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Ion-coupling Determinants of Na^+-driven and H^+-driven Flagellar Motors

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The bacterial flagellar motor is a tiny molecular machine that uses a transmembrane flux of H^+ or Na^+ ions to drive flagellar rotation. In proton-driven motors, the membrane proteins MotA and MotB interact *via* their transmembrane regions to form a proton channel. The sodium-driven motors that power the polar flagellum of Vibrio species contain homologs of MotA and MotB, called PomA and PomB. They require the unique proteins MotX and MotY. In this study, we investigated how ion selectivity is determined in proton and sodium motors. We found that *Escherichia coli* MotA/B restore motility in Δ*pomAB Vibrio alginolyticus*. Most hypermotile segregants isolated from this weakly motile strain contain mutations in *motB*. We constructed proteins in which segments of MotB were fused to complementary portions of PomB. A chimera joining the N terminus of PomB to the periplasmic C terminus of MotB ($PotB7^E$) functioned with PomA as the stator of a sodium motor, with or without MotX/Y. This stator (PomA/$PotB7^E$) supported sodium-driven motility in *motA* or *motB E. coli* cells, and the swimming speed was even higher than with the original stator of *E. coli* MotA/B. We conclude that the cytoplasmic and transmembrane domains of PomA/B are sufficient for sodium-driven motility. However, MotA expressed with a B subunit containing the N terminus of MotB fused to the periplasmic domain of PomB ($MomB7^E$) supported sodium-driven motility in a MotX/Y-dependent fashion. Thus, although the periplasmic domain of PomB is not necessary for sodium-driven motility in a PomA/B motor, it can convert a MotA/B proton motor into a sodium motor.

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Keywords: flagella; motor proteins; ion channels; ion selectivity; Vibrio

Introduction

Many species of bacteria monitor their chemical environment and swim toward favorable conditions by rotating helical organelles known as flagella.[1–3] The energy source for flagellar rotation is the electrochemical potential of a specific ion (H^+ or Na^+) across the cytoplasmic membrane. The most commonly studied bacteria, e.g. *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*, have proton-driven motors. The polar flagellum of *Vibrio* spp. (*V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae*) and the peritrichous flagella of alkalophilic Bacillus species, in contrast, have sodium-driven motors.[4,5]

The *pomA*, *pomB*, *motX* and *motY* genes, whose protein products are membrane-associated proteins that are essential for sodium-driven torque generation, have been cloned.[6–10] PomA and PomB are homologous to MotA and MotB, respectively, of the proton-driven motor.[10] MotX and MotY are unique to the sodium-driven motor. Little is known about the function of MotX and MotY, except that the C terminus of MotY contains a consensus peptidoglycan-binding motif[7,9] and that both proteins localize to the outer membrane.[11]

MotA and PomA each have four membrane-spanning regions, a large cytoplasmic loop and a cytoplasmic C-terminal tail. MotB and PomB each have a single membrane-spanning region.[10] PomA

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Abbreviations used: TM, transmembrane segment; Pof, polar flagellum.

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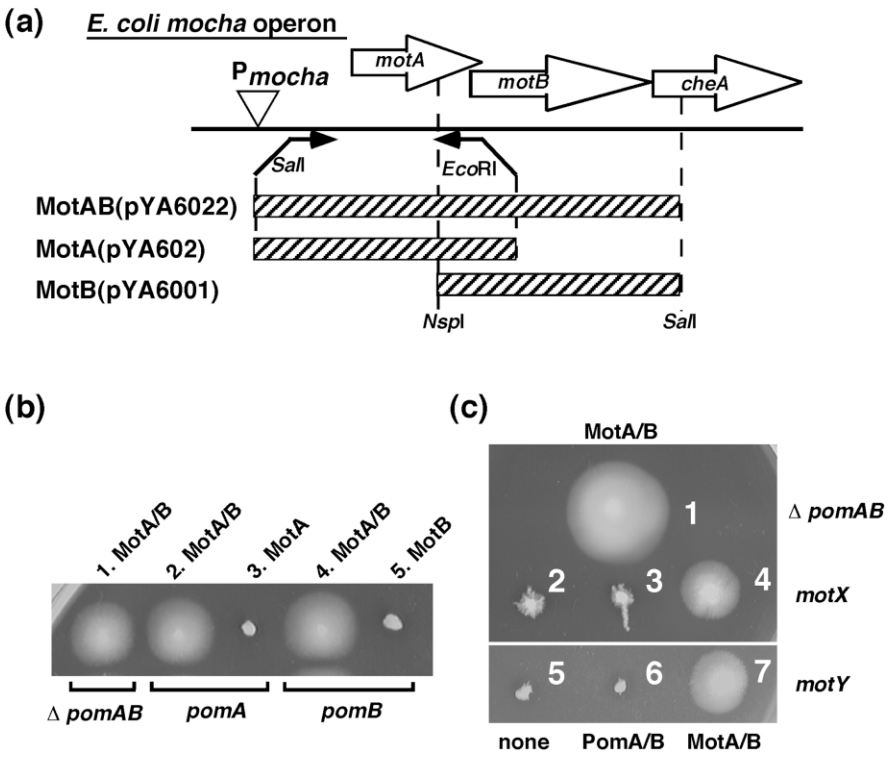


Figure 1. (a) Restriction maps of plasmids. Inserted fragments of each plasmid are indicated as hatched bars. Filled arrows show PCR primers. The inverted triangle shows the location of the promoter of the *E. coli mocha* operon. The open arrows delineate the coding region of *motA*, *motB* and *cheA*. (b) and (c) Complementation of *pom* mutants by *E. coli* MotA and/or MotB. (b) Stator proteins expressed are: 1, MotA/B; 2, MotA/B; 3, MotA; 4, MotA/B; 5, MotB. The host strains are: 1, NMB191 (Δ*pomAB* mutant); 2 and 3, NMB190 (*pomA* mutant); 4 and 5, NMB192 (*pomB* mutant). (c) Stator proteins expressed are: 1, 4 and 7, MotA/B; 2 and 5, none; 3 and 6, PomA/B. The host strains are: 1, NMB191 (Δ*pomAB* mutant); 2–4, NMB119 (*motX* mutant); 5–7, VIO542 (*motY* mutant).

