Molecular Microbiology (2009) 71(4), 825–835 ■
doi:10.1111/j.1365-2958.2008.06569.x
First published online 13 January 2009

Sodium-dependent dynamic assembly of membrane complexes in sodium-driven flagellar motors

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Summary

The bacterial flagellar motor is driven by the electrochemical potential of specific ions, H^+ or Na^+. The motor consists of a rotor and stator, and their interaction generates rotation. The stator, which is composed of PomA and PomB in the Na^+ motor of *Vibrio alginolyticus*, is thought to be a torque generator converting the energy of ion flux into mechanical power. We found that specific mutations in PomB, including D24N, F33C and S248F, which caused motility defects, affected the assembly of stator complexes into the polar flagellar motor using green fluorescent protein-fused stator proteins. D24 of PomB is the predicted Na^+-binding site. Furthermore, we demonstrated that the coupling ion, Na^+, is required for stator assembly and that phenamil (an inhibitor of the Na^+-driven motor) inhibited the assembly. Carbonyl cyanide *m*-chlorophenylhydrazone, which is a proton ionophore that collapses the sodium motive force in this organism at neutral pH, also inhibited the assembly. Thus we conclude that the process of Na^+ influx through the channel, including Na^+ binding, is essential for the assembly of the stator complex to the flagellar motor as well as for torque generation.

Introduction

Many kinds of proteins with diverse and essential cellular functions such as ion channels, transporters and receptors exist in the bacterial cell membrane. Many membrane proteins function in complexes that are localized at specific sites. Knowing how these membrane proteins assemble and localize at specific locations is essential to understand their functions, but little is known about the mechanisms responsible. The bacterial flagellum is a locomotive organelle and a supramolecular complex penetrating the bacterial cell envelope, including the cytoplasmic and the outer membranes. The flagellum contains a rotary motor driven by the electrochemical potential of H^+ or Na^+ (Yorimitsu and Homma, 2001; Blair, 2003). The assembly mechanism of the flagellum has been intensively studied by using the H^+-driven type present in *Salmonella* and *Escherichia coli.* The flagellum consists of a basal body (rotary motor), a helical filament (propeller) and a hook (universal joint), and cells swim towards favourable conditions by rotating the flagella (Macnab, 1996). The basal body consists of a rod structure and several rings, termed the L-, P-, MS- and C-ring, which are embedded in the outer membrane, peptidoglycan, cytoplasmic membrane, and attached on the cytoplasmic surface of the MS-ring respectively. In the Na^+-driven motor of *Vibrio alginolyticus*, a novel ring structure, called the T-ring, was recently found (Terashima *et al*., 2006). The T-ring structure is located beneath the LP-ring and is composed of MotX and MotY. Loss of this ring by mutation of the *motX* and *motY* genes does not affect the assembly of the flagellar structure but the flagellar motor does not rotate.

The motor consists of a rotor and stator, and their interaction is thought to generate the motor rotation. The stator is a complex of four molecules of an A subunit and two molecules of a B subunit (PomA and PomB in the Na^+-driven motor of *V. alginolyticus*, MotA and MotB in the H^+-driven motor of *E. coli*) and functions as an ion channel (Sato and Homma, 2000; Kojima and Blair, 2004). The proteins PomB and MotB each contain a large periplasmic domain that has a putative peptidoglycan-binding motif through which the stator unit is anchored around the rotor. PomB and MotB each also have an essential aspartic acid residue in the putative membrane spanning region that is likely to participate directly in ion conduction (Zhou *et al*., 1998; Yakushi *et al*., 2004). In PomB, the Asp-24 residue was shown to be the only essential negatively charged residue for motor function and this residue is thought to be a Na^+-binding site. On the other hand, Asp-32 of MotB is the predicted H^+-binding site in *E. coli*. It has been speculated that the substitution

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of Asp to Asn mimics the H^+-binding state, and the ion binding/dissociation at this residue triggers conformational changes in the stator that act on the rotor to drive rotation (Zhou *et al*., 1998; Braun *et al*., 1999; Kojima and Blair, 2001). It has been recently suggested that at least 11 independent torque-generating units or stator units, composed of the motor proteins, surround the basal body or the rotor (Reid *et al*., 2006).

