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Roles of Charged Residues in the C-Terminal Region of PomA, a Stator Component of the Na^+-Driven Flagellar Motor▽

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Bacterial flagellar motors use specific ion gradients to drive their rotation. It has been suggested that the electrostatic interactions between charged residues of the stator and rotor proteins are important for rotation in *Escherichia coli*. Mutational studies have indicated that the Na^+-driven motor of *Vibrio alginolyticus* may incorporate interactions similar to those of the *E. coli* motor, but the other electrostatic interactions between the rotor and stator proteins may occur in the Na^+-driven motor. Thus, we investigated the C-terminal charged residues of the stator protein, PomA, in the Na^+-driven motor. Three of eight charge-reversing mutations, PomA(K203E), PomA(R215E), and PomA(D220K), did not confer motility either with the motor of *V. alginolyticus* or with the Na^+-driven chimeric motor of *E. coli*. Overproduction of the R215E and D220K mutant proteins but not overproduction of the K203E mutant protein impaired the motility of wild-type *V. alginolyticus*. The R207E mutant conferred motility with the motor of *V. alginolyticus* but not with the chimeric motor of *E. coli*. The motility with the E211K and R232E mutants was similar to that with wild-type PomA in *V. alginolyticus* but was greatly reduced in *E. coli*. Suppressor analysis suggested that R215 may participate in PomA-PomA interactions or PomA intramolecular interactions to form the stator complex.

Many bacteria can swim by rotating helical flagellar filaments like a screw. At the base of each filament, a rotary motor is embedded in the cytoplasmic membrane. The fuel for rotation is the membrane gradient of ions, either H^+ or Na^+. The flagellar motor has been extensively studied in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, which have H^+-driven motors (5), and it is known that approximately 50 genes are required for flagellar assembly and rotation (4, 19). Proteins encoded by three of these genes, MotA, MotB, and FliG, are essential components for rotation (5). MotA has four transmembrane segments and a relatively large cytoplasmic domain between the second and third transmembrane segments (35). MotB has one transmembrane segment and a peptidoglycan-binding motif in its C-terminal region (7, 8). MotA and MotB form a complex and work as H^+ channels (27). They are thought to function as the stator part of the motor (11, 13, 34). In the rotor part of the motor, FliM and FliN are the components of the C ring structure, the MS ring is composed of FliF, and FliG is thought to form a ring structure between the MS ring and the C ring to connect them. It is thought that FliM and FliG are involved mainly in switching the rotation direction of the motor (26, 28) and in generating the torque of the motor (18), respectively.

In *Vibrio alginolyticus*, PomA, PomB, MotX, and MotY have been identified as components that are essential for torque generation in the Na^+-driven polar flagellum (20, 21, 22, 23). PomA (27 kDa) and PomB (35 kDa), which are homologous to MotA and MotB, respectively, form the stator complex (32). The molecular mass of the PomA/PomB complex has been estimated to be 175 kDa by gel filtration chromatography, suggesting that it forms a complex as oligomers, which may consist of four PomA molecules and two PomB molecules (24). PomA alone forms a stable dimer, and a genetically fused tandem PomA dimer functions in the Na^+-type motor. PomA may function as at least a dimer in the PomA/PomB complex (24, 25, 31). It has also been reported that MotA and MotB oligomerize as a complex of four MotA molecules and two MotB molecules in the H^+-driven flagellar motor of *E. coli* (6, 15).

In *E. coli*, mutational studies have shown that some crucial charged residues of MotA and FliG are involved in torque generation; these residues are R90 and E98 in the second cytoplasmic region of MotA and K264, R281, D288, D289, and R297 in the C-terminal region of FliG (17, 33). It has been suggested that these charged residues in MotA and FliG might engage in electrostatic interactions with each other (36). The charged residues of MotA, R90 and E98, are conserved in PomA as R88 and E96, and the charged residues of FliG in *E. coli*, K264, R281, D288, D289, and R297, are conserved in *V. alginolyticus* FliG as K284, R301, D308, D309, and R317. Although the coupling ions are different, it was expected that electrostatic interactions between charged residues in PomA and FliG in *V. alginolyticus* should be important for motor rotation, as found in *E. coli*. However, replacement of these charged residues, as well as residues adjacent to them in PomA and FliG, had little effect on motility based on swimming assays as well as swarming assays (30, 33). Electrostatic interactions among these charged residues may not be important. Thus, the possibility that other charged residues may be involved in flagellar rotation in *V. alginolyticus* was considered.

