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Flagellin-Containing Membrane Vesicles Excreted from *Vibrio alginolyticus* Mutants Lacking a Polar-Flagellar Filament[1]

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Polar flagellum-defective mutants (Pof$^-$ Laf$^-$) have been isolated from a lateral flagella-defective mutant (Pof$^+$ Laf$^-$). Among these Pof$^-$ Laf$^-$ mutants, polar-filamentless mutants, which have the hook structure but not the filament, were identified by electron microscopy. Their hooks were covered with a sheath structure which is contiguous to the outer membrane. The filament proteins, flagellins, were shed into the culture medium of these mutants. These flagellins could be sedimented by high-speed centrifugation even after heat or low pH treatment whereas the depolymerized flagellin of the Pof$^+$ strain was degraded by these treatments. After Triton X-100 treatment, most flagellin of the filamentless mutants could no longer be sedimented, and was degraded. We observed vesicle-like structures on the tips of the hooks and in the flagellin fraction sedimented by high speed centrifugation. These results suggest that flagellin of the filamentless mutants is not assembled into the tip of the hook, but is excreted together with a membrane structure which is probably the sheath of polar flagella.

Key words: flagella, flagellin, filament, outer membrane.

Vibrio alginolyticus has two types of flagella, polar flagella (Pof) and lateral flagella (Laf), which are driven by sodium-motive force and proton-motive force, respectively (*1, 2*). Under viscous conditions or on the surface of a solid medium, cells express many lateral flagella (*3, 4*). The cells sense either the rotation rate or the sodium flux in the polar-flagellar motor as a trigger for lateral-flagellar induction (*5, 6*). The lateral flagella work better than the polar flagellum for swimming in a high-viscosity environment (*7, 8*). However, at normal viscosities, cells can swim at a high speed of *ca.* 50-80 $\mu m \cdot s^{-1}$ using their polar flagellum. Under these condition, the rotation rate of the polar flagellum is very fast, over 1,000 r.p.s. (*9*). The rotation is stable at these high rotation rates and any fluctuation seems to be mainly caused by Brownian motion (*10*).

The flagellum consists of (i) the filament, which is a helical protein tubule, (ii) the motor, which is embedded in the cell membrane, and (iii) the hook, which is a structure joining the filament and the motor (*11-13*). The polar flagellum of *V. alginolyticus* is generated from the cell pole. The filament is sheathed with a membrane structure contiguous to the outer membrane (*14*), whereas the filaments of lateral flagella are not sheathed. The filament proteins, or flagellins, of lateral and polar flagella have different molecular weight and antigenicity (*15, 5*). The polar flagellar filament is about 30 nm in diameter (*14, 16*), making it easy to see in the dark-field microscope (*10*). In the polar flagellum, we previously found the five major proteins. Two of these were shown to be flagellins, and the other three might be sheath components (*17*).

We have previously isolated polar flagellum-defective (Pof$^-$ Laf$^-$) mutants from a lateral flagella-defective mutant (Pof$^+$ Laf$^-$) in *V. alginolyticus* (*18*). In the present study, to investigate how the polar flagellar filament is assembled in *V. alginolyticus*, we searched for and characterized mutants that lack the filament but have hooks attached to the basal body.

EXPERIMENTAL PROCEDURES

Bacterial Strains—*V. alginolyticus* strains used were derived from the lateral-flagella defective mutant (Pof$^+$ Laf$^-$) YM4 which was isolated from 138-2 (Pof$^+$ Laf$^+$).

Broth and Media—VC medium contains 0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.4% (w/v) K_2HPO_4, 0.2% (w/v) glucose, and 500 mM NaCl. V-buffer contains 25 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 10 mM $MgCl_2$.

Isolation of Polar-Flagellar Mutants—Mutagenesis was carried out using either ethyl methanesulfonate (EMS) or UV irradiation. Swarm-deficient mutants were isolated from Pof$^+$ Laf$^-$ strain YM4 in 0.25% agar VC plates as described previously (*2, 18*).

