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The Systematic Substitutions Around the Conserved Charged Residues of the Cytoplasmic Loop of Na^+-driven Flagellar Motor Component PomA

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PomA, a homolog of MotA in the H^+-driven flagellar motor, is an essential component for torque generation in the Na^+-driven flagellar motor. Previous studies suggested that two charged residues, R90 and E98, which are in the single cytoplasmic loop of MotA, are directly involved in this process. These residues are conserved in PomA of *Vibrio alginolyticus* as R88 and E96, respectively. To explore the role of these charged residues in the Na^+-driven motor, we replaced them with other amino acids. However, unlike in the H^+-driven motor, both of the single and the double PomA mutants were functional. Several other positively and negatively charged residues near R88 and E96, namely K89, E97 and E99, were neutralized. Motility was retained in a strain producing the R88A/K89A/E96Q/E97Q/E99Q (AAQQQ) PomA protein. The swimming speed of the AAQQQ strain was as fast as that of the wild-type PomA strain, but the direction of motor rotation was abnormally counterclockwise-biased. We could, however, isolate non-motile or poorly motile mutants when certain charged residues in PomA were reversed or neutralized. The charged residues at positions 88–99 of PomA may not be essential for torque generation in the Na^+-driven motor and might play a role in motor function different from that of the equivalent residues of the H^+-driven motor.

Keywords: energy transduction; flagella; motor protein; sodium-driven motor; *Vibrio alginolyticus*

Introduction

Bacterial flagella derive their energy for rotation from the electrochemical potential of a specific ion, either H^+ or Na^+, across the cell membrane.[1,2] This energy is harnessed by a motor complex located at the base of the flagellum. From studies of *Salmonella typhimurium* and *Escherichia coli*, which have H^+-driven flagellar motors, three proteins, MotA, MotB, and FliG, are thought to be involved directly in the energy conversion to generate the rotation from the ion flux.[3–6]

MotA has four transmembrane segments and a single large cytoplasmic loop,[7] and MotB has a single transmembrane segment.[8] The C-terminal domain of MotB appears to be bound to peptidoglycan, and the MotA/B complex is proposed to act as a stator.[8–11] The MotA/B complex functions as a H^+ channel, converting the H^+ flux into mechanical power for flagellar rotation.[12–16] It is speculated that conformational changes are induced by ion flow and are transferred to the cytoplasmic region of MotA, and that interactions between this region of MotA and FliG of the rotor complex drive rotation. FliG is part of the switch complex, which includes the FliM and FliN proteins, which together make up the C-ring.[6,17,18]

Unlike *E. coli* and *S. typhimurium*, some Vibrio species, including *Vibrio alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*, and alkalophilic Bacillus species are known to have Na^+-driven flagella.[19,20] In Vibrio, PomA and PomB, which are homologs of MotA and MotB, respectively, are essential for the function of the Na^+-driven motor. Like MotA and MotB, PomA and PomB have been shown to

Abbreviations used: CCW, counterclockwise; CW, clockwise.

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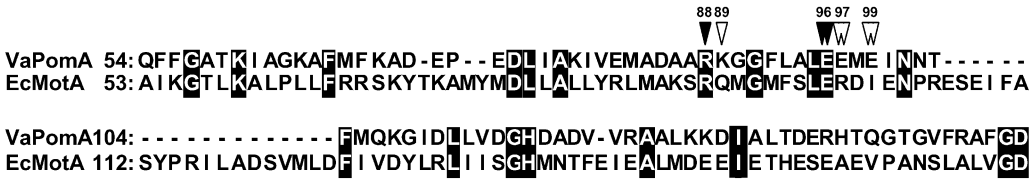

Figure 1. Alignment of the sequences comprising the cytoplasmic loops of the stator components, PomA of *V. alginolyticus* (VaPomA) and MotA of *E. coli* (EcMotA). White letters in black boxes indicate the conserved residues between PomA and MotA. The charged amino acid residues mutagenized in this study are indicated by single arrowheads (positively charged) and double arrowheads (negatively charged). Filled arrowheads show the conserved charged residues, and open arrowheads show the non-conserved charged residues. The numbers over the arrowheads are the positions of the amino acid residues in *V. alginolyticus* PomA.

be functional partners.[21,22] Additional components, MotX and MotY, are needed for torque generation by the Na^+-type motor.[23–26] These two components are specific to the Na^+-motor, but their precise function is still unclear.