Recently, to clarify how ion selectivity is determined in proton and sodium motors, chimeric motors between the H^+ type of *E. coli* or *Rhodobacter sphaeroides* and the Na^+ type of *Vibrio* species were constructed (1). For the rotor, the N ter-

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minus of FliG from *E. coli* and the C terminus from *V. alginolyticus* were joined to create the chimeric protein $FliG_{EV}$. This protein functioned as a proton-driven motor in *E. coli* (10, 30). Furthermore, chimeric protein PotB, which combines the N-terminal domain of PomB containing the transmembrane segment and the C-terminal domain of MotB containing the peptidoglycan-binding motif, was constructed. When PomA and PotB were coexpressed, they functioned not only in *V. alginolyticus* but also in *E. coli* as an Na^+-driven motor (2). The Na^+-driven motor of *Vibrio* can be converted into an H^+-driven motor in *E. coli* without MotX and MotY, which are essential for the wild-type *Vibrio* wild-type sodium motor.

Using this chimeric motor system, a comparative study was carried out with *E. coli* (29). Neutral or charge reversal mutations were introduced into conserved charged residues in the cytoplasmic domain of PomA and the C terminus of FliG from *V. alginolyticus*. Patterns of synergism and suppression in rotor/stator double mutants indicated that proteins in *V. alginolyticus* appeared to engage in electrostatic interactions in essentially the same way that they do in *E. coli*. However, the motor in *V. alginolyticus* was relatively more robust in response to the mutations than the motor in *E. coli*, which means that there may be specific electrostatic interactions between the rotor and the stator for the Na^+-driven motor.

In this study, we focused on the conserved charged residues in the C terminus of PomA in *Vibrio* species, which has an Na^+-driven motor, that are not present in MotA of *E. coli*, which has the proton-driven motor. We made eight charge reversal mutants and measured their effects on swarming in *V. alginolyticus.* Furthermore, the mutant proteins were coexpressed with PotB in *E. coli* cells as an Na^+-type motor, and the effects on swarming were investigated.

MATERIALS AND METHODS

Strains, plasmids, and mutagenesis. Strains and plasmids are listed in Table 1. Mutations were introduced into PomA using the QuikChange procedure as reported previously (29). In *Vibrio*, PomA and its variants were produced from plasmid derivatives of pYA303, which contains *pomA* and *pomB*. Plasmids were selected or maintained in the presence of 100 μg ml^{-1} kanamycin.

In *E. coli*, the PomA proteins were produced from plasmid derivatives of pYS3, which contains *pomA* and *potB*. Plasmids were selected or maintained in the presence of 25 μg ml^{-1} chloramphenicol. PotB is a chimeric protein composed of residues 1 to 50 of *V. alginolyticus* PomB and residues 59 to 308 of *E. coli* MotB. $FliG_{EV}$ is a chimeric protein with residues 1 to 241 of *E. coli* FliG fused to residues 262 to 351 of *V. alginolyticus* FliG. These chimeric proteins were produced from plasmid derivatives of pTY402 following addition of 1 mM arabinose.

Culture of cells. *V. alginolyticus* was cultured at 30°C in VC medium (0.5% [wt/vol] polypeptone, 0.5% [wt/vol] yeast extract, 0.4% [wt/vol] K_2HPO_4, 3% [wt/vol] NaCl, 0.2% [wt/vol] glucose) or in VPG500 medium (1% [wt/vol] polypeptone, 0.4% [wt/vol] K_2HPO_4, 500 mM NaCl, 0.5% [wt/vol] glycerol). *E. coli* was cultured at 37°C in LB broth (1% [wt/vol] tryptone peptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl) or in T broth (1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] NaCl). For swarming assays, overnight cultures were spotted on VPG500 medium-0.25% agar plates containing 100 μg ml^{-1} kanamycin for *Vibrio*. For *E. coli*, overnight cells were cultured in T broth at 30°C for 4 h. When cultures reached the exponential growth phase, 1 mM arabinose was added, and incubation was continued for an additional 4 h. The cultured cells were diluted appropriately and inoculated onto T broth-0.27% agar plates containing 12.5 μg ml^{-1} chloramphenicol, 50 μg ml^{-1} ampicillin, and 1 mM arabinose.

Detection of proteins. *Vibrio* and *E. coli* cells were cultured at 30°C for 4 h in VPG500 medium and for 4.5 h in T broth. The cells from 25-μl portions of cultures (optical density at 660 nm, 10) were suspended in sodium dodecyl sulfate (SDS) loading buffer containing 5% (vol/vol) β-mercaptoethanol and boiled at 100°C for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as described previously. Antibodies against PomA (PomA91 for samples prepared from *Vibrio* and PomA1312 for samples prepared from *E. coli*), PomB (PomB93), and MotB were used as previously described (32).