Electrophoresis—SDS-polyacrylamide gel electrophoresis (PAGE) was performed as previously (*26*).

Preparation of Anti-PF45 Serum—Polar flagella were prepared from YM4 as described previously (*17*). The polar flagella were run on SDS-PAGE gels. The flagellin band at 45 kDa (PF45) was cut from the gel and injected into a rabbit. Antiserum was prepared as described previously (*26*).

Immunoblotting—Immunoblotting was performed as

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described previously (*26*), using anti-PF45 antibody as the first antibody and an affinity-purified anti-rabbit IgG conjugated with alkaline phosphatase as the second antibody.

Fractionation of Flagellin from Culture Medium—An overnight culture was inoculated into VC medium at 50-fold dilution, and incubated with shaking at 30°C for 5 h to allow it to grow to late log phase. Cells were pelleted by centrifugation at 10,000×*g* for 10 min. The supernatant (Medium) was centrifuged at 100,000×*g* for 60 min. The pellet (Ppt2) was suspended in V-buffer (25 mM Tris-HCl [pH 7.5], 300 mM NaCl, 10 mM $MgCl_2$) and Triton X-100 was added at 1% final concentration. The suspension was centrifuged at 100,000×*g* for 60 min. The pellet (Ppt3) and the supernatant (Sup3) were recovered.

Electron Microscopy—Samples were negatively stained with 2% potassium phosphotungstate (pH 7.0) and observed with a JEM-1200 EXII electron microscope (JEOL, Tokyo).

RESULTS

Isolation of Mutants Lacking the Filament of the Polar Flagella—Swarm-deficient mutants isolated previously from lateral flagellar defective mutants (Pof^+ Laf^-) were divided into 3 categories: those defective in the polar flagellum formation (Pof^-), those defective in the polar flagellar motor (Pom^-), and those defective in chemotaxis (Che^-) (*18, 17*, this study). Cells of fresh colonies from about 70 Pof^- mutants were negatively stained and observed by electron microscopy. In three of those mutants (YM51, NMB103, and NMB116), we could identify the hook structure at the cell poles even though the filament was missing (Fig. 1). The hooks of these mutants were covered with a sheath structure having a length of about 50 nm, similar to that of *Salmonella typhimurium* (*19*).

Detection of Flagellin in the Culture Medium—Cells were grown to late-log phase in VC medium, and harvested by low-speed centrifugation. Proteins in the supernatant were separated by SDS-PAGE and flagellin was detected by immunoblotting using an anti-PF45 flagellin antibody which also strongly reacts with PF47 flagellin (data not shown). The flagellin bands were detected with the three filamentless mutants, but not with other Pof^- mutants such as NMB70 and NMB79 (Fig. 2a). The amount of flagellin in the unsedimented culture of the mutants was almost the same as in that of the wild-Pof strain, YM4, and while the amounts in the culture supernatants were larger in the case of the mutants (data not shown). Since flagellin of the wild-Pof strain is presumably sedimented with cells as attached filaments, we conclude that the filamentless mutants excrete flagellin into the culture medium.

Unassembled Flagellin—The culture medium of each mutant was centrifuged at 100,000×*g*. Flagellin was detected in the pellet (Ppt2) of the mutants and the wild-Pof strain (Fig. 2b), but was not detected in the supernatant (Sup2) in either case (data not shown). The Ppt2 fraction of each strain was treated with Triton X-100, and centrifuged at 100,000×*g* (Fig. 2b). With the wild-type strain, flagellin was detected in the pellet (Ppt3), but not in the supernatant