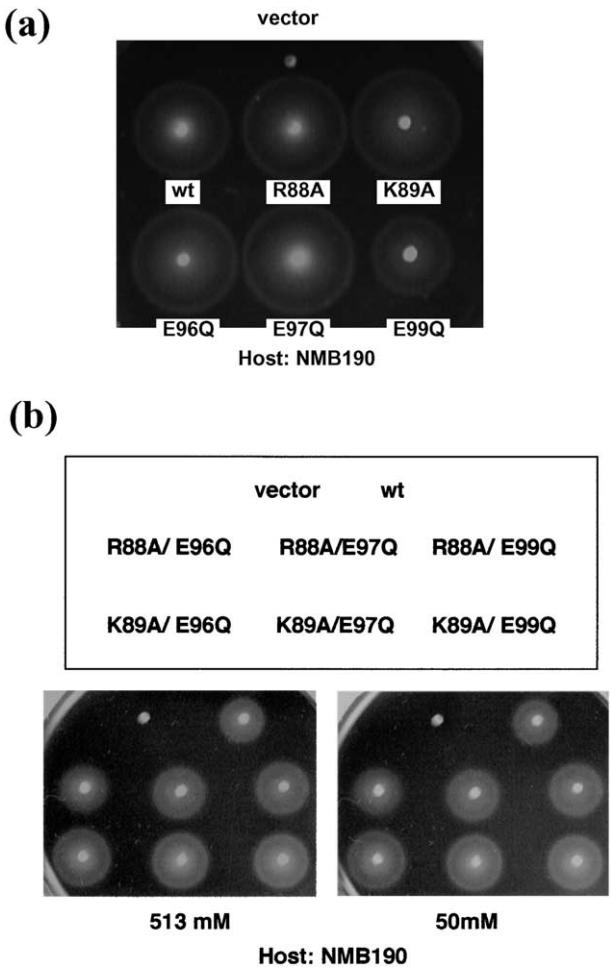

Figure 2. Effect on swarm formation of single substitutions of charged residues in the cytoplasmic loop of PomA. (a) Swarms of NMB190 cells containing vector, wild-type (wt), or single-substituted PomA (R88A, K89A, E96Q, E97Q, and E99Q). (b) Swarms of NMB190 cells containing vector, wild-type (wt), or double-substituted PomA on soft agar plates containing 513 mM (left) or 50 mM (right) NaCl. The pattern in which the various strains were inoculated is indicated in the square above the photograph.

When MotA of *Rhodobacter sphaeroides*, which has a H^+-type flagellar motor, is expressed in *V. alginolyticus* cells defective for PomA, the cells display Na^+-driven motility.[27] However, MotB from *R. sphaeroides* cannot functionally substitute for PomB, strongly suggesting that ion specificity is not mediated by the MotA/PomA component. In the MotB/PomB components of Na^+ and H^+ motors, a single negatively charged residue is conserved in the putative transmembrane segment. This charged residue is believed to be an ion-binding site.[28] PomB/MotB chimeras were produced to explore the mechanism of ion specificity. Surprisingly, in Vibrio cells, a chimeric protein composed of the periplasmic domain of PomB and the transmembrane segment of MotB functions as a Na^+-type motor, though the reverse chimera does not function at all.[29]

The PomA/PomB complex was purified and reconstituted into proteoliposomes. Na^+ uptake activity was observed in the absence of MotX and MotY when the PomA/B complex was present, but not with PomA alone. It has been suggested that PomA and PomB form a complex as oligomers. By gel-filtration chromatography, the molecular mass of the PomA/B complex was estimated to be 175 kDa. PomA alone forms a stable dimer, and a genetically fused tandem PomA dimer functions in the Na^+-type motor. The molecular mass of the tandem dimer was estimated by gel-filtration chromatography to be the same as that of the wild-type PomA.[30] Moreover, when either one or both of the PomA subunits in the homodimer is mutated, the resulting complex is completely defective in motor function.[31] From these lines of evidence, PomA is inferred to function as at least a dimer in the PomA/B complex. This inference is supported by cross-linking experiments using Cys-substituted mutants of PomA.[32] Many Cys residues were introduced into the two periplasmic loops, with the result that the two PomA proteins were cross-linked at certain sites. Function was lost upon cross-linking and restored by treatment with reducing reagent. This finding suggests that a specific interaction of two PomA subunits is important for function.

A model has been presented in which electrostatic interactions between charged residues in MotA and FliG, is involved in torque generation.[33]