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description[a]	Reference or source
V. alginolyticus strains		
VIO5	Laf^- Pof^+ Rif^r	23
NMB191	VIO5 Δ*pomAB*	32
E. coli DFB245	*motA* Δ*fliG*	36
Plasmids		
pSU41	Km^r P_{lac} *lacZ*α	3
pYA303	pSU41, 1.9-kb BamHI-SacI (*pomAB*$^+$)	14
pJN152	His_6-*pomA*/*pomB* in pSU41	H. Fukuoka
pBAD24	pBR322 derived, P_{BAD} Amp^r	11
pBAD33	pACYC184 derived, P_{BAD} Cm^r	11
pTY402	*fliG*$_{EV}$ gene in pBAD33	30
pYA25	pBAD24 derivative with inverted multiple cloning sites	29
pYS3	*pomA potB* in pYA25	29

[a] Km^r, kanamycin resistant; Rif^r, rifampin resistant; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; P_{BAD}, *araBAD* promoter; P_{lac}, *lac* promoter.

Coelution assay. Cells of *Vibrio* strain NMB191 (Δ*pomAB*) expressing a His_6-tagged PomA derivative were cultured in VC medium at 30°C for 4 h, harvested by centrifugation, washed with V buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 5 mM $MgCl_2$), resuspended in TMPD (20 mM Tris-HCl [pH 8.0], 5 mM $MgSO_4$, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT]) containing 5 μg ml^{-1} of DNase I, and then sonicated. After undisrupted cells were removed by low-speed centrifugation (4,000 × *g* for 5 min at 4°C), the membrane fraction was recovered by ultracentrifugation (110,000 × *g* for 1 h at 4°C). The pellet was homogenized with TPD (20 mM Tris-HCl [pH 8.0], 0.5 mM PMSF, 1 mM DTT, 20% [wt/vol] glycerol). The total cell membrane (5 mg ml^{-1} of protein) was suspended in TNPD (20 mM Tris-HCl[pH 8.0], 0.5 mM PMSF, 0.15 M NaCl, 1 mM DTT, 10% [wt/vol] glycerol) containing 5 mM imidazole and 2.5% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) for solubilization, incubated on ice for 10 min, and centrifuged at 150,000 × *g* for 30 min at 4°C. The supernatant was mixed with Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) and incubated for 1 h at 4°C with gentle mixing. The Ni-NTA beads were washed three times with TNPD containing 5 mM imidazole and 2.5% (wt/vol) CHAPS. TNPD containing 200 mM imidazole and 2.5% (wt/vol) CHAPS was added to the beads to elute His-tagged PomA and PomB. Eluates and other samples were analyzed by SDS-PAGE following immunoblotting as described above.

RESULTS

Mutagenesis of PomA. As previous studies showed, it is likely that electrostatic interactions of the conserved charged residues between the rotor and stator (MotA or PomA and FliG) contribute to the rotation of the motor (28, 36). However, the *V. alginolyticus* motor seemed to contain other important charged residues in addition to the conserved residues because it was more resistant to mutations in the conserved residues than the *E. coli* motor. We first aligned the amino acids of the C-terminal domains of the PomA orthologues in various bacteria to find specific charged residues that are conserved in most *Vibrio* species but not in *E. coli* MotA; this search resulted in identification of PomA K203, D209, E211,