In *V. alginolyticus*, the Na^+-driven motor rotates a single polar flagellum at the cell pole. Therefore, we can easily monitor the stator assembly to determine its polar localization. In fact, we have shown that green fluorescent protein (GFP)-fused stator proteins, GFP-PomA and GFP-PomB, localize at the base of the polar flagellum with the partner protein, PomB or PomA and found that their localization depends on the presence of the flagellar rotor (Fukuoka *et al*., 2005). Furthermore, MotX and MotY of the T-ring are also required for localization of the stator proteins (Terashima *et al*., 2006), suggesting that the T-ring is involved in the incorporation of the stator complex into the flagellar motor, or involved in the stabilization of the stator complex in the flagellar motor. We have shown that MotX probably interacts with PomB and that the N-terminal region of MotY is essential for the association of the stator complexes around the rotor (Okabe *et al*., 2005; Kojima *et al*., 2008). These stator units are able to assemble to the basal structure after the flagellar structure is built, and are able to exchange with the new unit in the functioning motor, suggesting that the stator behaves more dynamically than previously expected (Leake *et al*., 2006). In this study, we showed that mutation of the putative Na^+-binding site in PomB affected the assembly of stator complexes into the polar flagellar motor, and that the coupling ion, Na^+, was required for the stator assembly around the flagellar rotor. The evidence provides a new concept: the coupling ion is not only used for generating rotational force but also required for motor assembly.

Results

GFP-fused motor proteins

To express GFP-fused stator proteins, the *gfp* gene was genetically fused to the 5′ end of the *pomA* or *pomB* gene to form *gfp-pomA* and *gfp-pomB* respectively (Fig. 1A). The genes were placed under the control of an arabinose-inducible promoter in pBAD33 derivative plasmids. When GFP-PomA was produced in Δ*pomA* cells, a single punctate fluorescent signal was observed at the base of the polar flagellum (Fig. 1B). Similarly, GFP-PomB localized at the base of the polar flagellum of Δ*pomB* cells. In Δ*pomAB* cells producing both GFP-PomB and wild-type PomA from a plasmid, cells swarmed slightly in semisolid agar (Fig. 1C and D). The swimming speed and fraction of