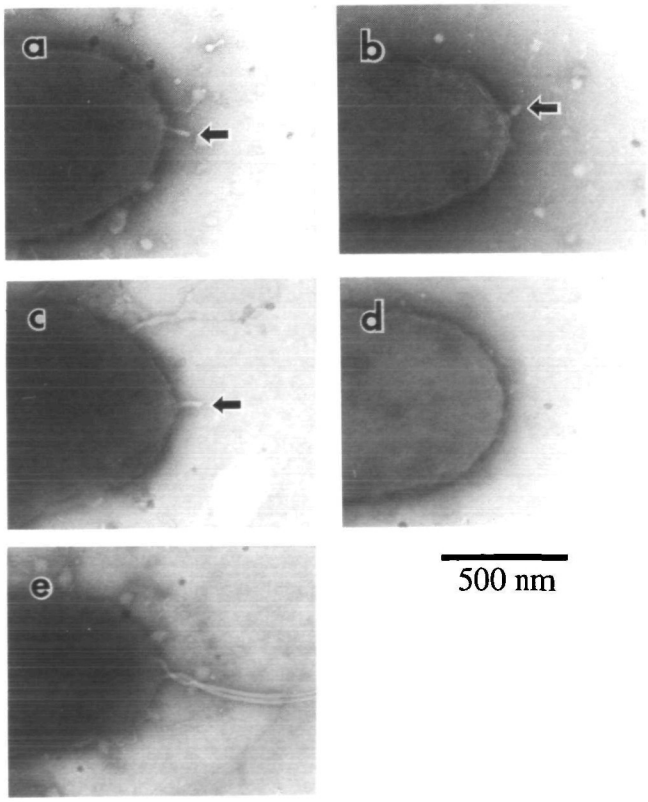

Fig. 1. **Electron micrographs of cells.** YM51 (a), NMB103 (b), NMB116 (c), NMB79 (d), and YM4 (e), were negatively stained with potassium phosphotungstate. Arrows indicate the hooks of the filamentless mutants.

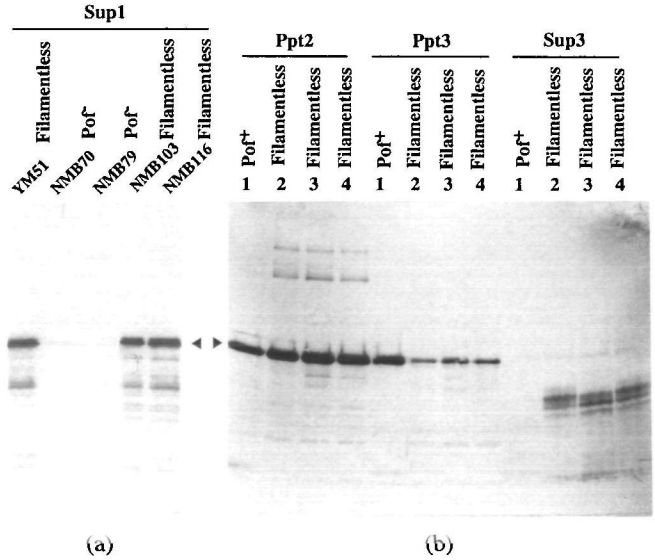

Fig. 2. **Detection and fractionation of flagellin.** (a) Detection of flagellin from culture media (Sup1) from YM51, NMB70 (Pof^-), NMB79 (Pof^-), NMB116, and NMB103, separated by SDS-PAGE and immunoblotted using anti-flagellin antibody. (b) Fractionation for flagellin. The culture medium was centrifuged at 100,000×*g* for 1 h, and the pellet (Ppt2) was suspended in a buffer. Triton X-100 was added, and the suspension was centrifuged again at 100,000×*g* for 1 h. The pellet (Ppt3) and the supernatant (Sup3) were recovered. Proteins in Ppt2, Ppt3, and Sup3 from 1, YM4 (Pof^+); 2, YM51; 3, NMB103; 4, NMB116 were separated by SDS-PAGE and immunoblotted using anti-flagellin antibody. Arrowheads indicate the position of flagellin.

