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Isolation of the Polar and Lateral Flagellum-Defective Mutants in *Vibrio alginolyticus* and Identification of Their Flagellar Driving Energy Sources

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***Vibrio alginolyticus* has two types of flagella (polar and lateral) in one cell. We isolated mutants with only a polar flagellum (Pof^+ Laf^-) or only lateral flagella (Pof^- Laf^+). Using these mutants, we demonstrated that the energy sources of the lateral and polar flagellar motors in *V. alginolyticus* are H^+ and Na^+ motive forces, respectively, as in the related species *V. parahaemolyticus*.**

Vibrio alginolyticus and *V. parahaemolyticus*, which are closely related, have similar flagellar systems. They have two types of flagella, named polar flagella (Pof) and lateral flagella (Laf), in one cell (1, 4). When they are grown in a liquid environment, they express mainly a single polar flagellum at their cell poles and swim by it rapidly. When they are transferred to a solid agar medium, they express lateral flagella peritrichously in addition to polar flagella, and the cells elongate and begin to move and spread on the surface (3, 9, 12, 13). Recently, by using those flagellar mutants with only polar flagella and with only lateral flagella, it has been shown that in *V. parahaemolyticus*, the energy sources of polar and lateral flagellar motors are Na^+ and H^+ motive forces, respectively (2). Results of experiments using wild-type cells have suggested that the polar and lateral flagella of *V. alginolyticus* are also powered by different ion motive forces (2, 5). Although some flagellum-defective mutants have been isolated from *V. alginolyticus* (12), the organism has not yet been characterized in this regard. In this study, we isolated mutants with only polar or with only lateral flagella from *V. alginolyticus*, which is less pathogenic than *V. parahaemolyticus* (4) and is well characterized in terms of respiration-coupled primary sodium pumps (10, 11). Furthermore, an energetic analysis of the polar and lateral motors was carried out.

Isolation of lateral or polar flagellum-defective mutants. The parental strain *V. alginolyticus* 138-2 (Pof^+ Laf^+) (11) was exposed to UV light at the 0.01% survival level. The cells were spotted on the center of 1.5% agar HI plates (1.5% agar in HI broth, which contained [per liter of distilled water] 25 g of heart infusion broth [Difco] and 20 g of NaCl) and incubated at 30°C overnight. To enrich nonswarming cells on 1.5% agar, the cells from the original spot location were grown in HI broth to stationary phase. After the enrichment procedure was repeated, the diluted cultures from the center were spread on 2.5% agar HI plates (2.5% agar in HI broth). As a result, we isolated the three independent mutants which could not swarm on the 1.5% agar plate. The lateral flagellum-defective mutants (Pof^+ Laf^-) were named YM4, YM5, and YM6. Strains YM4 and YM6 did not give rise to Laf^+ revertants when the cells were inoculated on 1.5% agar plates and incubated at 30°C for 3 days, but YM5 did.

Next, we tried to isolate polar flagellum-defective mutants (Pof^- Laf^+), which were expected to swarm on either 1.5 or 0.3% agar plates by using lateral flagella. Therefore, first we isolated Pof^- Laf^- mutants from the Pof^+ Laf^- mutants by a procedure similar to that used for the Pof^- Laf^+ isolation but using 0.3% agar HI plates (0.3% agar [Difco] in HI broth). After enrichment procedures, the swarming ability of colonies was tested in 0.3% agar plates. We obtained the following Pof^- Laf^- mutants, which could be regarded as independent mutants: YM14 from YM4; YM17, YM18, and YM32 from YM5; and YM44 from YM6. Next, the Pof^- Laf^- mutants were inoculated in lines on HI 1.5% agar plates and incubated at 30°C. After 2 or 3 days, we observed in YM17 and YM32 several spontaneous swarming revertants which recovered the same swarming ability by lateral flagella as wild-type cells. The Pof^- Laf^+ mutants from YM17 and YM32 were named YM19 and YM38, respectively.

In *V. parahaemolyticus*, polar and lateral flagellar genes have been characterized by using transposon mutagenesis and conjugation (8). The mutants isolated in this study were not genetically characterized because of the lack of a good genetic system for *V. alginolyticus*. However, a reliable transformation procedure for *V. alginolyticus* has been established recently by using a P15A replicon-based plasmid vector system and electroporation (7). This development promises to improve the genetic analysis of the mutants.