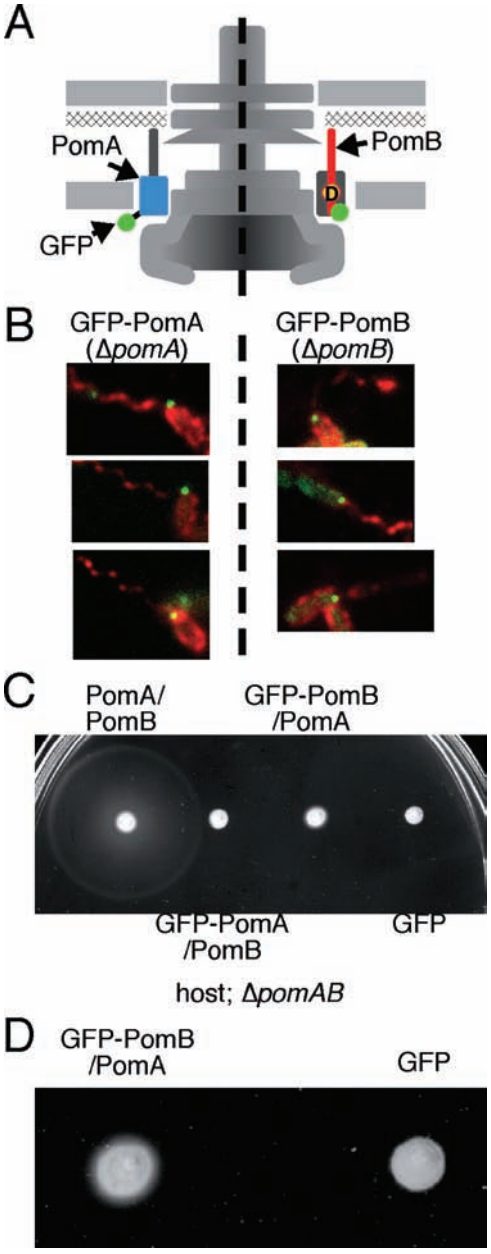

Fig. 1. GFP-fused stator proteins.
A. Diagram of the flagellar motor structure of *Vibrio alginolyticus* with GFP-fused stator proteins. Blue, red and green indicate PomA, PomB and GFP respectively. The orange circle in PomB indicates the aspartic acid residue that serves as the Na^+-binding site.
B. Colocalization of the polar flagellum and GFP-fused stator proteins. Cells were treated with rabbit anti-polar flagellum antibody and rhodamine-conjugated anti-rabbit secondary antibody (red). The green spots localized at the base of polar flagellum represent GFP-PomA in Δ*pomA* cells (left) and GFP-PomB in Δ*pomB* cells (right) respectively.
C. Swarming behaviour of cells producing GFP-fused stator proteins. Δ*pomAB* cells producing PomA and PomB, GFP-PomA and PomB, GFP-PomB and PomA, and GFP were grown on VPG500-0.25% agar plates containing 0.006% arabinose at 30°C for 7 h.
D. Magnified image of C. The small faint ring around the tightly packed colony is swarmed cells producing GFP-PomB and wild-type PomA (left). On the right is the colony of cells producing GFP.

Δ*pomAB* cells producing GFP-PomB and wild-type PomA in 500 mM Na^+ were 16 μm s^{-1} and 20% respectively. On the other hand, the swimming speed and fraction of Δ*pomAB* cells producing wild-type PomA and PomB from a plasmid were 74 μm s^{-1} and 64%. Using oblique epi-illumination fluorescence microscopy, GFP-PomB appeared to localize at the base of the polar flagellum in cells rotating about tethered flagella (see Movies S1 and S2). Thus GFP-PomB is incorporated into the flagellar motor and retains weak function. However, cells producing GFP-PomA did not confer any motility. This suggests that the fused GFP does not inhibit the assembly of the stator but it abolishes the function of PomA.

Effects of various PomB mutations on the polar localization of motor proteins

To investigate the effects of PomB mutations on the assembly of the stator, we mutated Asp-24, which is the putative Na^+-binding site (D24C, D24N), Phe-33, which is located in the putative transmembrane segment (F33C), and Thr-231 and Ser-248, which are located in the putative peptidoglycan-binding motif (T231I, S248F) based on previous information (Blair *et al*., 1991; Yakushi *et al*., 2004). First, we determined the effects of the mutations on motility. Mutant PomB proteins and PomA were coexpressed from a plasmid in the Δ*pomAB* strain and motility was investigated on soft agar plates (Fig. 2A). The D24C, D24N and S248F mutations did not confer any motility, whereas F33C showed very week motility as reported previously (Yakushi *et al*., 2004). T231I did not affect motility, which was unexpected because the corresponding mutation in MotB (T196I) caused a loss of motility (Blair *et al*., 1991). Each mutation was introduced into GFP-PomB or PomB and coexpressed with PomA or GFP-PomA from a plasmid in the Δ*pomAB* strain, and polar localization of the GFP-fused stator complex was observed by fluorescence microscopy. The fraction of

