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Roles of Charged Residues of Rotor and Stator in Flagellar Rotation: Comparative Study using H^+-Driven and Na^+-Driven Motors in *Escherichia coli*

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In *Escherichia coli*, rotation of the flagellar motor has been shown to depend upon electrostatic interactions between charged residues of the stator protein MotA and the rotor protein FliG. These charged residues are conserved in the Na^+-driven polar flagellum of *Vibrio alginolyticus*, but mutational studies in *V. alginolyticus* suggested that they are relatively unimportant for motor rotation. The electrostatic interactions detected in *E. coli* therefore might not be a general feature of flagellar motors, or, alternatively, the *V. alginolyticus* motor might rely on similar interactions but incorporate additional features that make it more robust against mutation. Here, we have carried out a comparative study of chimeric motors that were resident in *E. coli* but engineered to use *V. alginolyticus* stator components, rotor components, or both. Charged residues in the *V. alginolyticus* rotor and stator proteins were found to be essential for motor rotation when the proteins functioned in the setting of the *E. coli* motor. Patterns of synergism and suppression in rotor/stator double mutants indicate that the *V. alginolyticus* proteins interact in essentially the same way as their counterparts in *E. coli*. The robustness of the rotor-stator interface in *V. alginolyticus* is in part due to the presence of additional charged residues in PomA but appears mainly due to other factors, because an *E. coli* motor using both rotor and stator components from *V. alginolyticus* remained sensitive to mutation. Motor function in *V. alginolyticus* may be enhanced by the proteins MotX and MotY.

The flagellar motor of bacteria uses energy from either a H^+ or Na^+ ion gradient to drive rotation of the flagellar filament. Bacterial flagella contain about two dozen different proteins, most of which fulfill structural roles. Torque generation appears to involve only five proteins: the MotA and MotB proteins that form the stator and the FliG, FliM, and FliN proteins that form the switch complex on the rotor (see Fig. 1). The stator proteins go by different names in different motors. Those in the polar Na^+-driven motor of *Vibrio alginolyticus* are called PomA and PomB (1), those in a Na^+-driven variant of the *B. subtilis* motor are called MotP and MotS (12), and some variants that appear unique to *Pseudomonas* spp. and whose ion specificity is not yet certain are called MotC and MotD (9, 25). In spite of the different names, the proteins are closely related in sequence and are believed to carry out essentially similar functions. In addition to PomA and PomB, rotation of the polar flagellum of *V. alginolyticus* also requires the proteins MotX and MotY, which are in the outer membrane and are not closely related to the other Mot or Pom proteins (21).

The stator proteins function in complexes with composition A_4B_2 (15, 22). Several such complexes are arrayed in the membrane surrounding the basal body (7, 13), where they function to conduct ions and couple ion flow to rotation (4, 5, 8). The MotB and PomB proteins contain an invariant Asp residue that is essential for function and is believed to participate directly in ion conduction (30). Our working hypothesis is that ion binding/dissociation at this site drives conformational changes in the stator that work on the rotor to drive rotation (6, 14).

Proton-utilizing motors can rotate as fast as 300 revolutions per second (rps) (18), while some Na^+-driven motors have been clocked at 1,700 rps (19). The H^+- and Na^+-fueled motors appear to operate by fundamentally similar mechanisms. Orthologs of the rotor proteins FliG, FliM, and FliN occur in Na^+-utilizing motors, and the C-terminal domain of FliG is to a large extent interchangeable between species. A FliG chimera with N-terminal domain from *Escherichia coli* and C-terminal domain from *V. alginolyticus* can function well in the *E. coli* motor, and the complementary construct is functional in *V. alginolyticus* (26). The same is true of the stator components. PomA and an appropriately engineered PomB variant can function in the *E. coli* motor, where they use sodium ions and support rotation at speeds higher than normal (2). This hybrid Na^+-utilizing motor requires neither MotX nor MotY, in contrast to the motor of *V. alginolyticus*. Its Na^+ dependence has been exploited to allow detection of discrete stepping events in the motor (24). MotA and MotB can function in the motors of *Vibrio* spp., where they use protons and support relatively slow motility (2, 11).

Both the rotor protein FliG and the stator protein MotA contain well-conserved charged residues. Mutational studies in *E. coli* showed that these residues are important for function (16, 28) and that the charged residues of FliG interact with those of MotA (29) (Fig. 1). The precise function of these electrostatic interactions is not known. They could provide a

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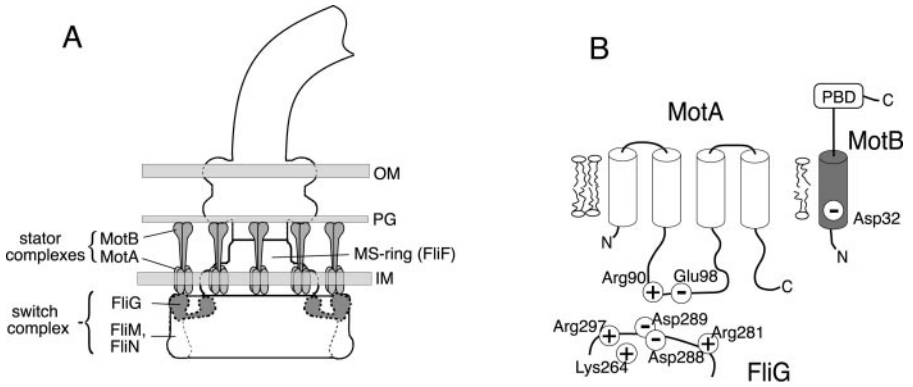