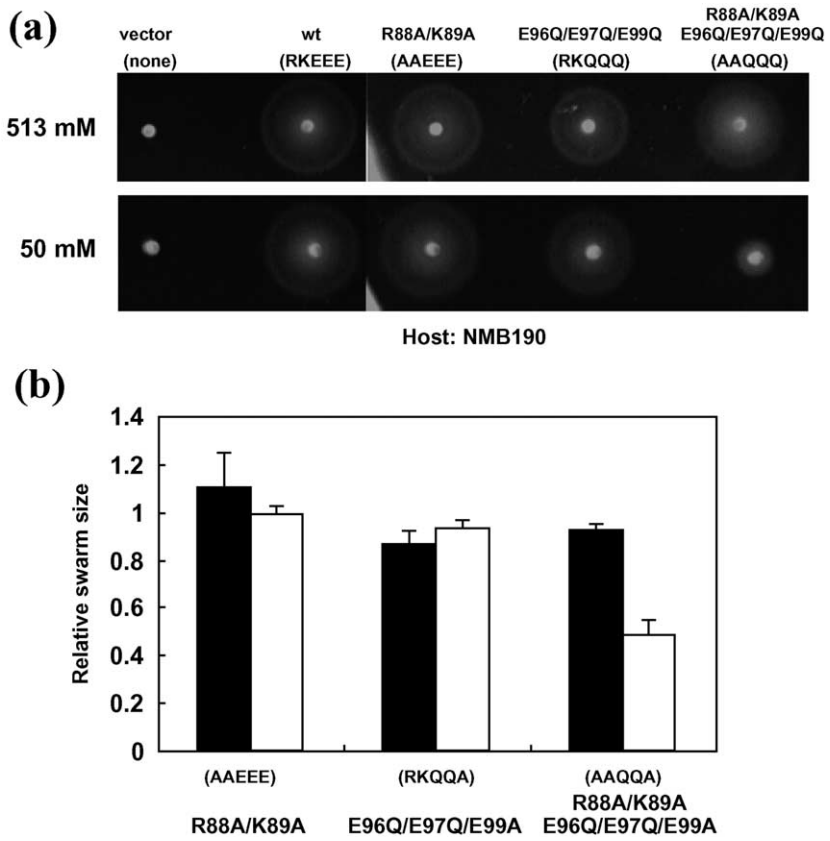

Figure 3. Effect on swarm formation of neutralizing both of the positively charged residues and all three of the negatively charged residues, separately and in combination. (a) Swarms of NMB190 cells containing vector, wild-type (wt) PomA, or PomA in which both of the charged residues and/or all charged residues were neutralized. Plates contained 513 mM (above) or 50 mM (below) NaCl. (b) Histogram showing the swarm sizes of NMB190 cells expressing mutant PomA proteins. The values shown are given relative to that of cells expressing wild-type PomA proteins at 513 mM NaCl (filled bar) or 50 mM NaCl (open bar).

The charged residues in the cytoplasmic region of MotA and FliG were analyzed by mutagenesis, and the residues crucial for torque generation were proposed to be R88 and E96 in MotA and R281, E288 and E289 in FliG.[34,35] When either R90 or E98 of *E. coli* MotA was substituted by alanine residue, motor function was preserved. However, when both of R90 and E98 were converted to Ala, or either residue was replaced with an oppositely charged amino acid, the motor was unable to rotate.

PomA shares those two conserved charged residues in the cytoplasmic loop with MotA, which correspond to R88 and E96 in PomA of *V. alginolyticus*. Other charged residues, namely K89, E97 and E99, are located near R88 and E96 of *V. alginolyticus* PomA. Substitutions at these sites have not been evaluated. Here, we examine by mutational analysis whether these residues are involved in the function of the Na^+-driven flagellar motor.

Results

Neutralizing the charged residues in the cytoplasmic loop of PomA

To study the role of the conserved R88 and E96 charged residues in the function of the Na^+-driven motor of *V. alginolyticus*, we replaced them with neutral amino acid residues (Figure 1). R88 was replaced with Ala (R88A) and E96 with Gln (E96Q). Both mutant proteins were expressed in a strain of *V. alginolyticus* that is defective for lateral flagella, and motility was examined by the swarm assay in a soft agar plate. As had been shown for MotA of the H^+-driven motor,[35] neither single mutation affected motility (Figure 2(a)). However, replacement of both of the charged residues eliminated the motor function of MotA completely. On the other hand, Vibrio cells expressing the R88A/E96Q double mutant PomA were still fully motile (Figure 2(b), left panel). This result suggests that these two conserved, charged residues of PomA are not essential for Na^+-driven torque generation.

In the primary sequence of PomA, there are other charged residues around R88 and E96: K89, E97, and E99 (Figure 1). These residues were replaced as above. Cells producing PomA containing either K89A, E97Q or E99Q were motile (Figure 2(a)). Mutants containing all possible combinations of two of the substitutions (positive and negative) in R88A, K89Q, E96Q, E97Q and E99Q were similarly functional (Figure 2(b), left panel).

Next, the two positively charged residues were neutralized, R88A/K89A (AAEEE), all three negatively charged residues were neutralized, E96Q/E97Q/E99Q (RKQQQ), and all five charged residues were neutralized, R88A/K89A/E96Q/E97Q/E99Q (AAQQQ). The swarm assay was carried out for cells expressing each of these