Characterization of isolated mutants. We determined that the polar and lateral flagella of the isolated mutants had the same appearances as those of the wild type by electron microscopy. Motility profiles of the flagellum-defective mutants on 1.5 and 0.3% agar plates are shown in Fig. 1. The swarm rings of the wild type (Pof^+ Laf^+) and YM4 (Pof^+ Laf^-) were similar in size on the 0.3% agar plate (Fig. 1A). However, the rings of YM19 (Pof^- Laf^+) on the 0.3% agar plate were smaller than those of the wild type and YM4. From these lines of evidence, it is inferred that wild-type cells swim mainly by using polar flagella on the 0.3% agar plate. Swarming ring profiles of the wild type (Pof^+ Laf^+) and YM19 (Pof^- Laf^+) on the 1.5% agar plate were identical (Fig. 1B), which suggests that polar flagella do not contribute to swarming on the 1.5% agar plate. The Pof^- Laf^+ mutants of *V. parahaemolyticus* have been isolated directly from wild-type cells by screening for

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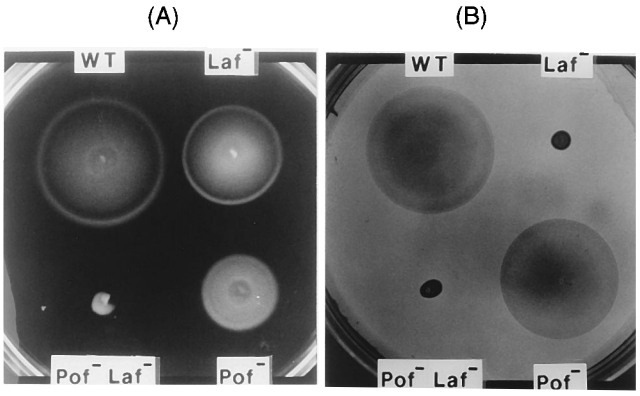

FIG. 1. Motility profiles of the flagellar mutants on 0.3% (A) and 1.5% (B) agar HI plates. Strains: wild type (WT), 138-2; Pof$^+$ Laf$^-$, YM4; Pof$^-$ Laf$^-$, YM18; Pof$^-$ Laf$^+$, YM19. Three microliters of an overnight culture of each strain was spotted on each plate and incubated at 30°C for several hours. Because swarming rings on 1.5% agar are made of thin layers of the cells and are hardly visible, cells were stained for about 3 min with 0.1% Coomassie brilliant blue in methanol-acetic acid-water (5:1:4) and destained with methanol-acetic acid-water.

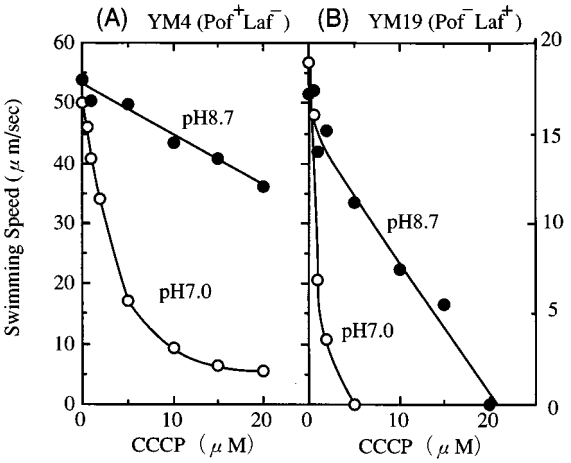

FIG. 3. CCCP sensitivity of swimming speeds of YM4 (Pof$^+$ Laf$^-$) and YM19 (Pof$^-$ Laf$^+$) cells. The swimming speed was measured in HEPES motility buffer containing 300 mM NaCl at pH 7.0 or pH 8.7 and supplemented with various amounts of CCCP.

motility-defective mutants in minimal medium solidified with 0.3% agar (9). Though we did not do such a selection to obtain Pof$^-$ Laf$^+$ mutants, their swarm size in the semisolid agar was smaller than those of the wild-type strain and the Pof$^+$ Laf$^-$ mutant (Fig. 1). This may be because the movement in semisolid agar by lateral flagella is slower than that by polar flagella in *V. alginolyticus* and *V. parahaemolyticus*, as observed for free-swimming cells.