and PomB interact with each other.[12] A reconstituted PomA/B complex is capable of supporting $^{22}Na^+$ uptake into proteoliposomes.[13] These results suggest that the PomA/B complex functions as a sodium-ion channel. MotB and PomB both have a highly conserved acidic residue that faces into the transmembrane channel. This residue is presumably crucial both for ion flux and for torque generation. Protonation of the acidic residue may induce conformational changes in the stator that drive rotation of the rotor. These conformational changes were detected by the differential protease sensitivities of MotA in membrane-bound MotA/B complexes in which the acidic residue was replaced with neutral or positively charged residues.[14]

The C-terminal regions of MotB and PomB contain a predicted peptidoglycan-binding motif[10] that has been implicated in anchoring the stator to the cell wall. The region between the membrane-spanning and peptidoglycan-binding domains is poorly conserved among B subunits (Figure 3) and is largely dispensable for function.[15] It may serve as a flexible linker.

It has been proposed that two charged residues in the cytoplasmic region of MotA interact with oppositely charged residues of FliG, a component of the rotor, and that these interactions are important for flagellar rotation.[16] On the other hand, it has been shown that these residues, which are conserved in sodium-driven motors, may not be essential for their function.[17] Thus, there are likely to be some fundamental differences in the operation of sodium-driven and proton-driven motors.

The Na^+-binding site of the motor was identified by mapping *pomA* and *pomB* mutations that confer resistance to phenamil,[18] an analog of the sodium-channel blocker amiloride. The mutations targeted residues near the cytoplasmic ends of the transmembrane segments of PomA or PomB, supporting the proposal that PomA and PomB combine to form a Na^+ channel.[19,20] The overlapping distributions of *pomA* and *motA* missense mutations that interfere with motor function suggest that PomA and MotA have similar structures and roles.[21] Periplasmic $loop_{1-2}$ of PomA, which connects transmembrane helices 1 and 2, might be in contact with other proteins, or it may interact with the pore region of the channel to contribute to the efficient transport of ions.[22–24] Crosslinking studies indicate that periplasmic $loop_{3-4}$, which joins transmembrane helices 3 and 4, of one PomA subunit is close to $loop_{3-4}$ of an adjacent PomA subunit within the stator complex.[24,25]

We have shown that MotA/B from the proton-driven motor of *Rhodobacter sphaeroides* do not complement a Δ*pomAB* mutant of *V. alginolyticus*,[26,27] nor do PomA/B support flagellar rotation without MotX and MotY. However, a Δ*pomABmotXY* strain of *V. cholerae* can swim using *E. coli* MotA/B and proton motive force.[28] In this study, we have addressed the question of what differences exist between PomA/B and *E. coli* MotA/B by determining whether hybrid stators that contain different combinations of elements from these proteins can support rotation of the sodium-driven polar flagellum of *V. alginolyticus*.

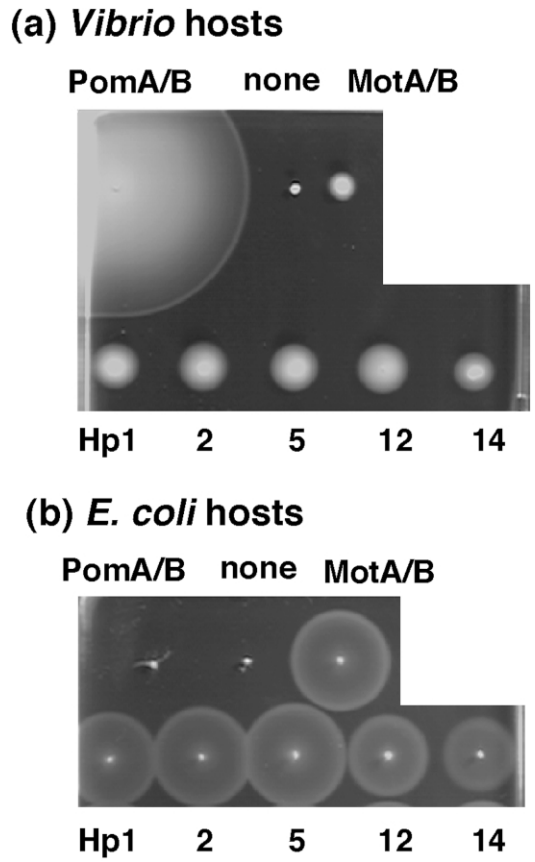


Figure 2. Complementation by the hyper-motile mutants. Fresh colonies of (a) the *V. alginolytucus pomAB* mutant or (b) the *E. coli motB* mutant carrying each plasmid, pYA303 (PomA/B), pSU41 (none), pYA6022 (MotA/B), and the hyper-motile mutant plasmids (HP) from pYA6022, were inoculated and incubated on 0.3% VPG (pH7.0) agar for 15 hours (for Vibrio cells) or on T broth agar for six hours (for *E. coli* cells) at 30 °C.