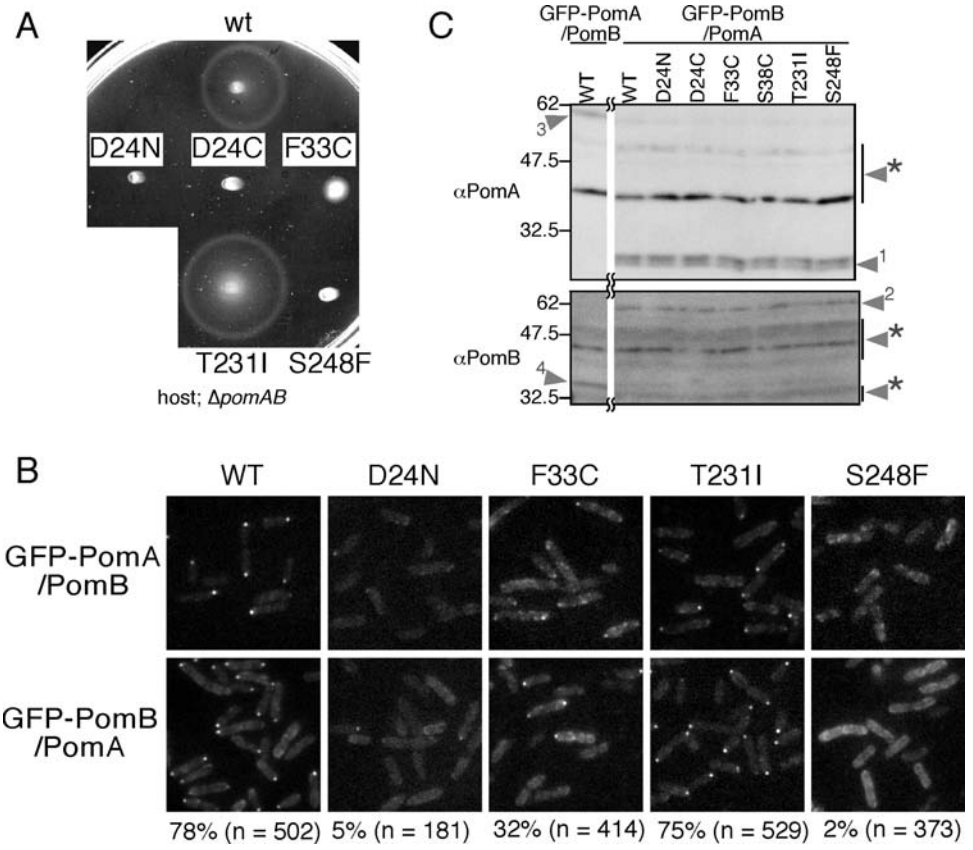

Fig. 2. A. Swarming behaviour of cells producing mutant PomB proteins. All mutant PomB proteins were produced in Δ*pomAB* cells with wild-type PomA. Cells were grown on VPG500-0.25% agar plates at 30°C for 5 h. Mutations in PomB are shown on the side.
B. The effects of the *pomB* mutations on the polar localization of stators. Δ*pomAB* cells producing GFP-PomA/PomB or GFP-PomB/PomA with various PomB mutations (D24N, F33C, T231I and S248F) were observed in 500 mM Na^+. Polar localized fractions of GFP-PomB are shown under the pictures. *n* is the total number of cells that were analysed to obtain the fractions.
C. Immunoblot of GFP-PomB variants and wild-type PomA in whole-cell extracts. Proteins were detected with anti-PomA1312 and anti-PomB93 antibodies respectively. Molecular mass values (kDa) are shown on the left side of the panels. Arrowheads marked with 1, 2, 3 and 4 indicate bands of PomA, GFP-PomB variants, GFP-PomA and PomB respectively. Arrowheads marked with an asterisk indicate non-specific bands detected by each antibody. *Vibrio* cells were grown in VPG500 medium containing 0.006% arabinose at 30°C for 4 h.