It has been proposed that the polar flagellum is a sensor of environmental conditions and controls lateral flagellar expression in *V. parahaemolyticus* (9). The Pof$^-$ Laf$^+$ cells (YM19 and YM38) expressed lateral flagella constitutively in liquid media without polyvinylpyrrolidone (PVP) and were not elongated even in viscous medium. A similar observation has been reported for the polar flagellum-defective mutants of *V. parahaemolyticus* (8), which suggests that the two species have the same sensor system by polar flagella.

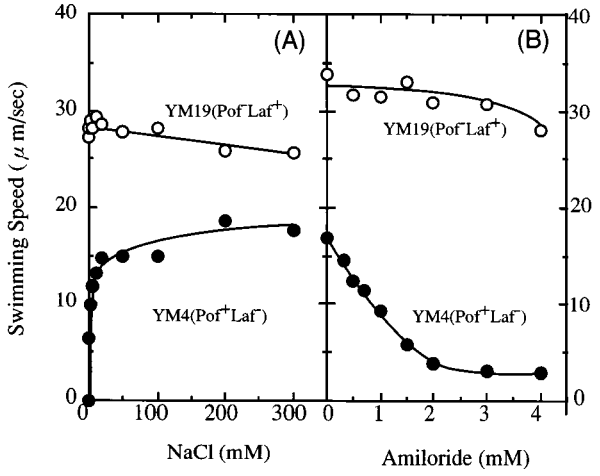

FIG. 2. Na^+ dependency and amiloride sensitivity of swimming speeds of YM19 (Pof$^-$ Laf$^+$) and YM4 (Pof$^+$ Laf$^-$) cells. Swimming speed was measured in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) motility buffer, which contained 50 mM HEPES-KOH (pH 7.0), 5 mM glucose, 10 mM $MgCl_2$, and various concentrations of NaCl in the presence of 3.5% PVP (A) and in HEPES motility buffer (pH 7.0) containing 50 mM NaCl and 3.5% PVP without $MgCl_2$ and supplemented with various amounts of amiloride (B).

Energy source of flagellar motors. We confirmed the energy source of flagellar motors in *V. alginolyticus* by using the mutants which possessed only polar or lateral flagella. The culture at the late log phase was directly diluted 100- or 500-fold into a motility medium, and the cell motility was observed under a dark-field microscope. Within 1 min after dilution, the swimming speed was measured at 25°C (maintained by air conditioning) by a photographic method (6). The motility medium was supplemented with 10 mM L-serine as an attractant. After the addition, the cells swim smoothly without directional change for at least 1 min (14). To improve the swimming speed by lateral flagella, PVP was added to the medium in some experiments, but the addition of PVP reduces the swimming speed by polar flagella. The motility of YM4 (Pof$^+$ Laf$^-$) cells was dependent on the Na^+ concentration and inhibited by amiloride, which is a specific inhibitor of Na^+-driven flagellar motors (Fig. 2). In contrast, YM19 (Pof$^-$ Laf$^+$) showed good motility even without Na^+ in the motility medium, and its motility was not affected by amiloride. *V. alginolyticus* has respiration-coupled H^+ and Na^+ pumps, and the Na^+ pump is only active in an alkaline pH range (10, 11). In the neutral to slightly acidic pH range, the Na^+ motive force is secondarily generated by the Na^+/H^+ antiporter from the H^+ motive force. Upon addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at pH 7.0, the H^+ motive force and hence the Na^+ motive force are lost. Under this condition, swimming speeds of both YM19 and YM4 were reduced drastically (Fig. 3). On the other hand, at the alkaline pH in the presence of CCCP, the H^+ motive force is lost but the Na^+ motive force is generated by the primary Na^+ pump. As shown in Fig. 3, at pH 8.7, the motility of YM19 was lost at high CCCP concentrations but YM4 remained motile in the presence of CCCP. These results showed that the polar flagellar motor is Na^+ driven and the lateral flagellar motor is H^+ driven in *V. alginolyticus*, as in the related species *V. parahaemolyticus* (2).

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