Results

Complementation of a *V. alginolyticus* Δ*pomAB* mutation by *E. coli motAB*

To examine whether *E. coli motA* and/or *motB* can complement *pomA*, *pomB* and *pomAB* null mutations in *V. alginolyticus*, we constructed plasmids containing *motAB* (pYA6022), *motA* (pYA601) and *motB* (pYA6001), as shown in Figure 1(a). The *motA* and/or *motB* genes were transcribed from the *lac* promoter, and the native sigma-28 promoter of the *mocha* operon was excluded from the plasmids. All of the *pom* mutants that expressed MotA/B were motile, although they formed very small swarms (Figure 1(b)). MotA alone did not restore motility to a *pomA* mutant, nor did MotB alone restore motility to a *pomB* mutant. Furthermore, the strains swarmed significantly when the *E. coli* MotA/B proteins were expressed in a *motX* or a *motY* mutant (Figure 1(c)). Motility was not restored by overexpression of PomA/B in either the *motX* or *motY* mutant. This result indicated that *E. coli* MotA/B functions as the proton-driven motor without MotX or MotY in the *V. alginolyticus* polar flagellar motor, as previously reported for *V. cholerae*.[28]

When the *pomAB* mutant expressing MotA/B from plasmid pYA6022 was incubated on semi-solid agar plates for a few days, some faster-swarming flares appeared. Thirty-eight of 45 isolates from independent flares contained plasmid-borne mutations that conferred hypermotility, and all of these hypermotile isolates formed swarms that were as large as those formed by wild-type *E. coli*. (Figure 2). The *motA* and *motB* genes of plasmids prepared from each strain were sequenced to find the mutations. Seventeen unique plasmid-borne mutations were found (Figure 3). All of them were located in *motB*, although both *motA* and *motB* are present in pYA6022. The mutations were distributed widely and some mutations were clustered in the periplasmic region near the putative transmembrane segment (TM). Most strains, except Hp14 and Hp26, which seem to be less motile than the others, showed similar sizes of swarming.

Properties of PomB/MotB chimeras

In a previous study, we constructed fusions between *R. sphaeroides* MotB and *V. alginolyticus* PomB.[27] One of these chimeras (MomB3^{R}) contains an N-terminal portion of *R. sphaeroides* MotB (from the first residue through the transmembrane region) and a C-terminal portion of PomB (the extracellular region from the end of the transmembrane region through the last residue). The fusion joint is at junction site 3 (Figure 3). MomB3^{R} functioned together with *R. sphaeroides* MotA in Vibrio, whereas intact *R. sphaeroides* MotB did not. MomB4^{R}, named in the same fashion for junction site 4, did not support swarming in combination with either *R. sphaeroides* MotA or PomA. Thus, the integrity of the region around junction site 4 may be important for the function of the B subunit.

For these reasons, and because of the strong similarity in the amino acid sequences of PomB, *R. sphaeroides* MotB and *E. coli* MotB around this site (Figure 3), we joined the extracellular domains of *V. alginolyticus* PomB and *E. coli* MotB at site 7. This chimeric B protein was named PotB7^{E} (Figure 4). Similarly, we constructed MomB7^{E} and MomB7^{R}. These chimeric B subunits were co-expressed with A subunits corresponding to the species that contributed the transmembrane region of the B subunits. All three chimeric stators function with the Vibrio rotor (Figure 5(a)). The PomA/PotB7^{E} stator supported the best swarming, which was much better than that of a motor containing the *E. coli* MotA/B stator. The reciprocal stator, MotA^{E}/MomB7^{E}, also functioned well.

We examined whether each B-subunit chimera could function in the absence of MotX or MotY. Although the MotA^{E}/MomB7^{E} and MotA^{R}/MomB7^{R} stators (data not shown) did not restore