FIG. 1. (A) Arrangement of proteins that function in rotation in the flagellar motor of *E. coli*. (B) Topology of the stator proteins MotA and MotB and functionally important residues of the rotor and stator. The indicated charged residues of MotA and FliG were shown to engage in functionally important interactions in the flagellar motor of *E. coli* (16, 28, 29). Asp32 of MotB is essential for rotation and has been implicated in proton conduction (30). PBD, peptidoglycan binding domain.

physical linkage between MotA and FliG to ensure that conformational movements in the stator are effectively transmitted to the rotor, or they might serve to signal the rotor position to the stator so that ion-gating events in the stator are appropriately timed (6, 14). Whatever their roles, the rotor-stator interactions are fairly robust, because mutations that neutralized any one of the charges had little effect on function. Mutations that reversed charge, or that neutralized two charges, were necessary to disrupt swarming ability in *E. coli* (16, 28, 29).

Mutational studies of charged residues in the motor of *V. alginolyticus* have given somewhat different results. Although the charged residues that are functionally important in *E. coli* are conserved in the *V. alginolyticus* FliG and PomA proteins, most mutations in them had little effect on *V. alginolyticus* motility in swarming or swimming assays (26, 27). Although certain combinations of mutations did impair motility, the charge alterations needed to impair motility were even greater than in *E. coli*, and because multiple mutations were necessary to give a strong motility impairment, the functional defects might conceivably have been due to alterations in protein structure rather than loss of functionally important charges per se. Thus, the possibility arose that electrostatic interactions between the rotor and stator do not occur in the *V. alginolyticus* motor or are relatively unimportant for function. Alternatively, such interactions might occur and make a significant contribution to motor function but be augmented by other features of the *V. alginolyticus* motor that make the rotor-stator interface more resilient. One might expect the rotor-stator interface to be more robust in the *V. alginolyticus* motor, because it rotates about five times faster than that of *E. coli* and the viscous load is borne by a single filament rather than a bundle.

Here, we have undertaken comparative studies of the *E. coli* and *V. alginolyticus* proteins that form the rotor-stator interface to understand better the differences noted in previous mutational studies. We engineered flagellar motors that were resident in *E. coli* cells but contained rotor components, stator components, or both stator and rotor components from *V. alginolyticus*. In each type of motor, mutations were made in the conserved charged residues and effects were measured in swarming and swimming assays. The results indicate that the *V. alginolyticus* proteins engage in electrostatic interactions that are similar to those occurring in *E. coli*. The *V. alginolyticus* motor must incorporate some additional features that make the rotor-stator interface more resistant to mutation. Some of this robustness appears due to additional charged residues that are present in PomA but not MotA, but most is due to factors besides the MotA (PomA) and FliG proteins themselves. The motor in *E. coli* remained relatively sensitive to mutation of the charged residues even when it used both rotor and stator elements from *V. alginolyticus*, implying that other factors, possibly including the MotX and MotY proteins unique to the sodium-driven motor, must contribute to the resiliency of the rotor-stator interface in the *V. alginolyticus* motor.

MATERIALS AND METHODS

Strains, plasmids, and mutagenesis. Strains and plasmids are listed in Table 1. MotA and its mutant variants were expressed from plasmid pDFB45. The chimeric FliG protein $FliG^{EV}$ consists of residues 1 to 241 of *E. coli* FliG fused to residues 262 to 351 of *V. alginolyticus* FliG. It was expressed from the arabinose promoter in plasmid pTY402. Mutations in $FliG^{EV}$ were made using the QuikChange procedure (Stratagene). Mutations in *E. coli* FliG were made using the Altered Sites procedure (Promega), as described previously (17).

PomA and its variants were expressed from either pYS3 or pYS13, depending on whether ampicillin or chloramphenicol resistance was needed. These plasmids also encode PotB, a fusion protein consisting of residues 1 to 50 of *V. alginolyticus* PomB fused to residues 59 to 308 of *E. coli* MotB. PotB allows PomA to function in *E. coli*, whereas PomB does not (2). Mutations in PomA were made by using the QuikChange procedure.

Motility assays. Assays of swarming in soft agar used TB (1% tryptone, 0.5% NaCl) and 0.27% Bacto agar. When needed, chloramphenicol was used at 12.5 μg/ml and ampicillin at 50 μg/ml. When cells contained pYS3 or pTY402 (or mutant variants), 1 mM arabinose was included to induce expression of the chimeric *fliG* gene. Single colonies of fresh transformants were picked onto swarm plates, plates were incubated at 32°C, and swarm diameters were measured at regular intervals. Swarm rates were calculated from linear fits to the data and are reported relative to wild-type controls included in the experiments.

To assay motility in liquid medium, a single colony of a fresh transformant was inoculated into TB plus appropriate antibiotics and cultured overnight with shaking at 32°C. Overnight cultures were diluted 100-fold into fresh TB and incubated for 4.5 h at 32°C. Motility was scored visually under a phase-contrast microscope.