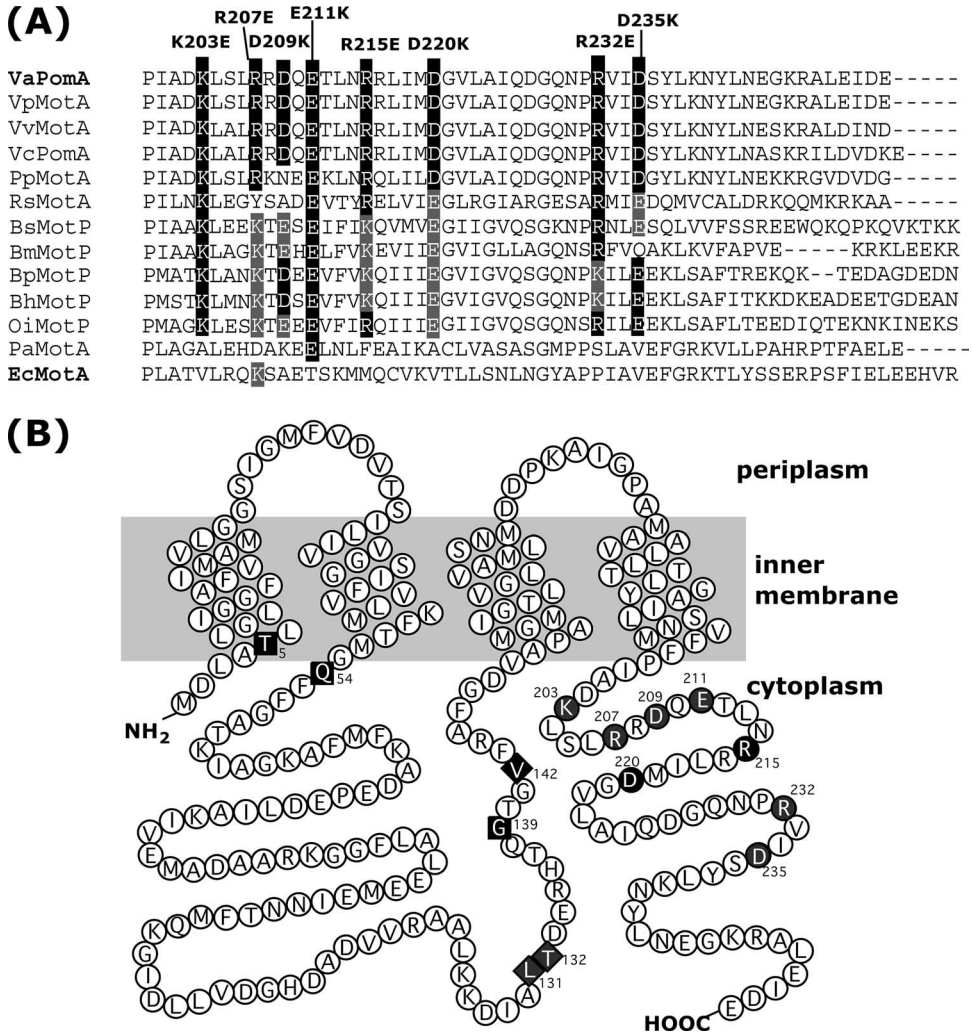

FIG. 1. (A) C-terminal amino acid alignment of PomA, PomA homologues, and *E. coli* MotA. A black background indicates residues identical to *V. alginolyticus* PomA residues, and a gray background indicates residues having the same charge as *V. alginolyticus* PomA residues. Abbreviations: VaPomA, *V. alginolyticus* PomA; VpMotA, *Vibrio parahaemolyticus* MotA; VvMotA, *Vibrio vulnificus* MotA; VcPomA, *Vibrio cholerae* PomA; PpMotA, *Photobacterium profundum* MotA; RsMotA, *R. sphaeroides* MotA; BsMotP, *Bacillus subtilis* MotP; BmMotP, *Bacillus megaterium* MotP; BpMotP, *Bacillus pseudofirmus* MotP; BhMotP, *Bacillus halodurans* MotP; OiMotP, *Oceanobacillus iheyensis* MotP; PaMotA, *Pseudomonas aeruginosa* MotA; EcMotA, *E. coli* MotA. (B) Mutations mapped in a schematic diagram of PomA. Black and gray circles show the residues that we individually replaced with residues with the opposite charge in this study; dominant mutations are indicated by black circles, and the other mutations are indicated by gray circles. R215E suppressor mutations are indicated by black squares. Temperature-sensitive mutations, as described previously (9), are indicated by gray diamonds. The suppressor mutation of the temperature-sensitive mutant is indicated by a black diamond.

R215, D220, R232, and D235 (Fig. 1). To examine whether these residues, which are not conserved in the *E. coli* H^+-utilizing motor, are involved in the function of the motor, they were individually replaced by amino acids with opposite charges, as follows: K203E, D209K, E211K, R215E, D220K, R232E, and D235K. Additionally, PomA R207 was also replaced by an amino acid with the opposite charge to obtain PomA(R207E). This charged residue is conserved in MotA and might be a common important charged residue.

Mutant profiles in *Vibrio* cells. The mutant *pomA* gene was introduced along with *pomB* into the Δ*pomAB* strain NMB191, and each transformant was inoculated onto a semisolid agar plate. We measured the diameter of the swarming ring at various times. Figure 2A shows the relative expansion values for the swarming rings of PomA variants compared with that of the wild type. The PomA(D209K), PomA(E211K), PomA (R232E), and PomA(D235K) mutants retained swarming ability comparable to that of wild-type PomA strain, but motility was completely eliminated by a mutation in residue K203, R215, or D220. The swarming ability of the R207E mutant was reduced moderately. The swimming speed was ca. 5 $\mu m\ s^{-1}$, while the fraction of the swimming cells was ca. 22%. The swimming speed of the wild type was ca. 63 $\mu m\ s^{-1}$.