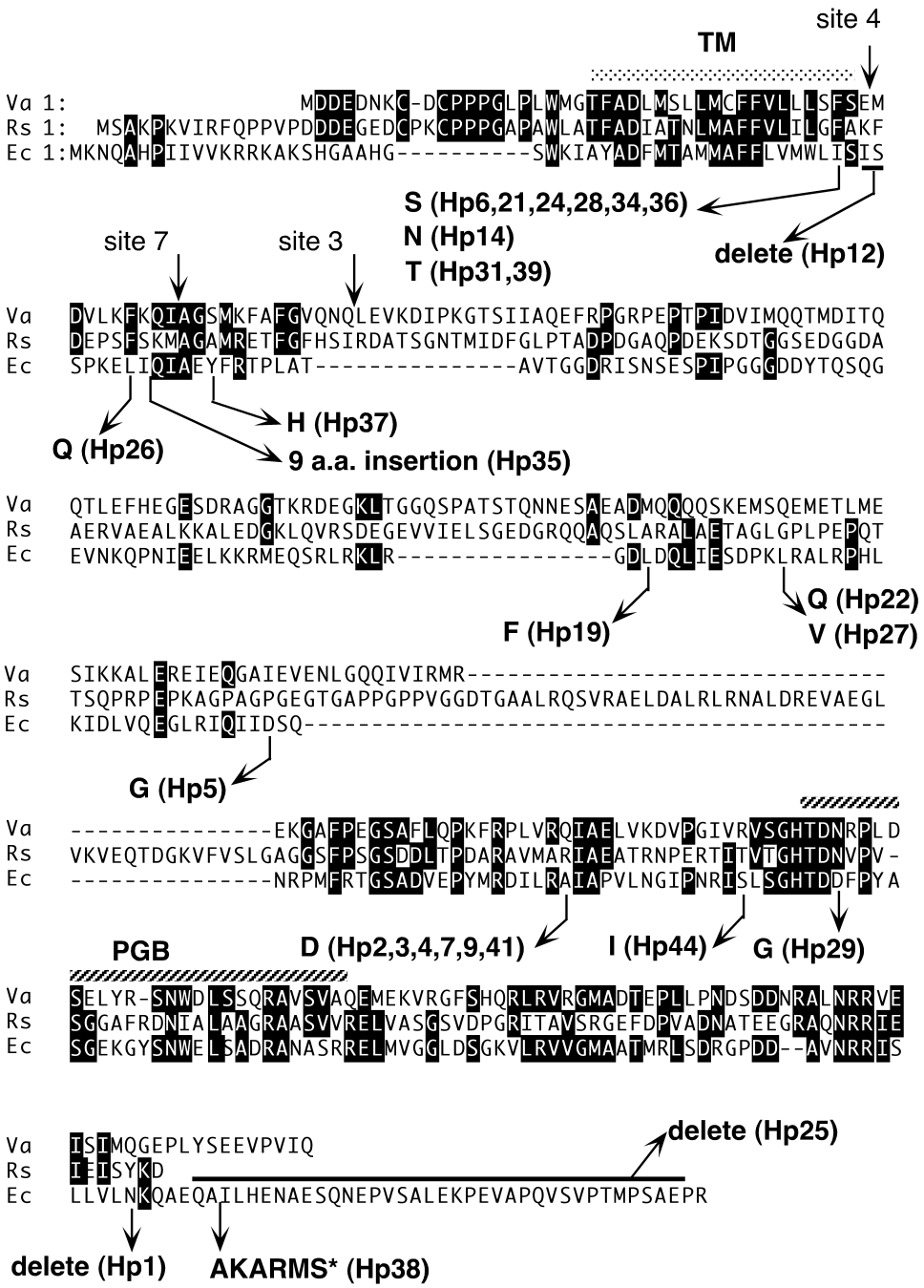


Figure 3. Amino acid alignments of *V. alginolyticus* PomB (Va), *R. sphaeroides* MotB (Rs), and *E. coli* MotB (Ec), and location of hypermotile mutations and junction sites of chimeric proteins. White letters in black boxes and arrows show identical residues and junction sites of chimeric proteins, respectively. Gray and hatched bars indicate putative transmembrane (TM) regions and peptidoglycan binding motif (PGB), respectively. Hypermotile mutations and strain numbers are indicated under the alignment.

motility to *motX* or *motY* mutants, which have MotY or MotX in addition to PomA and PomB, the PomA/PotB7^{E} stator allowed both mutants to swarm (Figure 5(a)). This result demonstrated that the PomA/PotB7^{E} stator functions without MotX or MotY. Furthermore, the PomA/PotB7^{E} stator conferred motility to a *motA* or *motB* mutant of *E. coli* (Figure 5(b)). On the other hand, neither the MotA^{E}/MomB7^{E} stator nor the MotA^{R}/MomB7^{R} stator functioned in *E. coli*.

Determination of the coupling ion in hybrid motors

PomA/PotB7^{E} worked without MotX and MotY in either *V. alginolyticus* or *E. coli*. We therefore examined whether hydrogen or sodium ions drive motor rotation in Vibrio and *E. coli* cells containing such hybrid stators (Figure 6). Vibrio cells can produce both a sodium-driven polar flagellum (Pof) and proton-driven lateral flagella (Laf) in the same

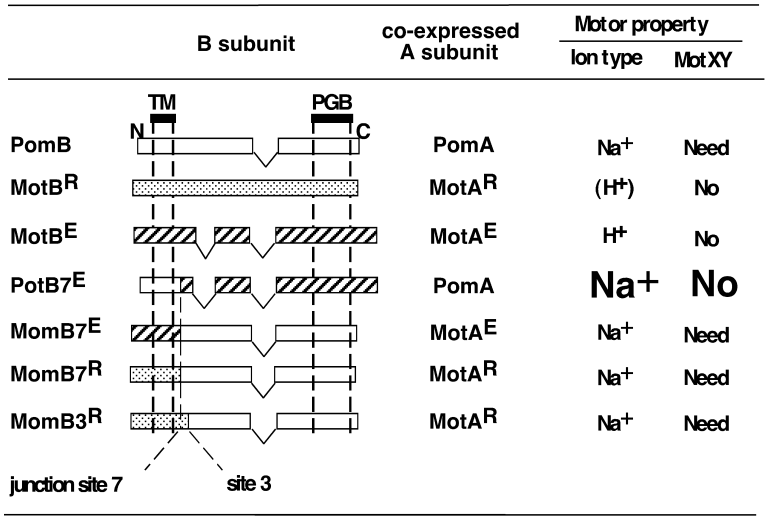
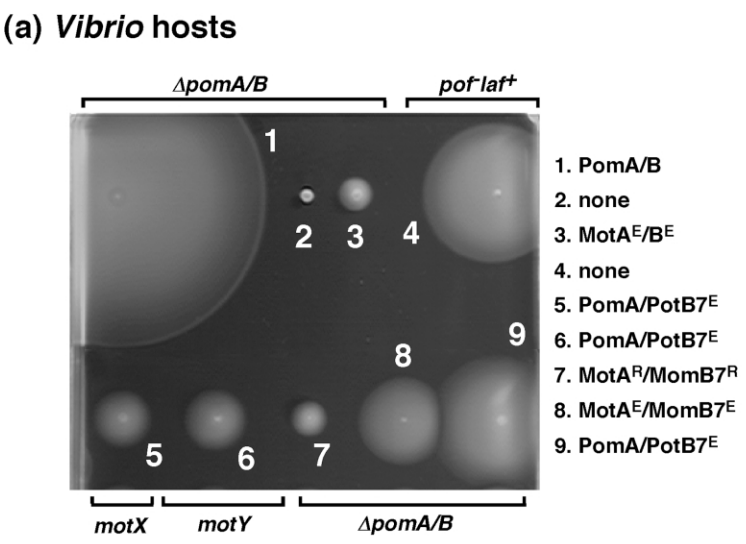


Figure 4. Diagram of the properties of each chimeric B subunit. White, gray and hatched bars show the contributions of PomB or MotB from *V. alginolyticus*, *R. sphaeroides* and *E. coli*, respectively. TM, transmembrane region; PGB, peptidoglycan-binding motif. The stator proteins were expressed in *V. alginolyticus* or *E. coli*. *R. sphaeroides* MotA/MotB do not function in *E. coli* or *V. alginolyticus*, and PomA/PomB function only in *V. alginolyticus*. MotB^E and PotB7^E function in *E. coli* and *V. alginolyticus*. The properties of motors containing either protein are essentially the same in both species. Stators containing MomB7^E or MomB7^R function only in *V. alginolyticus*.