RESULTS

Motor with stator from *E. coli* and FliG C-terminal domain from *V. alginolyticus*. Charged residues in the C-terminal do-

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
DFB245	*motAΔfliG*	30
pAlter-1	Tc^r; defective Ap^r; vector for mutagenesis	Promega
pBAD24	pBR322-derived, *araBAD* promoter, Ap^r	J. Beckwith, Harvard Medical School
pBAD33	pACYC184-derived, *araBAD* promoter, Cm^r	J. Beckwith
pDFB45	*motA motB* behind *trp* promoter	4
pDFB97	*E. coli fliG* behind *ara* promoter; Cm^r	D. Blair
pMMB206	*lacI, tac* promoter, Cm^r	20
pSL27	pAlter-1 derivative encoding *E. coli fliG*	17
pTY402	*fliG*^{EV} gene in pBAD33	26
pYA25	pBAD24 derivative with inverted multiple cloning site	Y. Asai, Nagoya University
pYS3	*pomA potB* in pYA25	Y. Sowa, Nagoya University
pYS13	*pomA potB* in pMMB206	Y. Sowa, Nagoya University

main of FliG were found to be important for motor rotation in *E. coli* (16) but less important in *V. alginolyticus* (26). A chimeric FliG protein (FliG^{EV}) consisting of residues 1 to 241 of *E. coli* FliG fused to residues 262 to 351 of *V. alginolyticus* FliG functions well in the motor of *E. coli* (26). To examine the role of electrostatic interactions in the motor using the chimeric rotor protein, we mutated charged residues in the C-terminal, *V. alginolyticus*-derived domain of FliG^{EV} and measured the effects on swarming and swimming. Results are summarized in Table 2.

In *E. coli*, three charged residues of FliG (Arg281, Asp288, and Asp289) were found to be of primary importance for motor rotation, while two others (Lys264 and Arg 297) made secondary contributions. The corresponding residues in *V. alginolyticus* FliG are Arg301, Asp308, and Asp309 (primary) and Lys 284 and Arg317 (secondary). In the *E. coli* motor using FliG^{EV}, most mutations in these residues caused severe motility defects. The charge-reversing mutations R301D and D309K in FliG^{EV} eliminated swarming in soft agar, and the cells were immotile under the microscope. Swarming was severely reduced by the charge-neutralizing mutation R301A. The charge reversals K284E and R317D also caused significant motility reductions (to about 40% and 30% of wild type, respectively); only the reversal D308K had no effect. Thus, although most of these mutations had only mild effects in the *V. alginolyticus* motor (an exception was R317D, which caused a severe motility impairment) (26), the charged residues of *V. alginolyticus* FliG were important for rotation when the domain was made to function in the *E. coli* motor.

Next, we examined the effects of mutations in charged residues of MotA in the motors using FliG^{EV}. In the native *E. coli* motor, rotation is prevented by mutations that neutralize both Arg90 and Glu98 of MotA or that reverse the charge of either (28). (The conserved charged residue Glu150 was shown to make a secondary contribution to rotation in *E. coli* and is important for rotation in *Sinorhizobium meliloti* [3], but Glu150 mutations were not tested here.) Arg90 and Glu98 also proved essential for rotation in the motor using FliG^{EV}. Swarming and swimming were eliminated by mutations that neutralized both Arg90 and Glu98 or that reversed the charge of either residue (Table 2). The single charge-neutralizing mutations had stronger effects in the motor using FliG^{EV} than in the native *E. coli* motor; swarming was prevented by the E98Q mutation and decreased to less than half the wild-type rate by the R90A mutation. Thus, the stator residues important for rotation in the native *E. coli* motor are also important in the motor using FliG^{EV}.

TABLE 2. Effects of charged-residue mutations in a motor using the *E. coli* stator and the chimeric rotor protein FliG^{EV}

FliG^{EV} mutation	Swarming rate for MotA mutation(s)[a]					
	wt	R90A	R90E	E98Q	R90A/E98Q	E98K
wt	1.00	0.44	0.0 (imm)	0.0 (w,f)	0.0 (imm)	0.0 (imm)
K284A	0.61	**0.0** (imm)		0		
K284E	0.41	**0.0** (imm)		0		
R301A	0.13	**0.0** (imm)		0		
R301D	0.0 (imm)	0.0		0		
D308A	0.88	**.14**		0		
D308K	1.04	**0.0** (imm)		0		
D309A	0.84	0.36		0		
D309K	0.0 (imm, t)	*0.27*	*0.22* (f)	0		
R317A	0.38	**0.0** (imm)		0		
R317D	0.32	**0.0** (imm)		0		

[a] Swarming rates in soft agar relative to controls expressing wild-type (wt) MotA and FliG^{EV} proteins. The measurements used cells of the *E. coli* strain DFB245 (*fliG motA*) transformed with a plasmid expressing MotA and a plasmid expressing the chimeric FliG protein FliG^{EV}, each with the mutations indicated. Numbers in FliG^{EV} correspond to numbers of the residues in the *V. alginolyticus* FliG protein to allow direct comparison with the *V. alginolyticus* FliG sequence; actual residue positions within the chimeric protein sequence are lower by 20. Instances of strong synergism are indicated in boldface and suppression by italics. Swimming behavior in liquid is indicated in parentheses: imm, immotile; w, weak motility; f, only a few cells were motile; t, trails seen in swarm plates indicating the rare occurrence of motile cells.