To detect mutant PomA and PomB proteins, we carried out an immunoblot analysis of whole-cell extracts. When the proteins were overexpressed from plasmids, we could detect PomA and PomB bands for all of the mutants, and some differences in the amounts of proteins among the mutants were observed (Fig. 2B). Although PomA(D235K) could restore the motility to almost wild-type levels, the amount of PomA(D235K)

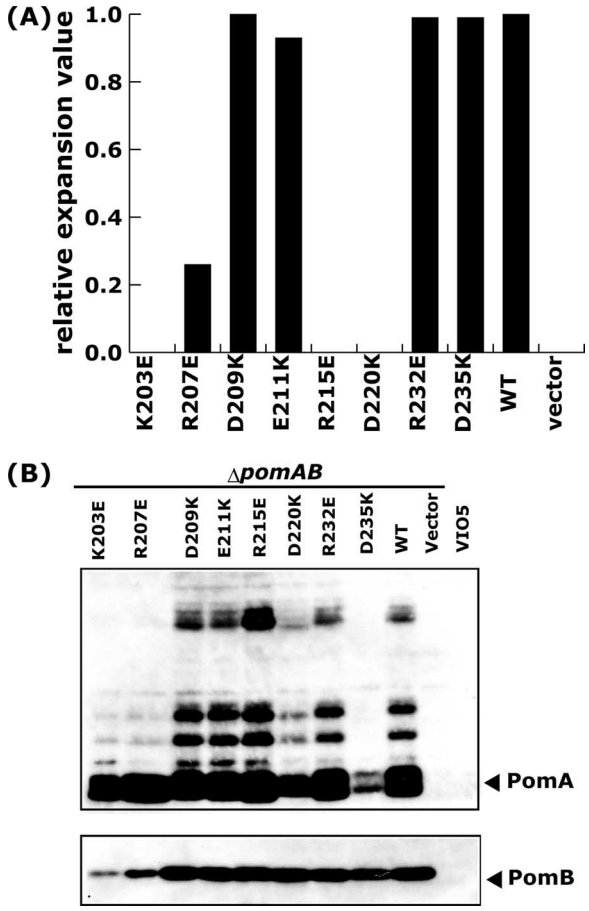

FIG. 2. (A) Relative colony expansion values for *Vibrio* PomA mutants. The relative colony expansion of NMB191 cells expressing no PomA (vector), wild-type PomA (WT), and PomA mutants with PomB was determined. Swarming assays were carried out at least three times, and the diameter of the swarming ring was measured at each time. The relative expansion value was calculated by normalizing the approximate swarm expansion rates of swarming rings of the mutants to that of the wild type (NMB191/pYA303). Cells expressing PomA(K203E), PomA(R215E), PomA(D220K), or PomA(R232E) did not form any swarming rings, like the cells possessed only the vector. (B) Immunoblotting of *Vibrio* cells. The cells described above and wild-type *V. alginolyticus* cells (VIO5) were harvested and suspended in distilled water. Each suspension was subjected to SDS-PAGE and immunoblotting with anti-PomA antibody and anti-PomB antibody.

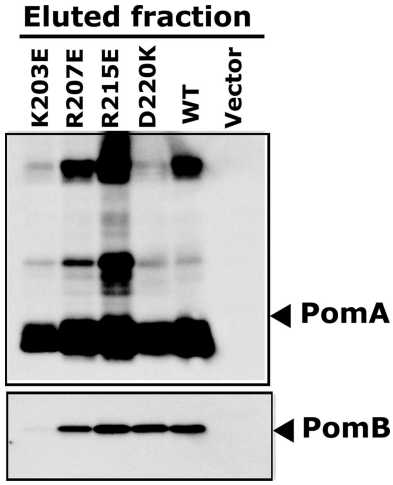

FIG. 3. Coelution analysis of PomB and PomA. The solubilized membrane extracts from NMB191 cells expressing PomA, PomA(K203E), PomA(R207E), PomA(R215E), or PomA(R220K) with PomB were incubated with Ni-NTA agarose and eluted by using imidazole as described in Materials and Methods. The eluted fraction of cells expressing PomA(R207E) was diluted 1:2 with SDS loading buffer, and the eluted fraction of cells expressing wild-type PomA was diluted 1:4 with SDS loading buffer. PomA and PomB proteins were detected by immunoblotting using anti-PomA and anti-PomB antibodies. WT, wild type.

expressed from the plasmid was significantly less than the amounts of the other PomA mutants and the wild-type PomA. Several bands with higher molecular masses than the PomA band were observed, and they may represent dimer forms and/or different conformational forms, as suggested previously (32). The intensities of these bands were reduced for PomA(K203E), PomA(R207E), and PomA(D220K). The chromosomal PomA expressed in VIO5 (*pomAB*$^+$) cells could not be detected. Therefore, the level of PomA in wild-type cells is quite low. The amount of PomB produced in the presence of PomA(K203E) and PomA(R207E) was significantly reduced even though the amount of PomA was not reduced. The K203E and R207E mutations may affect the interaction with PomB because PomB seems to be unstable in the absence of PomA (32).