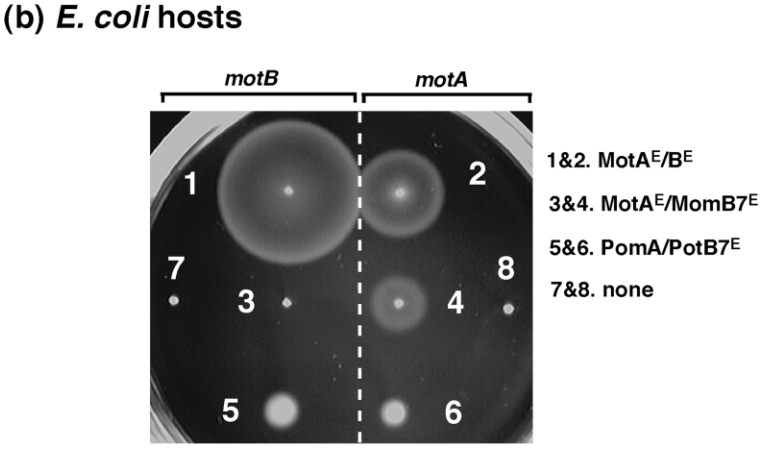


Figure 5. Complementation of (a) *V. alginolyticus* and (b) *E. coli* mutants by expression of chimeric proteins. Mutant cells expressing the indicated proteins were inoculated into 0.3% VPG (pH 7.0) agar and incubated at 30 °C for 15 hours (for *V. alginolyticus*) or 0.3% tryptone agar for six hours (for *E. coli*). (a) Stator proteins expressed from plasmids are: 1, PomA/B; 2, none; 3, MotA^E/B^E; 4, none; 5, PomA/PotB7^E; 6, PomA/PotB7^E; 7, MotA^R/MomB7^R; 8, MotA^E/MomB7^E; 9, PomA/PotB7^E. The host strains are: 1–3, and 7–9, NMB191 (Δ*pomAB* mutant); 4, YM19 (*pof laf*$^+$); 5, NMB119 (*motX* mutant); 6, VIO542 (*motY* mutant). (b) Stator proteins expressed are: 1 and 2, MotA^E/B^E; 3 and 4, MotA^E/MomB7^E; 5 and 6, PomA/PotB7^E; 7 and 8, none. The host strains are: 1, 3, 5, and 7, M254 (*E. coli motB* mutant); 2, 4, 6, and 8, M256 (*E. coli motA* mutant).

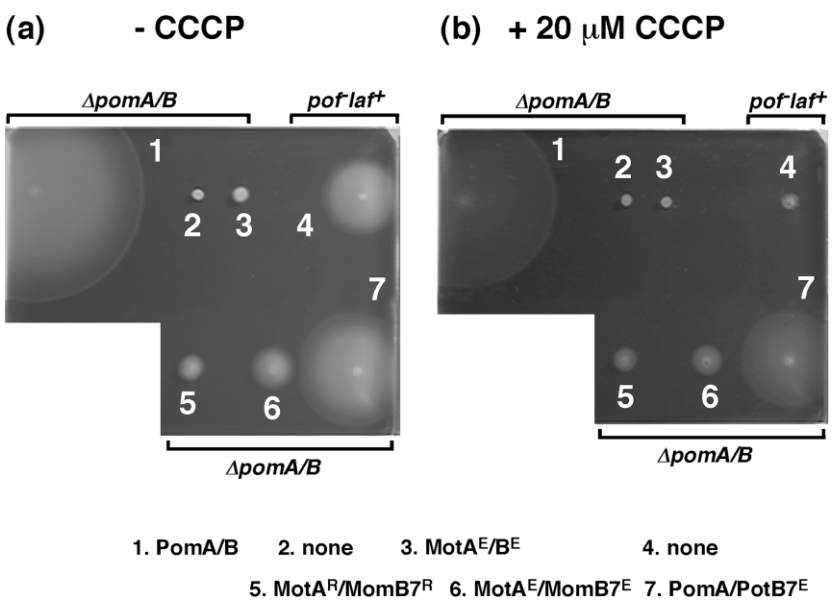


Figure 6. Effect of CCCP on swarming of *V. alginolyticus*. Fresh cells were inoculated into 0.3% VPG (pH8.5) agar (a) without or (b) with CCCP and incubated at 30 °C for 15 hours. Stator proteins expressed are: 1, PomA/B; 2, none; 3, MotA^{E}/B^{E}; 4, none; 5, MotA^{R}/MomB7^{R}; 6, MotA^{E}/MomB7^{E}; 7, PomA/PotB7^{E}. Strain NMB191 (Δ*pomAB* mutant) was used as host except in inoculum 4, for which strain YM19 (*pof laf*$^{+}$) was used.

cell.[29,30] The Pof^{-} Laf^{+} strain YM19 (Figure 6-4) did not swarm in the presence of CCCP, an H^{+}-shuttling uncoupler that specifically dissipates the proton motive force while leaving the sodium motive force intact. The Pof^{+} Laf^{-} strain PomA/B/NMB191 (Figure 6-1), in contrast, was unaffected by CCCP because *V. alginolyticus* can produce a sodium motive force directly in an alkaline environment.[31] The motility of *V. alginolyticus* cells expressing *E. coli* MotA/B was sensitive to CCCP like the Pof^{-} Laf^{+} strain (Figure 6-3).

Available evidence suggests that *E. coli* MotA/B function as an H^{+}-conducting channel in the proton-driven flagellar motor. Thus, substitution of PomA/B by *E. coli* MotA/B converted the sodium-driven polar motor of *V. alginolyticus* into a proton-driven polar motor. However, *V. alginolyticus* Δ*pomAB* mutants expressing any one of the chimeric stators MotA^{R}/MomB7^{R}, MotA^{E}/MomB7^{E} or PomA/PotB7^{E} were motile in the presence of CCCP at alkaline pH (Figures 6b-5, 6b-6 and 6b-7). Therefore, the polar motors in these strains must be driven by sodium motive force. We had confirmed that they did not move in a buffer containing no Na^{+}.