FIG. 2. Mutual suppression of charge-reversing mutations in the rotor and stator in the motor that uses the chimeric rotor protein $FliG^{EV}$. *E. coli* strain DFB245 (*fliG motA*) was transformed with one plasmid that expresses MotA and a second plasmid that expresses the chimeric FliG protein $FliG^{EV}$, each with the mutations indicated. The residue numbers given for $FliG^{EV}$ correspond to numbers in the *V. alginolyticus* FliG protein. Actual residue positions within the chimeric protein sequence are less, by 20. Fresh transformants were picked onto a plate containing TB and 0.28% agar. The plate was incubated at 32°C for 24 h.

Synergism and suppression in the motor using $FliG^{EV}$. Evidence for electrostatic interactions in the *E. coli* motor came from instances of synergism and suppression in MotA/FliG double mutants (29). To test for synergism and suppression in the motor using $FliG^{EV}$, we expressed various combinations of charged-residue mutations in MotA and $FliG^{EV}$ in the *motAfliG* double mutant strain and measured the effects on swarming and swimming.

In several cases, mutations in MotA and $FliG^{EV}$ were tolerated singly but not when paired. The R90A mutation in MotA acted synergistically with mutations in four charged residues of $FliG^{EV}$ (Table 2). Each of the residues showing synergism in the present experiments showed similar behavior in the previous study of the *E. coli* motor (29). Synergism involving Glu98 of MotA was also reported for the native *E. coli* motor (29) but could not be observed in the present case because the E98Q mutation alone was sufficient to prevent swarming of the cells using $FliG^{EV}$.

Instances of mutational suppression were also observed. The charge-reversing mutation D309K in $FliG^{EV}$ prevented motility when the stator was the wild type, but some swarming and swimming were restored when the R90A or R90E mutation was present in MotA (Table 2 and Fig. 2). The suppression seen here in the motor using $FliG^{EV}$ was not as strong as that reported previously in *E. coli* but was similar in involving the same pair of charged residues (28).

Chimeric motor with stator from *V. alginolyticus* and rotor from *E. coli*. Results above indicate that rotation of the motor using $FliG^{EV}$ depends upon electrostatic interactions similar to those occurring in the native *E. coli* motor. Although the motor using $FliG^{EV}$ contains a rotor element from *V. alginolyticus*, it did not exhibit the resiliency of the *V. alginolyticus* motor but was somewhat more sensitive to mutation than the motor of *E. coli*. To determine whether the stator from *V. alginolyticus* can confer greater resistance to mutation, we examined a motor using the stator proteins from *V. alginolyticus* and the native *E. coli* rotor. The *V. alginolyticus* stator proteins have previously been shown to function well in *E. coli*, provided that the C-terminal part of PomB is replaced with the corresponding part of MotB (2). The C-terminal domain of MotB is located in the periplasm and has not been implicated in direct rotor-stator interactions. We studied the effects of charged-residue mutations in PomA, coexpressed with the appropriate PomB-MotB fusion, termed PotB. Results of swarming and swimming assays are summarized in Table 3.

The relevant charged residues of PomA are Arg88 and Glu96, which correspond to residues Arg90 and Glu98 of MotA. In the *V. alginolyticus* motor, mutations of Arg88 or Glu96 had relatively weak effects (27). When PomA was resident in the *E. coli* motor, Arg88 and Glu96 were important for function. Swarming was eliminated by mutations that neutralized both residues (R88A/E96Q) or reversed the charge of either (R88E, E96K) (Table 3). Cells of the R88E and E96K mutants were immotile, and cells of the R88A/E96Q mutant showed only weak motility under the microscope. This weak motility was eliminated by the additional neutralization of other residues nearby in the sequence (K89, E97, and E99). Thus, although the PomA protein resident in *E. coli* motors is slightly more resistant to mutation than MotA (the R90A/E98Q mutant is immotile in *E. coli* [28]), Arg88 and Glu96 are clearly important for rotation.

Next, we examined the effects of mutations in FliG in the motor using the *V. alginolyticus* stator. Mutations in the charged residues of primary importance (Arg281, Asp288, and Asp289 [16]) caused severe defects in the motors using PomA. Swarming was prevented by mutations that neutralized any two of these residues or that reversed the charge of Arg281 or Asp289 (Table 3). Swimming in liquid was either eliminated or greatly weakened. Thus, the same charged residues in FliG are essential for rotation whether the stator is from *E. coli* or *V. alginolyticus*.

TABLE 3. Effects of charged-residue mutations in a motor using stator components from *V. alginolyticus* and the rotor of *E. coli*

Mutation(s)	Swarming rate[a]
PomA	
R88A	0.0 (w)
E96Q	0.9
R88A/E96Q	0.0 (w)
R88A/K89A/E96Q/E97Q/E99Q	0.0 (imm)
R88E	0.0 (f)
E96K	0.0 (f)
FliG	
R281D	0.0 (imm)
D288K	0.2
D289K	0.0 (imm)[b]
R281A/D288A	0.0 (imm)
R281A/D289A	0.0 (imm)
D288A/D289A	0.0 (w)
D288K/D289K	0.0 (imm)

[a] Relative to a control strain expressing wild-type PomA and FliG. The measurements used cells of the *E. coli* strain DFB245 (*fliG motA*) transformed with a plasmid expressing PomA and PotB, and another plasmid expressing *E. coli* FliG, each with the mutations indicated. Swimming behavior in liquid medium is indicated in parentheses: imm, immotile; f, a few cells show weak motility; w, weakly motile.

