated through a graded acetone series, instead of with ethanol, very distinct fibrillar appendages were observed (Fig. 1). These appendages formed two classes, according to relative diameter and points of insertion. The first type of structure was observed to occur singly at the pole of the organism and was notably thicker than the second type, which was inserted at various locations on the bacterium. The morphology and points of insertion suggest that the first type was the polar flagellum and that the second, thinner and more numerous, was the lateral flagella (Fig. 1e).

The edge of the spreading zone consisted of a mass of bacteria, apparently held together by lateral flagella (Fig. 1b). Bacteria at the edge of the spreading zone were approximately two to six times as long as organisms found closer to the colony center, which were 1.5 to 2.0 μm in length (Fig. 1a). Bacteria in front of the periphery (Fig. 1c to e) usually occurred singly or in groups of several organisms and were two to three times as long as those at the colony center (Fig. 1a). Single organisms possessed fewer lateral flagella than aggregates of four or five bacteria, and a single polar flagellum was present in these single bacteria (Fig. 1c). Figures 1c to e show the occurrence of lateral flagella between clumps of bacteria and single organisms lacking lateral flagella. Lateral flagella appeared to serve as bridges from organism to organism and from bacteria to the agar surface (Fig. 1d), a phenomenon which may occur from hydrophobic interactions between filaments and which is apparently responsible for the production of flagellar bundles reported for strains of *V. alginolyticus* (20). The flagella were often straight, without the wave pattern typically associated with these structures. We believe this phenomenon occurs when both the distal and proximal ends of the flagellum are in contact with a surface before fixation. For example, if a flagellum is inserted at the polar end of the bacterium and is also in contact with another surface, such as the agar matrix or another bacterium, the fixation procedure may cross-link the distal end of the flagellum to the other surface, forming a relatively secure linkage. The dehydration process can cause the flagellum to shrink and, as a result, straighten. We have found that this phenomenon occurs not only for bacteria grown on agar surfaces but also for flagellated organisms adsorbed to other surfaces, such as glass or chitin (submitted for publication). However, when the flagellum was attached only to the organism, the wave pattern was maintained in
FIG. 1. Scanning electron micrographs of swarming V. parahaemolyticus on solid agar medium. Bacteria were dehydrated through a graded acetone series. (a) Center of the spreading zone. Intertwining networks of lateral flagella filaments are present on 1.5- to 2.0-μm cells. Bar, 1 μm. (b) Periphery of the spreading zone. Organisms at the edge of the swarming colony possess lateral flagella which bridge bacteria and also occur at the bacterium-to-agar surface interface. Bar, 1 μm. (c) Organisms on the agar matrix approximately 10 μm away from the spreading zone periphery. Individual bacteria are shown which possess a polar flagellum but few lateral flagella. Organisms in groups at the upper left possess lateral flagella filaments. Bar, 2 μm. (d) Higher magnification of organisms in the upper left of (c). Bar, 1 μm. (e) Bacteria on the surface of the agar matrix. The arrow points to the polar flagellum pointing 45° off the agar surface. The thinner lateral flagella are observed between bacteria. Bar, 1 μm.
specimens prepared either for light microscopy or scanning electron microscopy (Fig. 1e).

The polysaccharide slime layer and fibrils were not observed. Also, fibrils or sheaths of polysaccharide-like material were not found when specimens were treated with a polysaccharide-staining fixative containing 0.05% (wt/vol) ruthenium red (5).

The use of scanning electron microscopy procedures to elucidate the swelling of *V. parahaemolyticus* suggests certain similarities with the swelling of bacteria of the genus *Proteus* (21, 22). The bacteria in the swelling colony appear to be of mixed sizes, ranging from organisms ca. 1.5 to 2.0 μm in length to those approximately two to six times as large at the periphery of the spreading zone. The production of swarm cells in *V. parahaemolyticus* is not as dramatic as in *Proteus* spp., where bacteria greater than 80 μm are observed, nor does *V. parahaemolyticus* form rafts of bacteria, as have been seen in *P. mirabilis* (21, 22). Production of lateral flagella appears to be greatest in the swelling zone itself and not in the bacteria found in front of the swelling zone. Movement of bacteria on agar may result in the loss of lateral flagella due to shearing, and this phenomenon may explain the lack of lateral flagella on bacteria in front of the swelling periphery. Uiltzur and Kessel (20) suggest that swelling is induced by metabolic waste products produced by the bacteria during growth on agar. It is possible that a metabolic product induces lateral flagella production in the swelling mass of bacteria, but once bacteria move away from the swelling periphery, lateral flagella cannot be replaced because the concentration of metabolic inducer has fallen below a critical threshold necessary to stimulate lateral flagella production.

The surface translocation events in *V. parahaemolyticus* are best defined as a swelling phenomenon, according to the definition proposed by Henrichsen (6), with spreading occurring with a concomitant increase in the numbers of flagella per unit surface area of the cell, i.e., with the production of at least 10 to 20 lateral flagella per cell on an agar surface compared with a single polar flagellum formed when bacteria are grown in broth. Mutants lacking lateral flagella but retaining the polar flagellum do not swarm, implicating the former in the swelling process (15).

The scanning electron microscopy procedure described has been successfully employed to examine lateral flagella production of both *V. parahaemolyticus* and *V. alginolyticus* adsorbed onto chitin particles and has been successful in studying the role of both lateral and polar flagella in the adsorption of these bacteria to the chitin surface (submitted for publication).

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