A current of Li ions can drive rotation of the wild-type polar flagellum of *V. alginolyticus*, although the maximal swimming speed in the presence of Li^{+} is only one-third that observed in the presence of Na^{+}.[27] K^{+} cannot be utilized by the polar flagellum. *E. coli motB* cells expressing MotA/B swam equally well in Tris motility buffer containing 100 mM KCl, NaCl or LiCl; these proton-driven motors are not affected by the presence

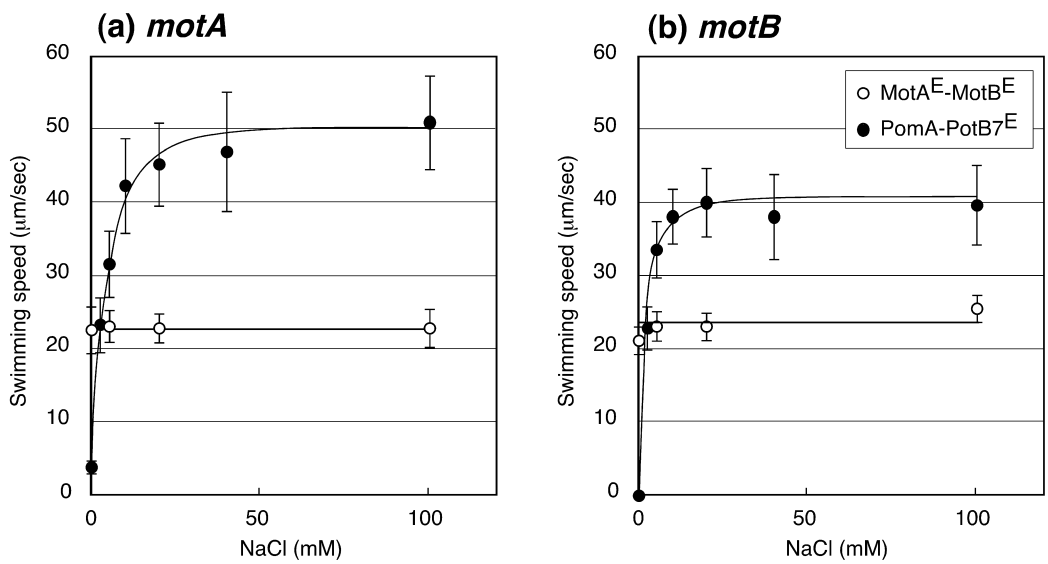


Figure 7. Swimming speed of *E. coli mot*-cells having MotA/B or PomA/PotB7^{E}. The swimming speeds of the (a) *E. coli motA* and (b) *E. coli motB* cells expressing *E. coli* MotA/B (open circles) or PomA/PotB7^{E} (filled circles) were measured at various concentration of NaCl as described in Materials and Methods.

of absence of these ions or by phenamil, a specific inhibitor for the sodium-driven motor. However, no motility was observed in the *motB* cells expressing the PomA/PotB7^{E} stator when only 100 mM K^+ was present, although these cells did swim in buffer containing 100 mM Na^+ or Li^+.

Next, we examined the dependency for Na^+ concentration in *E. coli mot* mutants expressing the MotA/B or the PomA/PotB7^{E} stator (Figure 7). The swimming speeds with the MotA/B stator did not change with the concentration of NaCl, at ca. 20–25 μm s^{-1}. On the other hand, the swimming speed with the PomA/PotB7^{E} stator increased with an increase of NaCl concentration and was saturated at about 50 mM NaCl. The speed profiles dependent on the external concentration of Na^+ were similar to those of *V. alginolyticus*.[26] The maximal swimming speed of cells expressing PomA/PotB7^{E} was at ca. 40–50 μm s^{-1}.

Those results demonstrate that an *E. coli* rotor equipped with PomA and PotB7^{E} stator elements functions as a sodium-driven, rather than a proton-driven, motor. It shows also that a sodium-driven motor can function without MotX and MotY, although the PomA/B-containing polar flagellum of *V. alginolyticus* absolutely requires both of those proteins for its sodium-dependent motility.

Discussion

The proton-driven motor of *E. coli* contains the MotA and MotB proteins in its stator complex, whereas the stator complex of the sodium-driven polar flagellum of *V. alginolyticus* consists of four components, including MotX and MotY in addition to the MotA/B homologs PomA and PomB. We showed previously that MotA and MotB of *R. sphaeroides* do not restore motility to a Δ*pomAB* null mutant of *V. alginolyticus*, whereas MotA together with an *R. sphaeroides*/*V. alginolyticus* chimeric MotB protein could function with MotX and MotY in the sodium motor.[26,27] It has been reported that a Δ*pomABmotXY* strain of *V. cholerae* could swim using a proton flux if *E. coli* MotA/B were supplied, although the swimming ability of the cells was very poor.[28]

Here, we have demonstrated that *E. coli* MotA/B can support rotation of the polar flagellum of *V. alginolyticus* in the absence of MotX and MotY. This motor is driven by a proton current, forcing the conclusion that the *E. coli* MotA/B proteins switch the Vibrio polar flagellum from the sodium-driven type to the proton-driven type. Although MotX and MotY were thought to be essential for operation of a sodium-driven motor, *pomA*$^{+}$*B*$^{+}$ *motX* or *motY* cells containing the *motAB* plasmid swarmed more poorly than did Δ*pomAB motX*$^{+}$ *motY*$^{+}$ cells containing this plasmid. The likely reason is that MotA/B compete with chromosomally encoded PomA/B for insertion into the motor in a *pomA*$^{+}$*B*$^{+}$ *motX* or *pomA*$^{+}$*B*$^{+}$ *motY* strain. In the presence of MotX and MotY, PomA/B are probably incorporated more efficiently into the polar motor than MotA/B are, because motility was not impaired when *E. coli* MotA/B were expressed in wild-type *V. alginolyticus* strain VIO5 (data not shown).