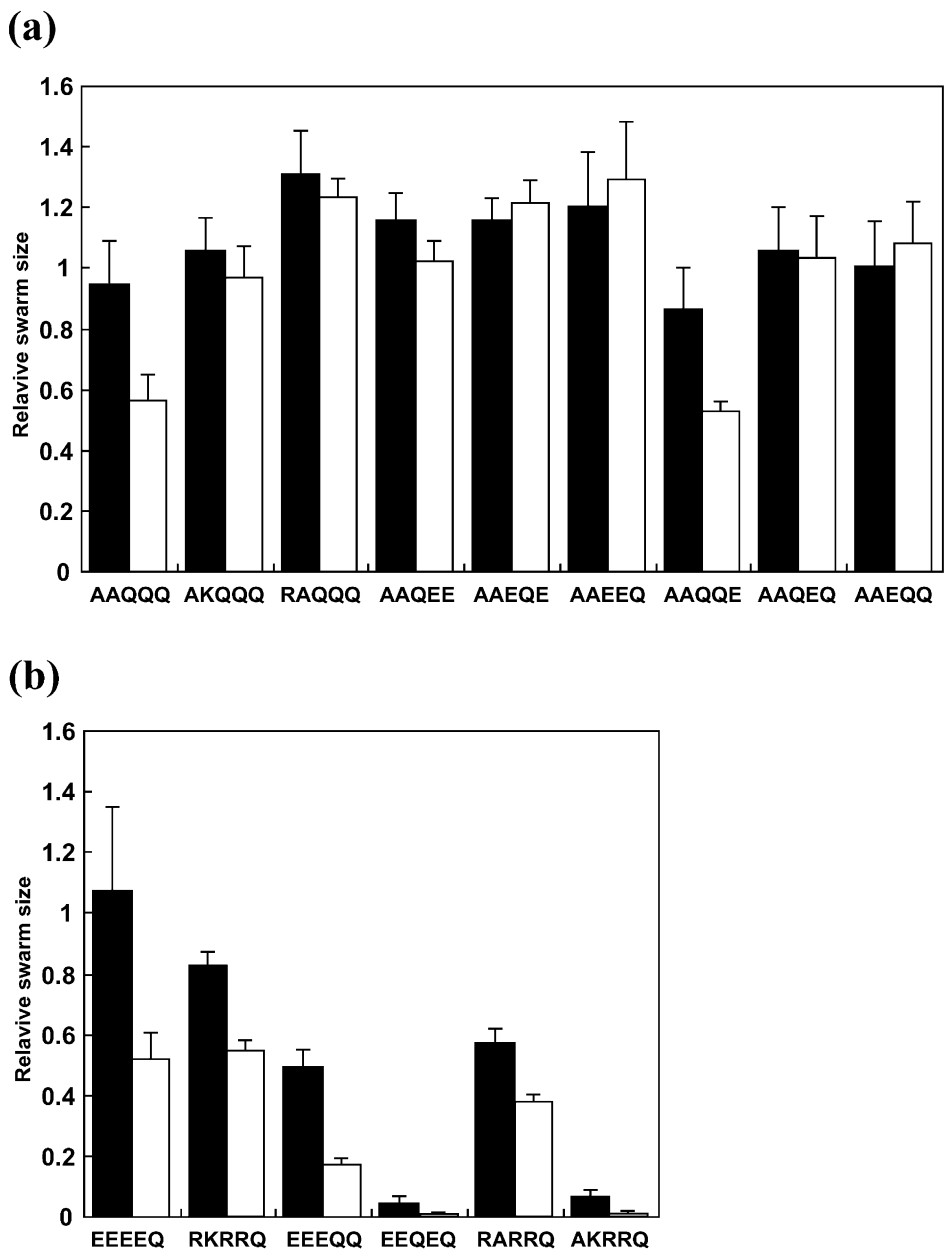

Figure 4. Effect of various combinations of the charge-substituting mutations on swarm behavior. Swarm diameters of NMB190 cells containing the various PomA mutants are shown in the histogram as a fraction of the diameters of swarms formed by cells expressing wild-type PomA at 513 mM NaCl (filled bar) or 50 mM NaCl (open bar). (a) Swarm behavior of the charge-neutralizing mutants. (b) Swarm behavior of the mutants reversing the charge of positive or negative residues.

mutant proteins. It was shown that even cells containing all five of the replacements swarmed as effectively as cells with the wild-type PomA (Figure 3(a), upper panel).

Effect of NaCl concentration on the motor function

The soft agar plate used normally for the swarm assay contains 513 mM (3%, w/v) NaCl. To test whether swarming is affected by the extracellular Na^+ concentration, we performed the swarm assay using soft agar plates containing only 50 mM NaCl (Figures 2(b), right panel, and 3(a), lower panel). The AAEEE and RKQQQ mutants seemed to function as well as wild-type PomA. However, the quintuple mutants of PomA formed swarms with only 50% of the diameter of the wild-type cells at the decreased Na^+ concentration (Figure 3(b), filled *versus* open bars). This decrease was not observed with any combinations of double mutation shown in Figure 2(b) (right panel). To determine whether the AAQQQ mutation is dominant, we expressed this mutant PomA in

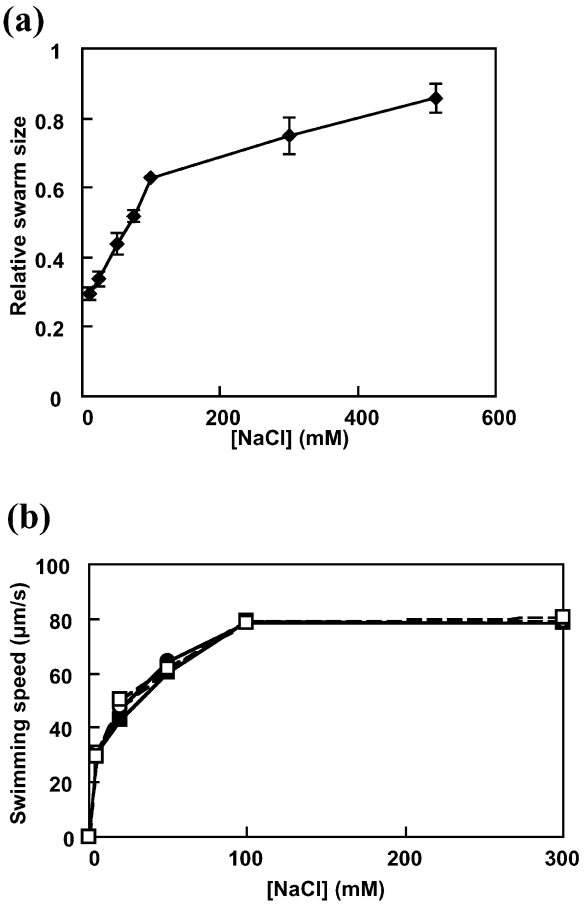

Figure 5. Effect of Na^+ concentration on the motility of cells expressing AAQQQ mutant PomA. (a) The relative swarm sizes compared with the diameters of NMB190 cells expressing AAQQQ PomA to those of cells expressing wild-type PomA are plotted as a function of NaCl concentration. (b) Cells expressing wild-type (circles) or AAQQQ mutant PomA (squares) were cultured in medium containing 50 mM NaCl (broken lines with open symbols) or 513 mM NaCl (continuous lines with filled symbols), collected at late logarithmic phase, and suspended in TMN medium. The swimming speeds were measured at various concentrations of NaCl as described in Materials and Methods.