(Sup3). With the mutants, on the other hand, some flagellin was detected in Ppt3 and smaller proteins, probably degradation products, were detected in Sup3. This suggests that flagellin of the filamentless mutants was a different, probably unassembled, form from that of the wild-type strain. Because this flagellin could be sedimented by high-speed centrifugation and degraded after Triton X-100 treatment (Fig. 2b), we thought that it might be associated with a membrane structure.

Membrane-Associated Flagellin—The Ppt2 fraction of the filamentless mutants was examined by electron microscopy, and exhibited amorphous membrane-like structures (Fig. 3), some of which were tubular in shape, resembling a piece of the polar-flagellar sheath. The Ppt2 fraction of the wild-type strain, on the other hand, contained sheathless filaments in addition to the amorphous membrane-like structures. They also sometimes contained tubular membrane-like structures similar to those seen with the filamentless mutants, and these were probably sheaths that had been separated from flagellar filaments during the fractionation procedure. Some of the hook or the short filament structures are shown in Fig. 3b. They might represent the assembled flagellin which was detected in Ppt3 of the filamentless mutants of Fig. 2b.

When the Ppt2 fraction was heated at 80°C and centrifuged again at high speed, flagellin from the wild-Pof strain was degraded. On the other hand, flagellin from the filamentless mutants was sedimented again. After the heat

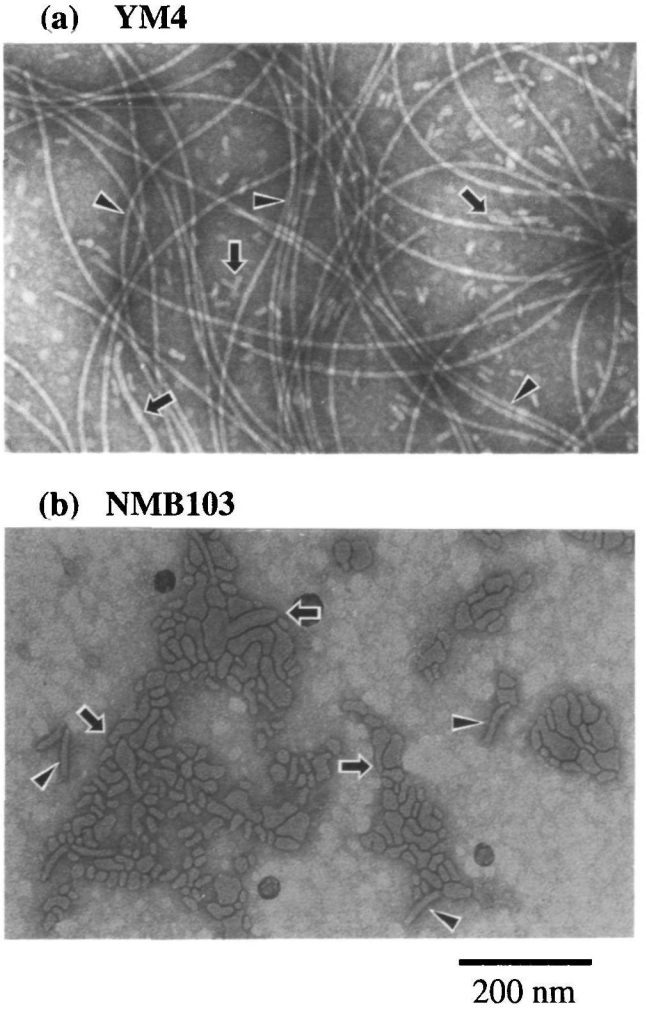

Fig. 3. **Electron micrographs of Ppt2.** Ppt2 from YM4 (a) and NMB103 (b), negatively stained with potassium phosphotungstate. Arrows indicate membrane-like structures, probably the sheath of polar flagella. The flagellar filaments and the short filament structures are indicated by arrowheads in (a) and (b), respectively.