[b] Becomes motile in the presence of attractant.

TABLE 4. Effects of charged-residue mutations in a motor using both stator and rotor elements from *V. alginolyticus*[a]

FliG^{EV} mutation	PomA mutation(s)											
	wt[b]	wt	R88A	K89A	E96Q	E97Q	E99Q	R88A/E96Q	R88A/K89A/E96Q/E97Q/E99Q	R88E	K89E	E96K
wt[b]	1.0		1.0	1.1	1.1	1.1	0.9	1.1	1.0	NM	NM	NM
wt		1.0	0.36	0.85	0.97	1.0	1.0	0.0 (w)	0.0 (imm)	0.0 (w)	0.75	0.0 (imm)
K284A	1.0	0.55	**0.0** (w)	**0.0** (w)	**0.0** (w)	0.48	0.62					
K284E	0.2	0.23										
R301A	0.7	0.33	**0.0** (f)	**0.0** (f)	**0.0** (f)	**0.0** (m)	0.1			NM	**0.0** (imm)	0.0 (imm)
R301D	0.4	0.0 (imm)								0.0 (NM)	0.0 (imm)	0.0 (imm)
D308A	1.1	0.98	*0.55*	1.11	0.91	1.18	1.18			*0.04*	0.76	0.0 (imm)
D308K	0.9	0.78								*0.08*	*0.85*	0.0 (imm)
D308K/E311Q	NM	1.08								*0.09*	0.68	0.0 (imm)
D309A	1.2	0.98	**0.19**	**0.38**	**0.07**	0.91	1.04			*0.04*	0.12	0.0 (imm)
D309K	1.1	0.16 (f)								*0.05*	0.0 (w)	0.0 (imm)
R317A	0.6	0.12	**0.0** (f)	**0.0** (w)	**0.0** (w)	0.06	*0.22*					
R317D	0.0 (imm)	0.0 (f)										

[a] Swarming rates of mutants relative to controls expressing wild-type (wt) PomA and FliG^{EV} proteins. The measurements used cells of the *E. coli* strain DFB245 (*motA fliG*) transformed with a plasmid expressing PomA and PotB and a second plasmid expressing the chimeric rotor protein FliGEV, each with the mutations indicated. Instances of strong synergism are indicated in boldface and suppression by italics. Swimming behavior in liquid is indicated in parentheses: m, motile; w, weakly motile; f, a few cells showed weak motility; imm, immotile; NM, not measured.

[b] Swarming rates of FliG mutants and of PomA mutants in the native *Vibrio* motor are from references 26 and 27, respectively.

***E. coli* motor that uses both stator and rotor components from *V. alginolyticus*.** The *E. coli* motor using either rotor or stator elements from *V. alginolyticus* remained relatively sensitive to mutation of the charged residues. To determine whether the *V. alginolyticus* stator and rotor components together can confer greater resistance to mutation, we examined a motor that uses both stator and rotor components from *V. alginolyticus*. PomA, PotB, and FliG^{EV} were expressed in the *E. coli motAfliG* strain, and the effects of various single or double mutations were studied in swarming and swimming assays as before. Results are summarized in Tables 4 and 5.

The motor using both rotor and stator components from *V. alginolyticus* showed mutational defects that were generally similar to the *E. coli* motor. Swarming was prevented by charge-reversing mutations in Arg88 or Glu96 of PomA or residue Arg301 or Asp308 of FliG^{EV}. Synergistic effects were also similar: although most single mutations were tolerated, swarming was prevented by several pairs of charge-neutralizing mutations, involving two residues of PomA or a residue of PomA in combination with a residue of FliG^{EV} (Table 4 and Fig. 3). Suppression was observed for the residue pair Arg88/Asp309, which correspond to Arg90/Asp289 in the *E. coli* proteins, and also for the pair Arg88/Asp308 (Table 4 and Fig. 4).

TABLE 5. Effects of double charge-neutralization mutations in PomA in the motor using both rotor and stator components from *V. alginolyticus*[a]

Second PomA mutation	First PomA mutation					
	wt	R88A	K89A	E96Q	E97Q	E99Q
wt	1.0	0.36	0.85	0.97	1.00	1.00
R88A	0.36		**0.0**	**0.0**	**0.17**	0.35
K89A	0.85			**0.40**	0.80	0.93
E96Q	0.97				NM	NM
E97Q	1.00					NM
E99Q	1.00					

[a] Swarming rates relative to wild-type (wt) controls. Boldface indicates cases of synergism in the double mutants. NM, not measured.