Segregants with improved motility (hypermotile strains) could be isolated easily from a *V. alginolyticus* Δ*pomAB* mutant containing plasmid YA6022, which encodes *E. coli* MotA/B. Thirty-eight of 45 mutations causing hypermotility were located within the *motB* gene on the plasmid, but the remaining mutations presumably map to the *V. alginolyticus* chromosome. The mutations of *motB* were distributed widely and some mutations were clustered in the periplasmic region near the putative transmembrane segment (TM). This might suggest that the periplasmic region of MotB forms a structure to support the function, such as the interaction with the other motor components or peptidoglycan. Garza *et al*. reported that a missense mutation that causes a residue substitution near the consensus peptidoglycan-binding motif of MotB could be suppressed by mutations in the gene encoding the intracellular rotor protein FliG.[32] They proposed that the original *motB* mutations misaligned the stator relative to the rotor, and that the suppressing residue substitutions in FliG caused compensating realignments. Thus, in Δ*pomAB V. alginolyticus* expressing *E. coli* MotA/B, mutations affecting the extracellular portion of MotB might improve motility by correcting a suboptimal interaction at the stator-rotor interface. If so, at least some of the chromosomal mutations that enhance motility might be located in the *V. alginolyticus fliG* gene.

We constructed various chimeric proteins whose extracellular domains contain different segments of *E. coli* or *R. sphaeroides* MotB combined with the complementing segment from PomB. The PomA/PotB7^{E}, MotA^{E}/MomB7^{E} and MotA^{R}/MomB7^{R} chimeric stator complexes all function as components of sodium-driven motors when they are expressed in the Δ*pomAB* mutant NMB191. The PomA/PotB7^{E} stator can function in a *motX* or *motY* mutant, meaning that this motor does not need MotX and MotY to function.

E. coli mot cells expressing PomA/B are not motile. However, if the extracellular domain of PomB is exchanged with that of MotB, the resultant stator (PomA/PotB7^{E}) functions with the *E. coli* rotor. This motor is driven by a sodium ion current. These results make it clear that there is no intrinsic necessity for MotX or MotY to be present for a flagellum to be powered by sodium ions, although they are essential for function, at least in *V. alginolyticus*.

The swarming ability of *E. coli mot* mutants expressing *E. coli* MotA/B was better than those expressing the PomA/PotB7^{E} stator. However, the maximal swimming speed of *E. coli mot* mutants expressing the PomA/PotB7^{E} stator was more

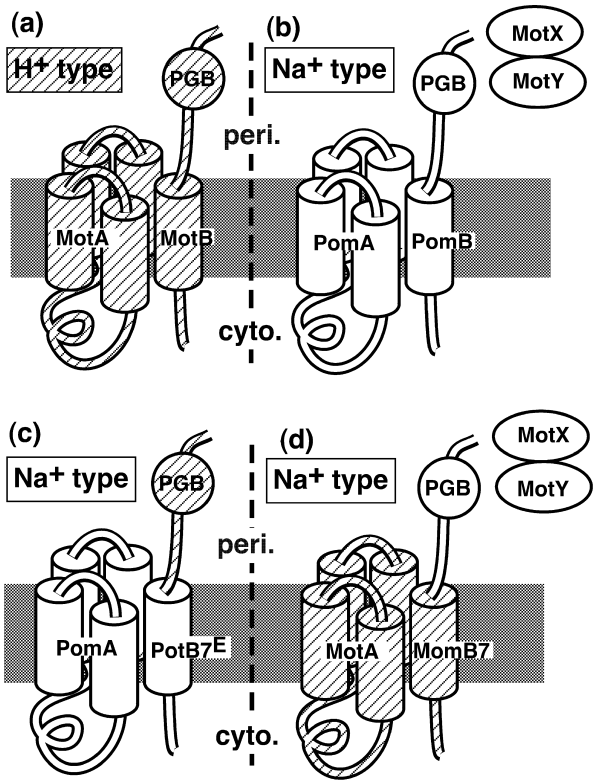


Figure 8. Diagrams for the summary of this study. The hatched and the open parts are derived from the components of the proton-driven (*E. coli*) and the sodium-driven (Vibrio) components, respectively. The dotted boxes and the columns indicate the cytoplasmic membrane and the transmembrane regions, respectively. PGB; peptidoglycan binding motif. The (a) MotA/B and (c) PomA/PotB7^{E} stator function in either *E. coli* or *V. alginolyticus* as the proton-driven motor and the sodium-driven motor, respectively. The (b) PomA/B and (d) MotA/MomB7 stator function in *V. alginolyticus* as the sodium-driven motor but they do not function in *E. coli*, though MotX and MotY are not co-expressed.

than twice that of those expressing the original *E. coli* MotA/B stator. This discrepancy is often observed in the *che* mutants, which cannot regulate the direction of the motor rotation, suggesting that the switching of clockwise/counterclockwise rotation was affected or was not suitable for the faster swimming speed. To investigate the reason for the faster swimming of the sodium-driven *E. coli* cells, the torque-speed relationship should be measured and the sodium motive force in *E. coli* cells should be estimated.

Our findings are summarized in Figure 8. In the native *V. alginolyticus* polar flagellum, the sodium-driven motor requires PomA, PomB, MotX and MotY. Motors containing *E. coli* MotA/B function as proton motors. If the extracellular region of MotB is exchanged with the corresponding region from PomB, as in the functional MotA/MomB hybrid stators, the hybrid motor is driven by sodium, and both MotX and MotY are essential for its function in Vibrio, as shown in this study and by our previous results.[27] MomB is not functional in *E. coli* under the present condition that MotX and MotY are not co-expressed. It will be interesting to determine whether the presence of MotX/Y in *E. coli* will allow PomA to function as part of a Na^+ motor in conjunction with PomB or MomB. Moreover, it will be informative to determine whether the sodium-driven flagella of Gram-positive alkalophilic Bacillus species require MotX and MotY. The PomA/PotB7^{E} stator, in contrast, can function in a sodium-driven motor without MotX and MotY in either *E. coli* or Vibrio. It thus appears that MotX and MotY are needed whenever the extracellular domain of the B subunit comes from PomB and some feature of the extracellular domain of *E. coli* MotB allows it to function in a sodium-ion motor in the absence of MotX and MotY. Although there is no direct evidence for interaction between PomA/B and MotX/Y, it may be that MotX and/or MotY stabilize the attachment of the extracellular domain of PomB to the cell wall. MotY has a peptidoglycan-binding motif in its C-terminal region similar to that of PomB, and MotX and MotY interact with each other.[6,8] Furthermore, we found recently that MotX and MotY appear to be outer membrane proteins.[11]