To test whether the mutations affect the PomA-PomB interaction, a His tag was fused to the N terminus of PomA(K203E), PomA(R207E), PomA(R215E), and PomA(D220K), and we performed a coelution assay. These PomA proteins were coexpressed with PomB in NMB191 cells. The amounts of PomA and PomB detected were similar to the amounts detected with cells producing tagless PomA and PomB (data not shown). The membrane proteins were solubilized with CHAPS, bound to Ni-NTA agarose, and eluted by using imidazole. The eluted proteins were detected by immunoblotting using anti-PomA and anti-PomB antibodies (Fig. 3). Almost the same amount of PomB protein coeluted with PomA(R215E) and PomA(D220K) as with wild-type PomA. However, an almost undetectable amount of PomB coeluted with the PomA(K203E) protein. Only a small amount of PomB protein was detected with PomA(K203E) with the whole-cell extract (Fig. 2B and data not shown). The level of eluted PomA(R207E) appeared to be the same as the level of wild-type PomA, but the amount of coeluted PomB was significantly reduced.

Mutant profiles in *E. coli* cells carrying a chimeric motor. Recently, a chimeric system has been established, which means that we can investigate the roles of PomA and FliG from *V. alginolyticus* in *E. coli* cells (2, 10, 30). To investigate the effects of PomA mutants on the motility of *E. coli* with the chimeric motor, the same PomA mutants that were examined in *V. alginolyticus* were made. Each of the PomA mutants was expressed together with PotB from pYS3 in motors possessing the chimeric rotor protein, $FliG_{EV}$. Motors of the transformants contained both MotB expressed from the chromosome and PotB expressed from the plasmid. The function of the B subunits of the transformants was thought to be due to PotB because PomA/MotB is not functional in *E. coli* (2).

The swarming ability was eliminated completely when any of the following four mutated PomA proteins was expressed: PomA

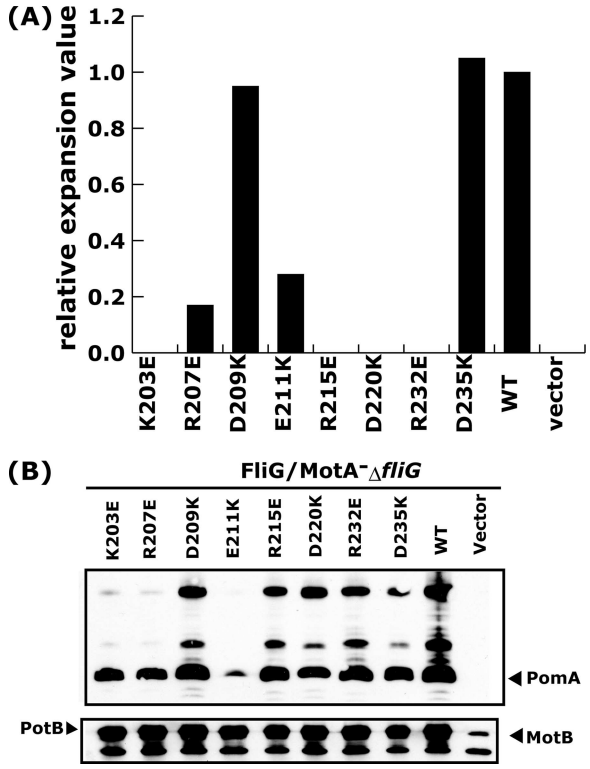

FIG. 4. (A) Relative colony expansion values for *E. coli* PomA mutants. The relative colony expansion values for DFB245 cells expressing no PomA (vector), wild-type PomA (WT), and PomA mutants with PotB and $FliG_{EV}$ were obtained by using the same method that was used for *Vibrio* cells (Fig. 2A). Cells expressing PomA(K203E), PomA (R215E), PomA(D220K), or PomA(R232E) did not form any swarming rings, like the cells possessing only the vector. (B) Immunoblotting of *E. coli* cells. The whole cells described above were harvested and subjected to SDS-PAGE and immunoblotting with anti-PomA antibody and anti-MotB antibody.