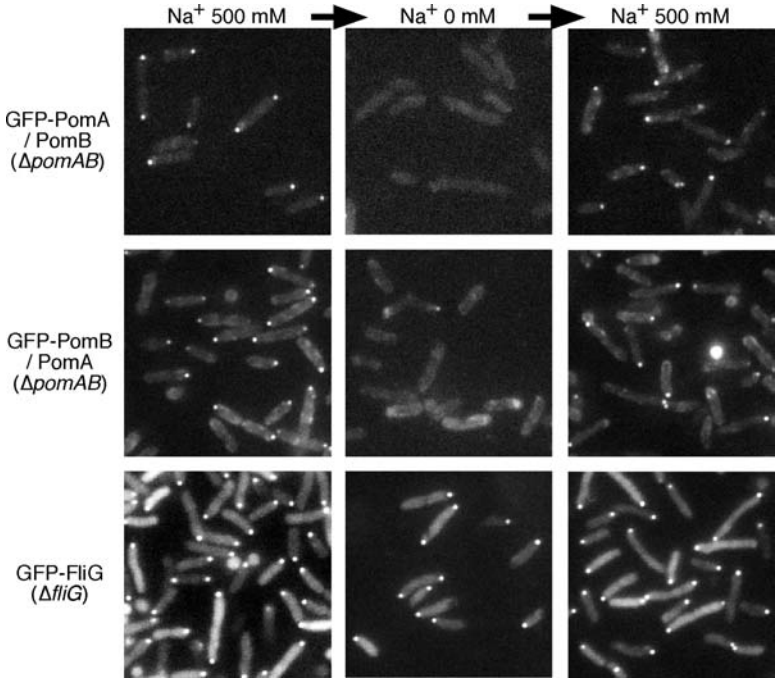

Fig. 3. Na^+-dependent polar localization of stators. Localization of GFP-fused stator proteins in 0 mM or 500 mM Na^+. Δ*pomAB* cells producing GFP-PomA and PomB (upper), GFP-PomB and PomA (middle), and Δ*fliG* cells producing GFP-FliG (bottom) were observed. The cells were suspended in 500 mM Na^+, resuspended in 0 mM Na^+ and resuspended again in 500 mM Na^+.

fluorescent dots that localized to the pole was drastically reduced by the D24 mutation; the fractions of GFP-PomB-D24N and GFP-PomB-D24C in the stator were 5% and 1%, respectively, in media containing 500 mM Na^+ (Fig. 2B, data not shown). In these strains, similar amounts of GFP-PomB variants and wild-type PomA were detected by immunoblotting (Fig. 2C). These results suggest that Asp-24 is essential for polar localization of stator proteins.

The polar localized fractions of GFP-PomB-F33C, GFP-PomB-T231I and, GFP-PomB-S248F with PomA were 32%, 75% and 2% respectively, and the polar localized fractions of GFP-PomA with the mutant PomB were similar (Fig. 2B). Similar amounts of GFP-PomB variants and wild-type PomA were detected in all strains by immunoblotting (Fig. 2C). There was good correlation between cell motility and the polar localization of the mutant PomB proteins investigated in this study. This correlation may suggest that the assembly of mutant PomB proteins into the flagellar motor was appropriately evaluated by determining the polar localization of GFP fusions.

Sodium-dependent polar localization of motor proteins

To examine the dependence of the polar localization of GFP stators on Na^+, localization was observed in the presence or absence of Na^+ using normal epifluorescence microscopy. In a medium containing 500 mM Na^+, polar localization of GFP-PomB was observed in approximately 80% of cells (Fig. 3, middle panels; Table 1). When the medium was replaced with Na^+-free medium (containing 500 mM K^+) following centrifugation, the polar localized fraction decreased to 5%. However, the polar localization was restored to 71% when cells were resuspended in 500 mM Na^+. Similar results were observed for GFP-PomA (Fig. 3, upper panels). In control cells producing GFP-FliG (Yorimitsu *et al*., 2003), which is one of the rotor components, fluorescent puncta were observed at the cell pole in about 80% of cells in either 500 mM or 0 mM Na^+ (Fig. 3, bottom panels). These results indicate that punctate fluorescent signals from GFP-stator proteins are dynamically localized and dispersed at the poles and the polar localization of PomA and PomB is dependent on Na^+. The relocalization was not affected by the addition of kanamycin, an inhibitor of new protein synthesis, at 1 mg ml^{-1} which is a concentration 10 times higher than the minimum inhibitory concentration for growth of *V. alginolyticus* cells (data not shown). Thus, new protein synthesis is not necessary to restore polar localization.