VIO5 cells, which is wild-type for motility. In swarm agar containing either 513 mM or 50 mM NaCl, the cells were equally motile, indicating that this mutant PomA is recessive with respect to its motility phenotype at reduced concentrations of Na^+ (data not shown).

Table 1. Direction of flagellar rotation in cells with wild-type (wt) or AAQQQ mutant PomA

	Dwell time (s)			
	CCW		CW	
[NaCl] (mM)	Wt	AAQQQ	Wt	AAQQQ
50	1.18 ± 0.85	3.25 ± 2.34	0.55 ± 0.20	0.64 ± 0.38
300	1.19 ± 0.84	2.33 ± 1.14	0.50 ± 0.22	0.66 ± 0.30

We further analyzed the motility phenotypes of cells expressing mutant PomA proteins containing various combinations of amino acid substitutions (Figure 4(a)). First, one of the two positively charged residues, R88 or K89, was neutralized together with all three negatively charged residues (E96Q/E97Q/E99Q), to generate either AKQQQ or RAQQQ PomA. Second, one of the three negatively charged residues, E96, E97 or E99, was neutralized together with both positive residues (R88A/K89A), generating AAQEE, AAEQE, and AAEEQ PomA. Finally, pairs of two of three negatively charged residues were neutralized in combination with the R88A/K89A mutation, generating AAQQE, AAQEQ, and AAEQQ PomA. Cells producing either of these mutant PomA proteins swarmed as well as the wild-type cells at high concentrations of Na^+ (filled bars). Among them, the AAQEE protein showed a little decrease in swarming at 50 mM Na^+ and the AAQQE protein showed a decrease in swarming at low concentrations of Na^+, to almost the same extent as the quintuple-mutant AAQQQ protein (open bars). These results demonstrate that only some combinations of charge loss at residues R88, K89, E96 and E97 inhibit swarming activity by a reduced concentration of Na^+.

We measured the swarm size of cells with the AAQQQ mutant PomA at various concentrations of Na^+ (Figure 5(a)). The relative swarm size decreased slowly from 513 mM to 100 mM NaCl, below which concentration the size decreased dramatically. To examine whether the swarming deficiency is caused by deficiency in torque generation, we measured the swimming speed of the cells (Figure 5(b)). Swimming speed profiles did not differ between wild-type and AAQQQ cells, and the cell growth is not different in these concentrations of NaCl. We therefore tested the possibility that the mutant PomA is not installed into the motor correctly when the cells are grown at a low concentration of Na^+. We cultured cells in medium containing 50 mM NaCl and measured the swimming speed (Figure 5(b), broken line). However, the swimming speed was not reduced. These results suggest that the decreased swarming ability witnessed in the AAQQQ PomA mutant is not due to a decrease in the torque generation.

Single flagellar rotation

Previous studies showed that *pomA*-deleted Vibrio cells expressing MotA of *R. sphaeroides*, whose flagellum is powered by H^+, show Na^+-dependent motility but exhibit a decreased tumble frequency.[27] This behavior led us to speculate that the AAQQQ PomA might inhibit CW–CCW switching. To investigate this possibility, we attached a bead to a single polar flagellar filament and monitored the direction of motor rotation (Table 1). The motor containing the AAQQQ mutant PomA rotated counterclockwise (CCW) for longer periods than motors containing

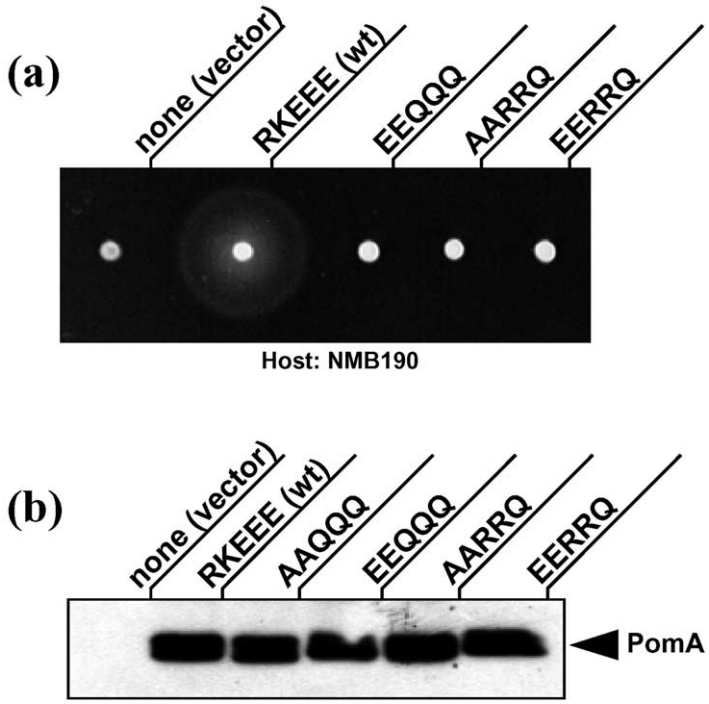

Figure 6. Properties of non-functional PomA mutant proteins created by combinations of sign reversal and neutralization of charged residues. (a) Swarms of NMB190 cells containing vector, wild-type PomA (wt), or the indicated PomA mutant proteins in the presence of 513 mM NaCl. (b) Immunoblot of PomA proteins in membrane fractions. Membrane vesicles (each containing 5 μg of total protein) from NMB190 cells harboring the vector plasmid, a plasmid encoding wild-type PomA, or a plasmid encoring the indicated PomA mutant protein, were subjected to SDS-PAGE and detected with anti-PomA antibody.