Additional charged residues in PomA contribute to function. The slightly greater resiliency of the motor using the *V. alginolyticus* stator suggests that PomA might contain one or more functionally important charged residues not found in MotA. Lys89 of PomA, in particular, was found to be important for optimal function of the motor that used *V. alginolyticus* components, and instances of synergism suggest that it might interact with a charged residue(s) of the rotor (Table 4). To identify other, potentially important charged residues present in PomA but not MotA, we aligned sequences of MotA from several H^+-utilizing motors and PomA proteins from several

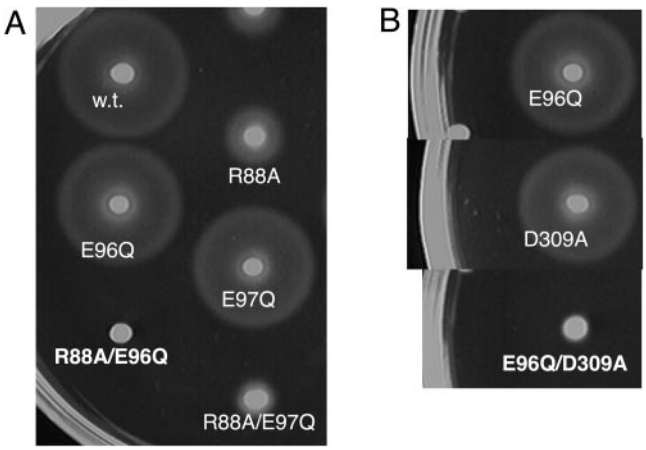

FIG. 3. Synergistic action of two charge-neutralizing mutations in the stator (A) or a charge-neutralizing mutation in the stator and another in the rotor (B) in the motor that uses both stator and rotor elements from *V. alginolyticus*. *E. coli* strain DFB245 (*fliGmotA*) was transformed with a plasmid that expresses PomA and PotB and a second plasmid that expresses FliG^{EV}, each with the mutations indicated. The residue number for FliG^{EV} is the position in the *V. alginolyticus* FliG protein. Transformants were cultured in liquid medium overnight, and aliquots were spotted onto a plate containing TB and 0.27% agar. Plates were incubated at 30°C for 10 h (most strains) or 10.8 h (the E96Q/D309A mutant). w.t., wild type.

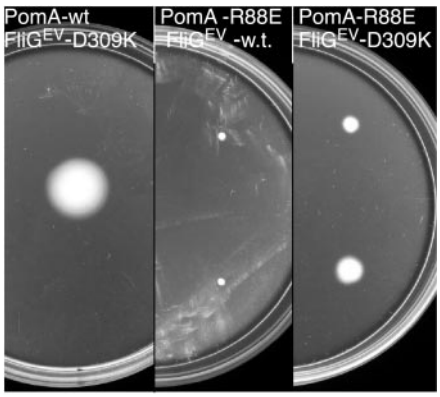

FIG. 4. Suppression of a charge-reversing mutation in the stator by a charge-reversing mutation in the rotor in the motor that uses both stator and rotor elements from *V. alginolyticus*. PomA, with the mutation R88E, and PotB were expressed from one plasmid, and $FliG^{EV}$ with the mutation D309K was expressed from a second plasmid. Procedures were as described in the legend to Fig. 2, except that the plates were incubated for 28 h.

(putatively) Na^+-using motors. Mutations were made in candidate charged residues that occur in PomA but not MotA, and function was assayed both in motors using *E. coli* FliG and motors using the chimeric rotor protein $FliG^{EV}$. Sixteen positions in PomA were tested. Motility was eliminated or greatly reduced by charge-reversing mutations in any of the six residues Asp114, Arg135, Lys203, Arg215, Asp220, and Arg232 (Table 6).

DISCUSSION

Because most mutations in the charged residues of rotor and stator components in *V. alginolyticus* had little or no effect on motility (10, 26, 27), the possibility arose that these charged residues, although conserved, do not make a critical contribution to function in the polar flagellar motor of *V. alginolyticus*. Alternatively, the residues might have important roles in both types of motor, but their action could be augmented by additional features of the *V. alginolyticus* motor that make its function more resistant to mutation. The present results argue that charged residues at the rotor-stator interface play similar roles in the flagellar motors of *V. alginolyticus* and *E. coli*. When the rotor and stator components of *V. alginolyticus* are made to function in an otherwise *E. coli*-based motor, mutations in the key charged residues cause motility defects that are similar to those seen in *E. coli*. Patterns of synergism and suppression in rotor/stator double mutants are also similar, indicating that the interactions between rotor and stator are basically the same whether the proteins come from *E. coli* or *V. alginolyticus*.

The previous mutational studies of PomA (27) and FliG (26) gave some indication that charged residues contribute to function of the *V. alginolyticus* motor, albeit to different extents than in the *E. coli* motor. The charge-reversing mutation R317D in *V. alginolyticus* FliG impairs motility strongly (more so than the corresponding mutation in *E. coli*) (26). Double mutations in PomA that neutralize one charge and reverse another charge cause a strong motility impairment (27); i.e., a change of 3 charge units in the *V. alginolyticus* stator had effects roughly equivalent to a 2-unit change in *E. coli*. Thus, we may suggest that electrostatic interactions between the rotor and stator contribute to motor rotation in both species and that the *V. alginolyticus* motor incorporates additional features that strengthen the rotor-stator interaction and make it more resistant to mutation.