Table 1. Polar localized fractions of GFP-PomB under conditions that inhibit polar flagellar rotation.

Conditions	Polar localized fraction (%)
Na^+ 500 mM	78 (n = 502)
Na^+ 0 mM	5 (n = 215)
0 μM CCCP	49 (n = 1257)
20 μM CCCP	11 (n = 2176)
0 μM phenamil	53 (n = 1142)
100 μM phenamil	28 (n = 1054)
300 μM phenamil	21 (n = 871)

n is the total number of cells that were analysed.

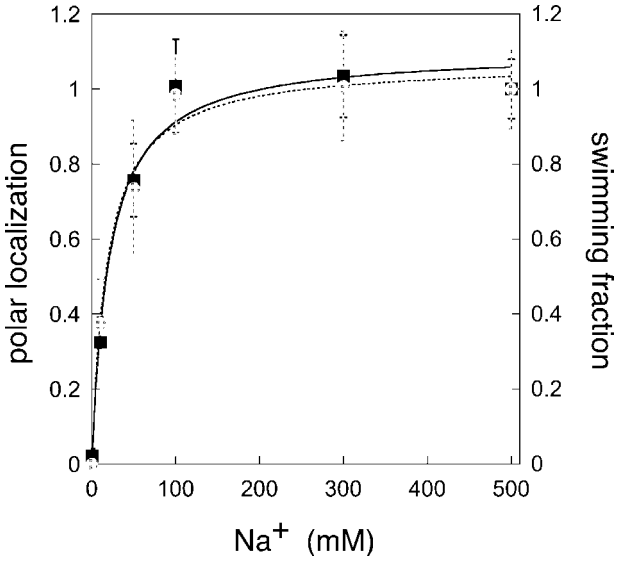

Fig. 4. Relationship between the polar localization of stators and the sodium ion concentration. The polar localized fraction of GFP-PomB (squares) and swimming fraction of VIO5 cells (circles), versus [Na^+], relative to values in 500 mM Na^+, are shown. GFP-PomB was produced in Δ*pomAB* cells with PomA. Michaelis–Menten fits for polar localization and swimming fractions are depicted by solid and dotted lines respectively.

To detect the motion of GFP-PomB at higher time resolution, we observed the localization by oblique illumination epifluorescence microscopy using an EMCCD camera. In this microscopic system, a fluorescent punctum was observed at the cell pole and we observed many fluorescent puncta diffusing along the cell body (see Movie S3). Thus it appears that stator complexes that are not incorporated into the flagellar motor diffuse in the membrane pool as proposed by Leake *et al*. (2006). Stator complexes diffusing in the cytoplasmic membrane in Na^+-free medium would relocalize to the cell pole upon addition of Na^+.

Relationship between cell motility and polar localization of motor proteins

If Na^+ affects the incorporation of stator complexes into the flagellar motor, there should be a correlation between the polar localization of stators and cell motility. We investigated the polar localization of GFP-PomB and the motility of VIO5 cells in various concentrations of Na^+. Polar localization of GFP-PomB and the motile fraction of VIO5 cells were approximately constant in media containing from 100 mM to 500 mM Na^+. In media with lower than 100 mM Na^+, the localization and fraction of swimming cells were coordinately reduced (Fig. 4). There seems to be a correlation between the polar localization of stator proteins and cell motility. We assumed that the reduction of the swimming fraction at lower concentrations of Na^+ is due to the dissociation of the stator proteins from the flagellar motor.

Stator complexes in the absence of sodium ions

Previously, we reported that individually, the stator proteins PomA and PomB do not localize at the cell pole but the stator complex PomA/PomB does (Fukuoka *et al*., 2005). Therefore, we examined the size of the PomA/His-GFP-PomB complex purified from the cell membrane in the presence and absence of Na^+ by size exclusion chromatography. In both ionic conditions, the elution profiles and the complex size of PomA and GFP-PomB appeared the same; GFP-PomB and PomA were mainly eluted in fractions 10–15, and in fraction 10 and 12 respectively (Fig. 5, bottom panels). These results suggest that the PomA/PomB complex is retained in the absence of Na^+, and that stator proteins move to the cell pole in the form of stator complexes.