wild-type PomA, although the duration of the intervals of rotation were not different in the two strains. The dwell time of CCW rotation was about twice as long at 300 mM NaCl and about three times longer at 50 mM NaCl for the AAQQQ mutant PomA compared to wild-type PomA. When we measured the tumble frequency of the swimming cells in various media, it also decreased to about 51% of the wild-type frequency in 50 mM NaCl and 57% of wild-type frequency at 300 mM. Thus, the decrease in swarming ability in semisolid agar containing low concentrations of salt may be a consequence of those effects. The AAQQQ mutant PomA biases the motor toward CCW rotation at any concentration of salt, but this decrease in tumbling may impair swarming only when the cells are swimming more slowly at low speeds (i.e. at low concentrations of NaCl) but have little effect on swarming when the cells swim at higher speeds. The rotation speeds of the 1.0 μm beads were not significantly different between the wild-type and the mutant motor under the high or low concentrations of NaCl, suggesting the generating torques in high load also are similar (data not shown).

Reversing the charges

Previous studies showed that reversing the charge of either residue R90 or E98 of MotA completely abolishes motility of the *E. coli* H^+-driven motor.[35] We replaced the R88 and K89 residues with Glu, or the E96 and E97 residues with Arg to generate R88E/K89E/E99Q (EEEEQ) PomA or E96R/E97R/E99Q (RKRRQ) PomA (Figure 4(b)). Cells expressing either of those mutant proteins swarmed as well as wild-type cells at 513 mM NaCl (filled bars). As seen with cells expressing the AAQQQ mutant PomA, the swarming ability of cells expressing these reversed-charge mutants was severely affected at low concentrations (50 mM) of NaCl (open bars). These results indicate that these charges are not essential for torque generation in the Na^+ motor.

To analyze these charged residues further, either R88 or K89 in the RKRRQ mutant and either E96 or E97 in the EEEEQ mutant were neutralized, resulting in the AKRRQ, RARRQ, EEQEQ and EEEQQ proteins. In the presence of 513 mM NaCl, the swarm size of the RARRQ and EEEQQ mutants was less than half that of wild-type PomA, whereas the swarms of cells containing the AKRRQ and EEQEQ proteins were very small (Figure 4(b)). At 50 mM NaCl, the diameters of the swarms of cells expressing the RARRQ and EEEQQ proteins decreased even more, to about 16% and 36%, respectively, of those containing wild-type PomA, whereas the AKRRQ and EEQEQ mutants did not swarm and no motile cells were observed under the microscope. We speculate that positive charge at R88 and K89 or negative charge at E96 and E97 might be required to compensate for any charge reversal.

Non-functional PomA protein

We examined the combination of charge-reversing and charge-neutralizing mutations by constructing the following PomA mutants: R88E/K89E/E96Q/E97Q/E99Q (EEQQQ) and R88A/K89A/E96R/E97R/E99Q (AARRQ). Neither protein supported any motor function (Figure 6(a)) although we could detect normal amounts of these mutant PomA proteins in the membrane

(Figure 6(b)). When these proteins were expressed in VIO5 cells, swarming was inhibited about 20–30%. When the extracellular concentration of Na^+ was decreased to about 50 mM, this dominant-negative effect was enhanced to be 40% (data not shown). As shown above, reversing only the positive or negative charges (EEEEQ or RKRRQ) did not affect swarming significantly. However, when both of the charges were reversed simultaneously to generate the EERRQ mutant protein (R88E/K89E/E96R/E97R/E99Q), cells displayed no motility (Figure 6(a)) and no swimming cells were observed under the microscope (data not shown). These proteins were also present in normal amounts in membrane (Figure 6(b)). These results support our idea that the effect caused by reversing charges is not seen when some of the original residues with opposite charge are retained but are observed when these additional charged residues are neutralized or substituted by residues of opposite charge.

Discussion

In the bacterial flagellar motor, the stator and rotor cooperate to generate torque from ion influx, resulting in motor rotation. Studies of the H^+-driven flagellar motor of *E. coli* led to a model for force generation in which the cytoplasmic region of MotA, a stator component, and the C-terminal region of FliG part of the rotor, interact electrostatically in the cytoplasm *via* specific charged residues; R90 and E98 in MotA and R281, E288, and E289 in FliG.[33] Similar functional charged residues have been identified in *Sinorhizobium meliloti*, whose flagellar motor is H^+-driven (R. Schmitt, personal communication). PomA, the MotA homolog in the Na^+-driven flagellar motor of *V. alginolyticus*, also has these conserved charged residues, R88 and E96. In this work, we focused on the role of these charged residues in torque generation. In the H^+-driven motor, when R90 and E98 in MotA are changed to Ala and Gln, respectively, MotA is not functional, although MotA with either single change still functions.[35] On the other hand, the same double-substitution of R88 and E96 in PomA does not affect motor function significantly. This result suggests that these charged residues themselves may not mediate the electrostatic interaction that generates force in the Na^+-driven motor of *V. alginolyticus*. Therefore, we suggest that R88 and E96 of PomA are less critical than the corresponding residues of the H^+-driven motor, because there are other charged residues nearby in PomA, namely K89, E97, and E99. Since these residues might mediate electrostatic interaction, we additionally neutralized one negative and one positive residue in all possible combinations, but none of these mutations impaired motility. We thought that more than one positively and negatively charged residue may be used to generate the torque in the Na^+ motor, though a single positively charged residue and a single negatively charged one are enough to generate torque in the H^+ motor. To test this hypothesis, we neutralized the positive charges R88 and K89 and/or the negative charges E96, E97 and E99. PomA proteins with either R88A/K89A-double or E96Q/E97Q/E99Q-triple mutations did not affect the motility of the cells. The Na^+-driven motor, which rotates about five times faster than H^+-driven motor, may have additional charged residues essential for energy coupling.