The robustness of the rotor-stator interaction in *V. alginolyticus* appears due in part to the presence of additional charged residues in PomA. These include residue Lys89, which is near the previously identified residues of importance, residues Asp114 and Arg135 in other segments of the first cytoplasmic domain, and residues Lys203, Arg215, Asp220, and Arg232 in the second cytoplasmic domain. We do not know whether all of these charged residues of PomA contribute to a single functionally important site on the protein, nor have we determined whether they interact with charged residues of FliG. Additional charged residues cannot be responsible for all of the robustness of the *V. alginolyticus* motor, however, because an *E. coli* motor using both rotor and stator components from *V. alginolyticus* remained sensitive to mutation. We have not yet identified all of the factors contributing to the robustness of the *V. alginolyticus* motor; among the possibilities are the proteins MotX and MotY, which are essential for rotation of the *V. alginolyticus* motor and might modulate the rotor-stator interaction so that it is more resistant to mutation.

A recent analysis of charged residues in the *S. meliloti* motor casts additional light on the variation that can occur at the rotor-stator interface (3). Unlike *E. coli*, which steers by means of reversals in motor direction, *S. meliloti* directs its movements by modulating the speed of exclusively clockwise-rotating motors, a behavior termed chemokinesis (23). The charged residues in MotA and most of those in FliG are conserved in *S. meliloti*, and detailed mutational analysis confirms that they are engaged in functionally important electrostatic interactions at the rotor-stator interface (3). The topology of the interactions and the relative importance of the various residues

TABLE 6. Effects of mutations in other charged residues of PomA[a]

Mutation	Swarming rate[a]	
	FliG rotor	$FliG^{EV}$ rotor
E97K	1.0	1.0
E99K	1.0	1.0
K107E	1.0	1.0
D114K	0.0 (imm)	0.0 (imm)
D117K	1.0	1.0
D119K	1.0	1.0
R122E	1.0	1.0
D128K	0.5	1.0
D133K	1.0	1.0
R135E	0.0 (imm)	0.0 (imm)
H136D	1.0	0.4
K203E	0.0 (imm)	0.0 (imm)
D209K	1.0	1.0
R215E	0.0 (imm)	0.0 (imm)
D220K	0.0 (imm)	0.0 (imm)
R232E	0.0 (f)	0.0 (w)

[a] Rates relative to wild-type controls included on the plates. Positions 97 through 136 are in the first cytoplasmic domain of MotA, and positions 203 and higher are in the second cytoplasmic domain (see topology in Fig. 1). Swimming behavior in liquid is given in parentheses: imm, immotile; w, weakly motile; f, a few cells showed weak motility.

were found to be somewhat different from either *E. coli* or *V. alginolyticus*. Most notably, the pattern of mutational defects in *S. meliloti* suggested that controlled modulation of the rotor-stator interface might be the basis of chemokinesis (3). Thus, it appears that the rotor-stator interface can vary not only between species but also with circumstances.

A stronger rotor-stator interaction in the *V. alginolyticus* motor may be an adaptation to the greater operational demands on the Na^+-driven polar flagellum. Owing to its much (ca. fivefold) greater rotation speed, the polar flagellum of *V. alginolyticus* is likely to operate against a greater viscous load than that of *E. coli*. Closer tolerances at the interface, and/or a larger number of participating groups, might be necessary to maintain efficient coupling between the rotor and stator in the face of this greater load.

In summary, electrostatic interactions between rotor and stator appear to be a general and functionally important feature of bacterial flagellar motors. The particular residues involved and the detailed relationship of the rotor and stator may vary, but the protein domains present at the interface and the electrostatic nature of the interaction seem to be conserved. The precise role(s) of the electrostatic interactions remains to be determined. Further comparative studies of the motors of different species should be helpful for addressing this question.