The discovery that a sodium-driven motor can function without MotX and MotY opens up new possibilities for investigating flagellar motors. For example, we can compare proton-driven motors containing MotA/B and sodium-driven motors containing PomA/PotB7^{E} in *E. coli* cells without the complication of having to supply MotX and MotY. The results presented here emphasize both the complexity of ion selectivity and the intrinsic similarity of proton-powered and Na^+-powered motors. The PomA/PotB7^{E} stator will be an excellent subject for research into the mechanisms of ion selectivity, energy coupling and torque generation by bacterial flagella.

Materials and Methods

Bacterial strains, plasmids, growth conditions and media

The strains and plasmids used in this study are listed in Table 1. The mutant and chimeric plasmids are described in the text. *V. alginolyticus* was cultured at 30 °C in VC medium or VPG medium, as described.[27] To adjust the pH of the media, HCl (for pH 7.0) and K_2CO_3 (for pH 8.5) were used. For culturing *E. coli* cells, LB broth (1% (w/v) Bactotryptone, 0.5% (v/v) yeast extract, 0.5% (w/v) NaCl) or tryptone broth (1% (w/v) tryptone, 0.5% NaCl) was used. When necessary, kanamycin was added to final concentrations of 100 μg/ml for *V. alginolyticus* and 50 μg/ml for *E. coli*. Cells examined for swimming behavior were prepared as described.[27]

DNA manipulations and sequencing

Routine DNA manipulations were carried out as described.[27]

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
A. V. alginolyticus *strains*		
VIO5	VIK4 *laf* (Rif^r Pof$^+$ Laf$^-$)	9
NMB191	VIO5 Δ*pomAB* (Rif^r Laf$^-$ Pom$^-$)	12
YM19	138-2 *pof* (Pof$^-$)	29
NMB190	VIO5 Δ*pomA* (Rif^r Laf$^-$ Pom$^-$)	26
NMB192	VIO5 Δ*pomB* (Rif^r Laf$^-$ Pom$^-$)	This work
NMB119	YM4 *motX* (Rif^r Laf$^-$ Pom$^-$)	8
VIO542	VIO5 *motY* (Rif^r Laf$^-$ Pom$^-$)	9
B. E. coli *strains*		
DH5α		33
M254	AW330 *motB*	34
M256	AW330 *motA*	34
C. *Plasmids*		
pSU41	*kan* (Km^r) P_{lac} *lac* Zα	35
pYA303	pSU41 1.9 kb *Bam* HI-*Sac* I (*pomAB*$^+$)	21
pGD2	pBR322 3.2 kb *Eco* RI-*Pst* I (*motAB*$^+$)	36
pMN1	pUC118 3.2 kb *Eco* RI-*Pst* I (*motAB*$^+$)	This work
pYA600	pSU41 2.6 kb *Sph* I-*Sal* I (*motAB*$^+$)	This work
pYA6001	pSU41 1.6 kb *Nsp*I-*Sal* I (*motB*$^+$)	This work
pYA602	pSU41 0.9 kb *Sal* I-*Eco* RI (*motA*$^+$)	This work
pYA6022	pSU41 2.4 kb *Sal* I-*Sal* I (*motAB*$^+$)	This work
pYA703	pSU41 1.9 kb *Bam* HI-*Sac* I (*motAB*$^+$)	27

Km^r, kanamycin-resistant; Rif^r, rifampicin-resistant; P_{lac}, *lac* promoter.

Plasmid construction and site-directed mutagenesis

Plasmid pYA602 contains a PCR-amplified fragment of *motA* that extends from upstream of the start codon to downstream of the stop codon. PCR amplification was done as described.[20] We used plasmid pMN1, whose *mot* genes are derived from plasmid pGD2, as the template. The end primers were Ec*motA*.S1 and Ec*motA*.E2. Ec*motA*.S1 has a *Sal*I site and was the sense primer that annealed with the sequence upstream of *motA*. Ec*motA*.E2 includes an *Eco*RI site and was the antisense primer that annealed downstream of the gene. This fragment was inserted into plasmid pSU41 between the *Sal*I site and the *Eco*RI site. The other plasmids were constructed as noted in Table 1. Two-step PCR for construction of chimeric genes was performed as described.[20]

Construction of a *pomB* null mutant

A *pomB*-deletion strain was constructed by a procedure similar to that described.[26] The 0.2 kb *Hpa*I-*Sna*BI region in the *pomB* gene was deleted from plasmid pYA303, and the fragment encompassing intact *pomA* and *pomB* containing the deletion was moved onto a suicide vector. The deleted *pomB* gene was then introduced *via* homologous recombination into the chromosome of strainVIO5, which produces a wild-type polar flagellum.

Transformation of Vibrio cells

Transformation of Vibrio cells was carried out by electroporation, as described.[37]

Measurement of swimming speed

An overnight culture of *E. coli* cells grown in LB at 30 °C was inoculated into TG medium (1% Bacto-tryptone, 0.5% NaCl, 0.5% (w/v) glycerol) at 1/50 dilution. Cells were harvested at late logarithmic phase, washed with Kpi buffer (10 mM potassium phosphate (pH 7.5), 10 mM DL-lactate), and suspended in Kpi buffer. The cell suspension was diluted 50-fold into Kpi buffer containing various concentrations of NaCl, and cells were observed at room temperature under a dark-field microscope and recorded on videotape. Their swimming speed was determined as described.[38]

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