In order to further analyze the role of the charged residues of the Na^+ motor PomA, we substituted the charged residues in the region of interest with residues of opposite charge. We expected to see more severe effects on the function of PomA than with substitutions of neutral amino acid residues. In MotA of *E. coli*, reversal of the charge of either R90 or E98 abolished the motor function completely.[35] In contrast, reversing any single positive or negative charge never completely disrupted motor function in the Na^+-driven flagellar motor, suggesting that these charged residues might not be very important in the Na^+-driven motor. Some charge-reversal mutations conferred reduced swarming at low concentrations of Na^+, as seen with the neutralized R88A/K89A/E96Q/E97Q/E99Q (AAQQQ) mutant PomA. These reversed-charge PomA proteins might interact less efficiently with FliG to change the direction of the motor rotation. We went on to combine the mutations that reverse and neutralize the charged residues of PomA. Motor function was lost completely in cells containing the EEQQQ and AARRQ PomA proteins, even though proteins were present in the membrane. Reversing the charges of R88, K89, E96 and E97 (EERRQ) also abolished motor rotation. However, EEEQQ or RARRQ PomA proteins support some motility, suggesting that the function can be maintained when one of the original charged residues remains, in which there is E96 or R88, respectively. These might indicate that E96 and R88 are as important as the corresponding residues of *E. coli*, though it is hard to interpret the effect of the charges that can affect the conformations indirectly.

When all five charges were neutralized in the AAQQQ mutant, swarming ability was impaired when the concentration of NaCl in the soft agar was lowered from 513 mM (3%) to 50 mM. On the basis of this result, we thought that the AAQQQ mutant PomA generates torque less efficiently than wild-type PomA. To examine this, we measured swimming speed in liquid medium containing various concentrations of Na^+. However, the swimming speed in response to Na^+ concentration was similar in cells containing the wild-type or the AAQQQ mutant PomA. This result shows that the neutralizing mutations do not block force generation. Secondly, we tested the possibility that the AAQQQ mutant PomA is not incorporated efficiently into the motor at the decreased concentration of Na^+. However, there

was no difference between swimming speeds of cells with wild-type and AAQQQ mutant PomA cultured in medium containing high (513 mM) or low (50 mM) concentrations of Na^+, indicating that motor assembly is not affected by low concentrations of Na^+.

In a previous study, Asai *et al.* demonstrated that Vibrio cells expressing MotA of the H^+-driven motor of *R. sphaeroides* use Na^+ to rotate the flagellar motor.[27] Cells with this hybrid motor could swarm, although very poorly, and the swarm size did not correspond to the swimming speed of the cells. This discrepancy arises presumably because the hybrid motor is CCW (smooth)-biased and rarely rotates clockwise (CW), even if the cells are stimulated with a chemical repellent. If MotA in the stator interacts with FliG in the rotor to rotate the motor, and this interaction is abnormal in the hybrid motor due to a poor match between the cytoplasmic loop of MotA of *R. sphaeroides* and FliG of *V. alginolyticus*, a directional bias could result. To examine whether this phenomenon occurs with the AAQQQ PomA mutant in bias, we monitored flagellar rotation directly by attaching a bead to a single filament. The flagella of cells containing AAQQQ PomA had a longer CCW-rotating dwell time than did cells containing wild-type PomA, a result consistent with the lower tumble frequency of cells containing AAQQQ PomA. Although there is no significant difference between the CW-rotating time of cells with wild-type and AAQQQ mutant PomA, the CCW-rotating time of the motor with the mutant PomA was longer than that containing wild-type PomA. In other words, the CW mode is rare in the motor but, once changed to CW, it can maintain the direction stably. Garza *et al.* reported that *E. coli* MotA containing a single residue substitution in its periplasmic region conferred a CW-bias on the motor.[36] As far as we know, the AAQQQ form of PomA represents the first example of an alternation in the cytoplasmic region of a stator component that displays an altered CCW/CW ratio. The swarming ability of the most severe mutants, the reversed-charge mutants, was reduced at low concentrations of NaCl. This may suggest that the reversed charges affect the interactions between PomA and FliG at low rotation speed.

It is known that the direction of flagellar rotation is ruled by CheY in *E. coli*. Chemotactic stimuli are transduced into the cell, CheA is activated, the activated CheA phospholylates CheY,[37,38] and the phospho-CheY binds to the C-ring of the flagellar motor.[39] Consequently, the conformation of the rotor probably changes and the direction of the flagellar motor changes from CCW to CW.[40] It has been speculated that the conformation of FliG changes reversibly between CCW and CW states of the motor, although there is little direct supporting evidence for this proposition. One model has been proposed to describe how a conformational change in FliG could change the direction of motor rotation. FliG is involved in three processes: assembly of the flagella; rotational switching; and torque generation.[41] In the H^+ motor, multiple copies of FliG compose a ring structure with FliM and FliN.[18,42,43] Each C-terminal region of the assembled FliG might interact with the cytoplasmic region of MotA in the stator. Lloyd *et al.* obtained a high-resolution structure of the C-terminal domain of FliG (FliG-C) from the hyperthermophilic bacterium *Thermotoga maritima*, which has a H^+-driven motor.[44] FliG-C of *T. maritima* contains the conserved charged residues essential for torque generation in *E. coli*. Two subsets of charged residues of MotA might be accessed differently upon conformational changes of FliG-C and used to switch from CCW to CW rotation. Each arrangement of these important charged residues might be involved in interacting with residues in the stator subunit, MotA.