Requirement of sodium motive force for the polar localization of motor proteins

In *V. alginolyticus* cells, at neutral pH, sodium motive force (smf) is secondarily generated by the H^+/Na^+ antiporter using proton motive force (pmf) (Yorimitsu and Homma, 2001). Under these conditions, addition of the proton-ionophore CCCP (carbonyl cyanide

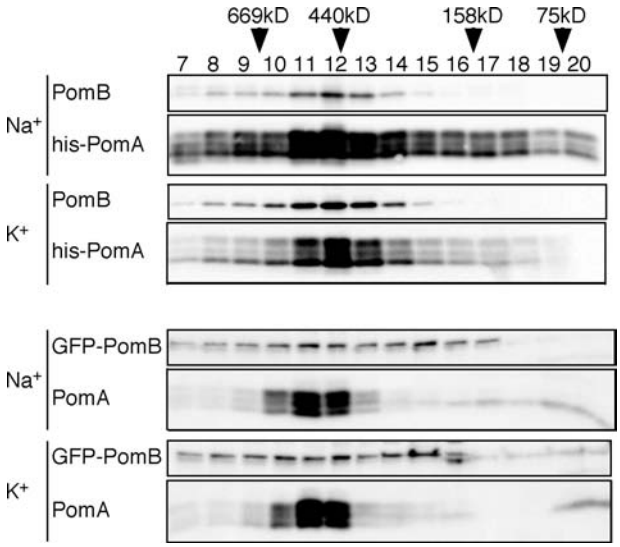

Fig. 5. Size exclusion chromatography. Proteins eluted from the Superdex 200 10/300 GL column in fractions were condensed by precipitation with trichloroacetic acid and were analysed by SDS-PAGE followed by immunoblotting. PomA was detected with anti-PomA1312 antibody, and PomB and GFP-PomB were detected with anti-PomB93 antibody. Numbers shown on top of the columns indicate fraction numbers from the size exclusion column elution. Arrowheads indicate molecular markers used in the size exclusion experiment.

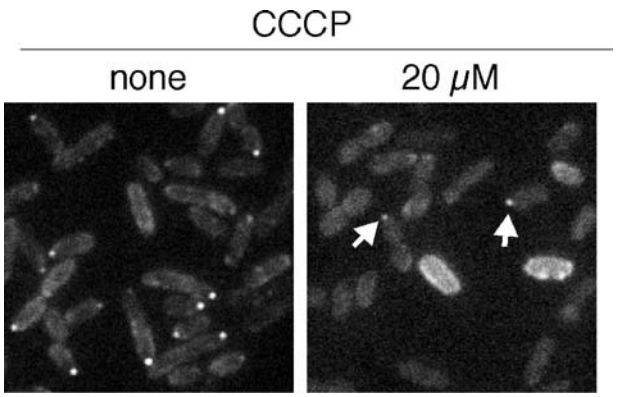

Fig. 6. Effect of CCCP on the Na^+-dependent polar localization of stators. Δ*pomAB* cells producing GFP-PomB and PomA in 50 mM Na^+ (pH 7.0) were observed in the presence of 0 μM, 20 μM CCCP. White arrowheads indicate polar localization in the presence of CCCP.

m-chlorophenylhydrazone) collapses pmf and hence smf. In medium containing 50 mM Na^+ (only this experiment was carried out at pH 7.0), the polar localized fraction of GFP-PomB was 49% in the absence of CCCP. On the other hand, the polar localized fraction was drastically decreased to 11% in the presence of 20 μM CCCP (Fig. 6, Table 1). In the presence of CCCP, most VIO5 cells did not swim, although a small fraction of cells was very slowly motile. smf seemed to be required for stator assembly around the rotor in the sodium-driven motor.