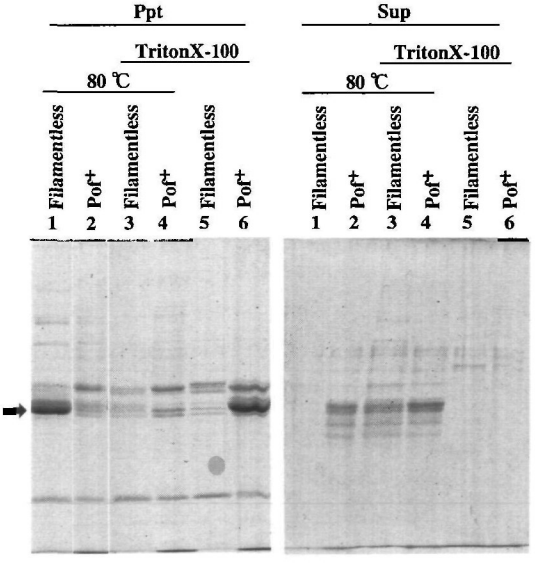

Fig. 4. **Effect of heat treatment of the Ppt2 fraction.** The Ppt2 fraction was prepared from NMB103 (1, 3, 5) and YM4 (2, 4, 6). The Ppt2 fraction was treated with heating at 80°C for 5 min (1, 2), with heating at 80°C for 5 min and then 1% (w/v) Triton X-100 (3, 4), or with 1% Triton X-100 (5, 6), then centrifuged again at 100,000 × *g* for 1 h. The pellet (Ppt) and the supernatant (Sup) were recovered and their proteins were separated by SDS-PAGE and stained with Coomassie Blue R250. An arrow indicates the position of flagellin.

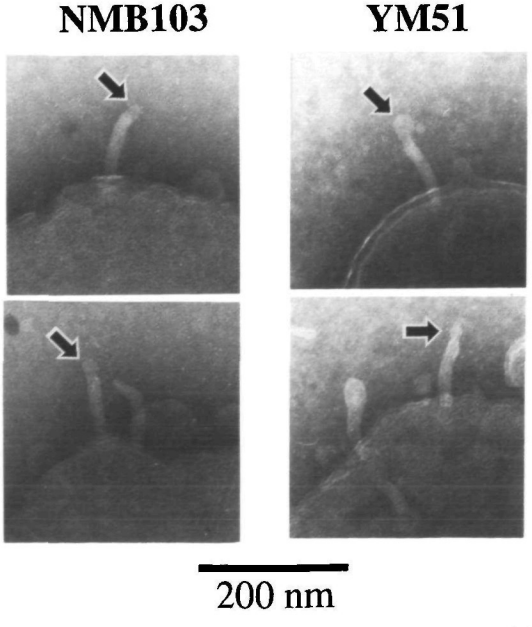

Fig. 5. **Structure of hook tips in filamentless mutants.** Cells of NMB103 and YM51 were negatively stained by potassium phosphotungstate. Arrows indicate vesicle-like structures of the hook tips.

and the Triton X-100 treatment, flagellin from both the wild-Pof and mutant was degraded (Fig. 4). A low pH treatment gave similar results to the heat treatment (data not shown). The heat or low pH treatment probably causes disassembly of the flagellar filament, and the unassembled flagellin became accessible to a proteolytic enzyme. On the other hand, the heat or low pH treatment seems essentially not to affect the membrane structure, because flagellin from the filamentless mutants could be sedimented after the treatment and was degraded by Triton X-100 treatment.

Tips of Hooks—When we observed carefully the tips of hooks in cells of the filamentless mutants, it was seen that the tips bore the membrane-like structures which are probably a part of the sheath (Fig. 5). The sheath of the outer membrane of *V. alginolyticus* is very flexible. It is inferred that flagellin exported through the flagellar structure is sealed by the sheath structure.