The *fliG* gene of the Na^+-driven motor has been characterized in *V. alginolyticus* (unpublished results), *V. cholerae*,[45] and *V. parahaemolyticus*,[46] which are closely related. The charged residues functionally important in FliG-C of the H^+-driven motor are conserved in the FliG proteins of the Vibrio species. If the model MotA–FliG interactions in the H^+-driven motor applies to the Na^+-driven motor, the charges in the cytoplasmic loops of PomA should interact with charged residues in FliG in either of two conformations. It may seem surprising that the charge-neutralized mutant AAQQQ PomA can support rotation in either the CCW or CW direction, although the frequency of motor switching is lower. In the Na^+-driven motor, these charge interactions are not likely to determine the direction of rotation against the H^+-driven motor model. On the other hand, a chimeric FliG protein composed of the C terminus of *E. coli* FliG and the N terminus of *V. cholerae* FliG is functional in *V. cholerae*, although full-length *E. coli* FliG does not function in these cells.[35] *V. cholerae* has a Na^+-driven motor similar to that of *V. alginolyticus*[47,48] and PomA is functionally interchangeable between the two species (unpublished results). Similarly, a FliG chimera composed of the C terminus of *V. cholerae* FliG and the N terminus of *E. coli* FliG was functional in *E. coli*.[45] According to this evidence, the rotor-stator interface cannot be very different between the Na^+-driven and the H^+-driven motors. However, PomA of the Na^+-driven motor may have additional essential charged residues necessary for the high-speed rotation. To understand the mechanism of force generation, it will be necessary to model the interface between the stator and the rotor in the two types of flagellar motors.

Materials and Methods

Bacterial strains, plasmids, growth conditions and media

V. alginolyticus strains VIO5 (Rif^r, Pof^+, Laf^-), NMB190 (Rif^r, Pof^+, Laf^-, Δ*pomA*), and NMB194 (Rif^r, Pof^+, Laf^-,

Che$^-$ Δ*pomA*) were used.[8,11] *E. coli* strain JM109 (*recA1, endA1, gyrA96, thi*$^-$, *hsdR17, relA1, supE44*, λ$^-$,Δ(*lac-proAB); (F′, traD36, proAB, lacI*^{q}, *lacZ*Δ*M15*)) was used for DNA manipulations. *V. alginolyticus* cells were cultured at 30 °C in VC medium (0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.4% (w/v) K_2HPO_4, 3% (w/v) NaCl, 0.2% (w/v) glucose); or VPG medium (1% (w/v) polypeptone, 0.4% (w/v) K_2HPO_4, 3% (w/v) NaCl, 0.5% (w/v) glycerol). For the swarm, overnight cultures of cells were spotted onto 0.25% agar-VPG plates containing 100 μg/ml of kanamycin and incubated at 30 °C. If the Na^+ concentration was changed in a VPG agar, the total salt concentration was held at 513 mM by the addition of a suitable amount of KCl. *E. coli* cells were cultured at 37 °C in LB medium. When necessary, kanamycin was added to a final concentration of 100 μg/ml for *V. alginolyticus* cells or 50 μg/ml for *E. coli*. Plasmid pYA301, a pSU41-based plasmid, carries the *pomA* gene under the control of the *lac* promoter.[49]

Site-directed mutagenesis

To introduce substitutions into PomA, we used a PCR method described elsewhere.[49] We synthesized pairs of mutant primers homologous to either the sense or antisense strand of the *pomA* gene, with a 1–3 base mismatch at the mutation site. We amplified the full plasmid sequence containing the *pomA* gene. The presence of the *pomA* mutations was confirmed by DNA sequencing.

Measurement of swimming speed

Cells were harvested at late logarithmic phase and suspended in TMN buffer (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 250 mM KCl). The cell suspension was diluted 100-fold in TMN buffer, and cells were observed at room temperature under a dark-field microscope and recorded on videotape. Their swimming speed was determined as described.[50]

Measurement of flagellar rotation

Cells were prepared as for measurements of swimming speed, and 1.0 μm latex beads (Molecular Probes) coated with poly-L-lysine (15–30 kDa; Sigma Chemical Co.) was mixed with cells and placed on a glass coverslip. Rotation of the bead spontaneously attached to the filament of a cell that was immobilized to the glass was measured by projecting the phase contrast image of the bead onto a quadrant photodiode through a 60× oil immersion objective lens. The outputs from the quadrant photodiode were recorded on a digital data recorder (TEAC, RD-125T).

Detection of PomA proteins

NMB190 (Δ*pomA*) cells producing wild-type or various mutant PomA proteins were cultured in VPG medium and collected by centrifugation at mid-log phase. Membranes were prepared from sonicated cells as described.[32] The membranes were solubilized with SDS-sample buffer and subjected to SDS/12% polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with antibody against PomA, as described.[32]

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