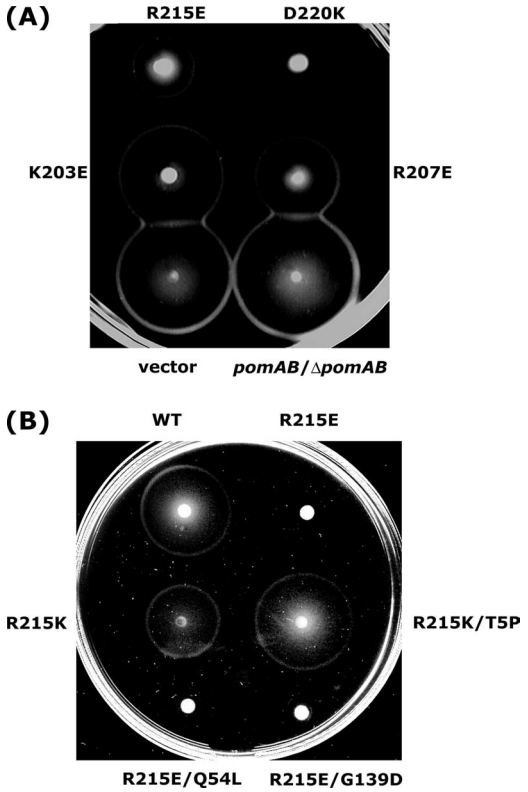

FIG. 5. (A) Dominance of PomA mutants. Wild-type *V. alginolyticus* cells (VIO5) expressing no motor protein (vector), PomA(K203E), PomA(R215E), or PomA(D220K) cells with PomB, and Δ*pomAB* cells expressing wild-type PomA with PomB were incubated on semisolid agar at 30°C for 4.5 h. (B) Motile suppressor of the R215E mutant. Swarms of NMB191 cells expressing wild-type PomA (WT), PomA(R215E), or the PomA(R215K), PomA(R215K/T5P), PomA(R215E/Q54L), or PomA(R215E/G139D) suppressor mutant are shown. The plasmids were extracted from the revertant cells and reintroduced into NMB191 cells. The retransformants were incubated overnight in the liquid cultures and inoculated onto semisolid agar plates. Cells were grown at 30°C for 6 h.

(K203E), PomA(R215E), PomA(D220K), or PomA(R232E) (Fig. 4A). These results are consistent with those obtained with *Vibrio* except for the PomA(R232E) mutant results. PomA(D209K) and PomA(D235K) mutants restored the motility to the wild-type levels. Interestingly, unlike the results obtained with *Vibrio*, the PomA(R207E) and PomA(E211K) mutant proteins restored the swarming ability slightly. Although the R232E mutant had no swarming ability, we observed some swimming cells (the fraction of swimming cells was ca. 20%) by dark-field microscopy. We have recently found that the torque at low speed was reduced by the R232E mutation (12). The PomA(R207E) mutation reduced the motility of both *V. alginolyticus* and *E. coli*. Without arabinose, which acts as an inducer, all of the strains remained completely immotile (data not shown).

The immunoblot analysis of whole-cell extracts of *E. coli* was carried out using anti-PomA and anti-MotB antibodies (Fig. 4B). All PomA proteins expressed from plasmids were present in the same amount as the wild-type protein except for the PomA(E211K) protein. The E211K mutant, which could be detected as a faint single band and caused reduced swarming ability, may be rapidly degraded or may be produced at lower levels. It is worth noting that the levels of the bands with molecular masses higher than that of PomA were significantly reduced in the K203E and R207E mutants. PotB, which is a chimeric protein composed of PomB and MotB, was detected using anti-MotB antibody. PotB proteins from all PomA mutants were detected at almost the same level as wild-type PomA at a higher position than MotB expressed from the chromosome.

Dominance of *pomA* mutations in *Vibrio*. We investigated the dominant-negative effects of the PomA(K203E), PomA (R207E), PomA(R215E), and PomA(D220K) variants. These PomA mutants were introduced into VIO5 ($pomAB^+$) cells, and each transformant was inoculated onto a semisolid agar plate. Motility was greatly reduced by PomA(R215E) and PomA(D220K) even with the wild-type motor, indicating that the proteins were integrated into the *Vibrio* motor and were important residues for rotation (Fig. 5A).

Motile suppressors of the R215E mutant. To investigate potential residues that have the ability to interact with either R215 or D220, we obtained motile suppressor mutants by using long incubation on semisolid agar plates. The nucleotide

changes in *pomAB* for all of the suppressor mutants were determined by sequencing. Eleven motile suppressors were obtained from NMB191 with PomA(R215E) but not from NMB191 with PomA(D220K). In 8 of the 11 revertants, R215E substitutions were changed to lysine, and in each case GAA was changed to AAA (the wild-type codon is CGC). In one revertant, the R215E substitution was changed to lysine, and the T5 residue of PomA was changed to proline. In two of the revertants, a Q54L mutation in PomA (single isolate) and a G139D mutation in PomA (two isolates) were found in the plasmids in addition to the original R215E mutation (Fig. 1B and 5B).