DISCUSSION

Mutants which have the hook but not the filament of the polar flagellum were selected by electron microscopy among Pof$^-$ Laf$^-$ strains. These filamentless mutants produce the filament protein, flagellin, in amounts comparable to the wild-Pof strain. We found that the flagellin was excreted into the culture medium and could be sedimented by high-speed centrifugation even after heat or low pH treatments, which usually cause dissociation of flagellar filaments into flagellin monomers (*11*). After treatment with Triton X-100, most of the flagellin from the filamentless mutants was degraded. This suggests that some of the flagellin may exist in an assembled form although most of it does not. Hooks of these mutants were covered with a sheath structure contiguous to the outer membrane and some generated vesicles which are probably made from the sheath. These results strongly suggest that in these mutants the flagellin is not polymerized, but is excreted together with a membranous structure composed of the polar-flagellar sheath (Fig. 6).

Flagellar biogenesis has been extensively studied in *Salmonella typhimurium* (for review, see Refs. *12, 13*, and *20*). The MS ring, which is part of the motor, is constructed in the cytoplasmic membrane first and the switch complex and the export apparatus assemble on its cytoplasmic face. The rod components assemble on the periplasmic face of the MS ring. The components of the P and L rings, which interact with the outer membrane, are transported by a signal peptide-dependent pathway. After the motor has been constructed, the hook and filament structures are sequentially assembled on it. Their components are believed to be transported by a flagellum-specific export apparatus through a central channel in the flagellar structure-motor, hook, and filament. To assemble the filament on the hook, three minor proteins, the hook-associated proteins (HAP1, HAP2, and HAP3), are essential and are located on the distal end of the hooks of flagellin mutants. HAP1 and HAP3 are hook-filament junction proteins. HAP2 is the capping protein of the filament and is required to polymerize flagellin *in vivo* (*21, 22*). HAP mutants and flagellin-defective mutants do not assemble filaments (*23*). HAP mutants excrete unassembled flagellin molecules into the culture medium (*24*).

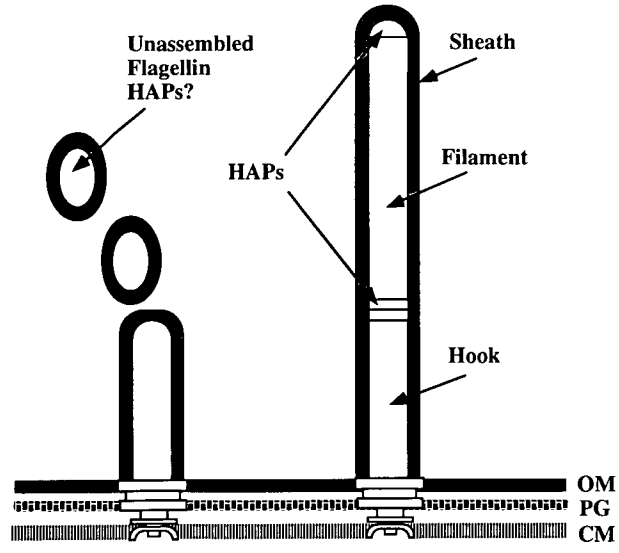

Fig. 6. **Schematic diagrams of polar-flagellar structure and the flagellin excretion.** The right and left indicate the polar-flagellar structures of the wild-type and the filamentless mutant, respectively. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; HAPs, hook-associated proteins.

Based on the above description of flagellar biogenesis in *S. typhimurium*, it seems likely that the polar-filamentless mutants of *V. alginolyticus* characterized in this study are HAP mutants. In *V. parahaemolyticus* which is closely related to *V. alginolyticus*, three genes homologous to HAP1, HAP2, and HAP3 have been cloned (*25*). HAP1 (FlaE) and HAP3 (FlaF) mutants were non-motile, but it seemed that they excreted truncated filaments. The HAP2 (FlaH) mutant, however, was motile and its ring size on motility plates was smaller than that of the wild type. It was assumed that the sheath could trap excreted flagellin sufficiently to permit polymerization to some degree without HAP2. Based on these observations, the filamentless mutants isolated in our study may not include HAP2 mutants.

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