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REFERENCES

1. **Asai, Y., S. Kojima, H. Kato, N. Nishioka, I. Kawagishi, and M. Homma.** 1997. Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium. J. Bacteriol. **179:**5104–5110.
2. **Asai, Y., T. Yakushi, I. Kawagishi, and M. Homma.** 2003. Ion-coupling determinants of Na(+)-driven and H(+)-driven flagellar motors. J. Mol. Biol. **327:**453–463.
3. **Attmannspacher, U., B. Scharf, and R. Schmitt.** 2005. Control of speed modulation (chemokinesis) in the unidirectional rotary motor of *Sinorhizobium meliloti*. Mol. Microbiol. **56:**708–718.
4. **Blair, D. F., and H. C. Berg.** 1990. The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor. Cell **60:**439–449.
5. **Blair, D. F., and H. C. Berg.** 1991. Mutations in the MotA protein of *Escherichia coli* reveal domains critical for proton conduction. J. Mol. Biol. **221:**1433–1442.
6. **Braun, T. F., S. Poulson, J. B. Gully, J. C. Empey, S. Van Way, A. Putnam, and D. F. Blair.** 1999. Function of proline residues of MotA in torque generation by the flagellar motor of *Escherichia coli*. J. Bacteriol. **181:**3542–3551.
7. **Coulton, J. W., and R. G. E. Murray.** 1978. Cell envelope associations of *Aquaspirillum serpens* flagella. J. Bacteriol. **136:**1037–1049.
8. **Dean, G. E., R. M. Macnab, J. Stader, P. Matsumura, and C. Burke.** 1984. Gene sequence and predicted amino acid sequence of the MotA protein, a membrane-associated protein required for flagellar rotation in *Escherichia coli*. J. Bacteriol. **143:**991–999.
9. **Doyle, T. B., A. C. Hawkins, and L. L. McCarter.** 2004. The complex flagellar torque generator of *Pseudomonas aeruginosa*. J. Bacteriol. **186:**6341–6350.
10. **Fukuoka, H., T. Yakushi, and M. Homma.** 2004. Concerted effects of amino acid substitution in conserved charged residues and other residues in the cytoplasmic domain of PomA, a stator component of Na^+-driven flagella. J. Bacteriol. **186:**6749–6758.
11. **Gosink, K. K., and C. C. Hase.** 2000. Requirements for conversion of the Na^+-driven flagellar motor of *Vibrio cholerae* to the H^+-driven motor of *Escherichia coli*. J. Bacteriol. **182:**4234–4240.
12. **Ito, M., D. B. Hicks, T. M. Henkin, A. A. Guffanti, B. D. Powers, L. Zvi, K. Uematsu, and T. A. Krulwich.** 2004. MotPS is the stator-force generator for motility of alkaliphilic *Bacillus*, and its homologue is a second functional Mot in *Bacillus subtilis*. Mol. Microbiol. **53:**1035–1049.
13. **Khan, S., M. Dapice, and T. S. Reese.** 1988. Effects of *mot* gene expression on the structure of the flagellar motor. J. Mol. Biol. **202:**575–584.
14. **Kojima, S., and D. F. Blair.** 2001. Conformational change in the stator of the bacterial flagellar motor. Biochemistry **40:**13041–13050.
15. **Kojima, S., and D. F. Blair.** 2004. Solubilization and purification of the MotA/MotB complex of *Escherichia coli*. Biochemistry **43:**26–34.
16. **Lloyd, S. A., and D. F. Blair.** 1997. Charged residues of the rotor protein FliG essential for torque generation in the flagellar motor of *Escherichia coli*. J. Mol. Biol. **266:**733–744.
17. **Lloyd, S. A., H. Tang, X. Wang, S. Billings, and D. F. Blair.** 1996. Torque generation in the flagellar motor of *Escherichia coli*: evidence of a direct role for FliG but not for FliM or FliN. J. Bacteriol. **178:**223–231.
18. **Lowe, G., M. Meister, and H. C. Berg.** 1987. Rapid rotation of flagellar bundles in swimming bacteria. Nature **325:**637–640.
19. **Magariyama, Y., S. Sugiyama, K. Muramoto, Y. Maekawa, I. Kawagishi, Y. Imae, and S. Kudo.** 1994. Very fast flagellar rotation. Nature **371:**752.
20. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide host-range low-copy number vectors that allow direct screening for recombinants. Gene **97:**39–47.
21. **Okabe, M., T. Yakushi, and M. Homma.** 2005. Interactions of MotX with MotY and with the PomA/PomB sodium ion channel complex of the *Vibrio alginolyticus* polar flagellum. J. Biol. Chem. **280:**25659–25664.
22. **Sato, K., and M. Homma.** 2000. Functional reconstitution of the Na(+)-driven polar flagellar motor component of *Vibrio alginolyticus*. J. Biol. Chem. **275:**5718–5722.
23. **Schmitt, R.** 2002. Sinorhizobial chemotaxis: a departure from the enterobacterial paradigm. Microbiology **148:**627–631.
24. **Sowa, Y., A. D. Rowe, M. C. Leake, T. Yakushi, M. Homma, A. Ishijima, and R. M. Berry.** 2005. Direct observation of steps in rotation of the bacterial flagellar motor. Nature **437:**916–919.
25. **Toutain, C. M., M. E. Zegans, and G. A. O'Toole.** 2005. Evidence for two flagellar stators and their role in the motility of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**771–777.
26. **Yorimitsu, T., A. Mimaki, T. Yakushi, and M. Homma.** 2003. The conserved charged residues of the C-terminal region of FliG, a rotor comonent of the Na^+-driven flagellar motor. J. Mol. Biol. **334:**567–583.
27. **Yorimitsu, T., Y. Sowa, A. Ishijima, T. Yakushi, and T. Homma.** 2002. The systematic substitutions around the conserved charged residues of the cytoplasmic loop of Na^+-driven flagellar motor component PomA. J. Mol. Biol. **320:**403–413.
28. **Zhou, J., and D. F. Blair.** 1997. Residues of the cytoplasmic domain of MotA essential for torque generation in the bacterial flagellar motor. J. Mol. Biol. **273:**428–439.
29. **Zhou, J., S. A. Lloyd, and D. F. Blair.** 1998. Electrostatic interactions between rotor and stator in the bacterial flagellar motor. Proc. Natl. Acad. Sci. USA **95:**6436–6441.
30. **Zhou, J., L. L. Sharp, H. L. Tang, S. A. Lloyd, S. Billings, T. F. Braun, and D. F. Blair.** 1998. Function of protonatable residues in the flagellar motor of *Escherichia coli*: a critical role for Asp 32 of MotB. J. Bacteriol. **180:**2729–2735.