DISCUSSION

In *E. coli*, which has proton-driven motors, it has been thought that electrostatic interactions between charged residues in the rotor protein FliG and charged residues in the stator protein MotA are important for rotation (36). A mutational study of PomA and FliG provided an indication that in *E. coli* the interactions between the rotor and the stator seemed to be electrostatic (29), but other mutational studies have shown that the homologous charged residues in PomA and FliG are apparently not essential for rotation in *Vibrio* (30, 33). The possibility that the *V. alginolyticus* motor might incorporate interactions similar to those in the *E. coli* motor was considered, as was the possibility that the other charged residues not homologous to *E. coli* residues are important and that specific electrostatic interactions between the rotor and stator proteins might occur in the *V. alginolyticus* motor.

In this study, eight charged residues (K203, R207, D209, E211, R215, D220, R232, and D235) present in the C terminus of PomA and its homologues, but not in *E. coli* MotA (except for R207), were replaced with residues with the opposite charge to search for the important charged residues. E211K and R232E substitutions did not affect the swarming and swimming ability of only *Vibrio* cells; on the other hand, D209K and D235K substitutions did not affect either *Vibrio* or *E. coli* cells. The results may also suggest that the different charged residues contribute to the motor function in the *E. coli* and *Vibrio* motors. The amount of the PomA(D235K) protein was significantly reduced when this protein was expressed in *Vibrio* cells, suggesting that the mutation affects the protein stability but not its function. We could not detect the PomA protein expressed from the wild-type cells (*pomAB*$^+$) by the same method. The stator complex seems to function at a level that cannot be detected by available methods, as previously shown (16).

The results for *V. alginolyticus* and *E. coli* cells showed that K203, R215, and D220 appear to be important residues for motor function, such as torque generation or assembly. The R215E and D220K mutations showed dominant-negative effects in *Vibrio* cells, suggesting that mutant proteins are individually inserted into the motor but do not function properly and cause a motility defect. Mutations in M219 and D220, which are adjacent to each other, were identified as dominant double mutations (M219I/D220N) by random mutagenesis of *pomA* (16). The strong dominant effect of the D220 mutation may indicate that this region is very important for motor function but not for assembly or protein interactions.

The R215E mutation was suppressed by mutations in other residues, including Q54L and G139D in PomA. Furthermore, we found a suppressor mutant in which R215 and T5 were replaced by lysine and proline, respectively. The mutations, in addition to the original R215 mutation, occurred near the boundaries of the cytoplasmic and transmembrane domains (Fig. 1B and 5B). In particular, the suppressor mutation G139D is interesting because the mutation at one charged residue was suppressed by another residue that was changed to a charged residue. We speculate that this mutation might change the structure around this residue and rescue the interaction between the C-terminal region of PomA and the region around G139.

It has been suggested that the charged residues in MotA (R90 and E98) and FliG (K264, R281, D288, D289, and R297) might engage in electrostatic interactions with each other and that a charge-neutralized mutant of MotA was nonfunctional (36). However, a charge-neutralized mutant of PomA with residues corresponding to the critical charged residues of MotA was functional. From the charge-neutralized mutant of PomA, we could isolate mutants with a temperature-sensitive phenotype whose mutations were mapped to the L131 or T132 residue. Furthermore, a suppressor mutant with the temperature-sensitive phenotype was isolated and mapped to the V142 residue, which is close to G139 (9). This implies that an interaction between the critical charged residues and the C-terminal charged residues occurs, although it is not necessarily a direct interaction.

The mutations in K203 and R207 seem to affect dimer formation in *E. coli*, as well as in *V. alginolyticus*. This region may be involved in PomA-PomA interactions or may confer proper conformation of the protein. When PomA(K203E) and PomA(R207E) were individually coexpressed with PomB, the amount of PomB protein was significantly reduced only in *Vibrio* cells. It has been reported that in *Vibrio* PomB is very unstable without PomA (32). This may indicate a difference in the protein interactions in *Vibrio* and *E. coli*, or it may simply indicate that PotB is more stable than PomB. A coelution assay showed that the motility defect of R215E and D220K mutants is not due to the defect in the interaction between PomA and PomB.

We think that the C-terminal region of PomA may be partially involved in protein-protein interactions to form the stator complex, or it may be important for protein conformation rather than for the PomA-FliG interaction to generate torque. We identified important charged residues in the C-terminal region but do not know if these residues contribute to electrostatic interactions between the rotor and the stator in *V. alginolyticus*. Clarification of the precise role of the C terminus of PomA would help define the mechanism of the rotor-stator interaction that generates torque.

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