Effect of sodium-driven motor inhibitor on the polar localization of motor proteins

The rotation of Na^+-driven flagellar motors is specifically inhibited by the non-competitive inhibitor phenamil (Atsumi *et al*., 1990; Kojima *et al*., 1999). We investigated whether phenamil affects the polar localization of the stator in medium containing 500 mM Na^+. In the absence of phenamil, the polar localized fraction of GFP-PomB was 53% (Fig. 7, Table 1). This reduced polar localization compared with the control experiments is probably due to the solvent for phenamil, dimethyl sulphoxide (DMSO). With 100 μM and 300 μM phenamil, the polar localized fraction of GFP-PomB was reduced to 28% and 21% respectively (Fig. 7, Table 1). In the presence of phenamil, most VIO5 cells did not swim but we observed a few moving cells. Phenamil affected the Na^+-dependent assembly of stator proteins into the motor. Polar localization of GFP-PomB was rarely observed in Na^+-free medium with or without 100 μM phenamil (data not shown). The results indicate that the sodium-channel inhibitor prevents the polar localization of the stator complex, although the effect is not perfect.

Discussion

The bacterial flagellar motor, which is driven by the electrochemical potential of specific ions, H^+ or Na^+, is a supramolecular complex embedded in the cell membrane. The stator, which is composed of PomA and PomB, functions as an ion channel and is thought to be a torque generator converting the energy of ion flux into mechanical power via the interaction with the rotor. Multiple stator units assemble around the rotor and these units are able to exchange with new units even after the flagellar structure has been built. Leake *et al*. (2006) reported that the stator complex can be exchanged in the functional motor, indicating that each stator behaves more dynamically than previously expected. When a mutation is introduced at the Asp-24 residue of PomB, which is the putative Na^+-binding site, the polar localization of the fluorescent punctum was drastically reduced even in media containing 500 mM Na^+. It is likely that in this mutant, the stator complex might not be assembled into the motor because Na^+ ions do not bind to their binding site. In the proton-driven motor of MotB, which is a PomB homologue, the substitution of Asp to Asn in the putative ion-binding site is assumed to mimic a H^+-binding state (Zhou *et al*., 1998; Kojima and Blair, 2001). If this assumption is applied to the Na^+-driven motor, the polar localization of

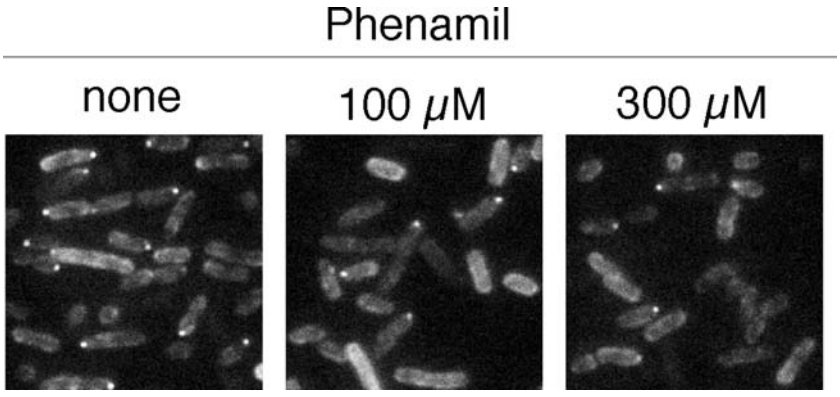

Fig. 7. Effect of phenamil on the Na^+-dependent polar localization of stators. Δ*pomAB* cells producing GFP-PomB and PomA in 500 mM Na^+ were observed in the presence of 0 μM, 100 μM and 300 μM phenamil.

the stator complex may not be reduced by the D24N substitution. Our data suggest that the substitution of Asp-24 to Asn may not mimic the ion-binding state in the sodium motor because the size of a sodium ion is larger than that of a proton. The polar localization was reduced by the mutation of PomB-F33 which is a bulky side-chain that is located in the transmembrane segment of PomB. However, the complex containing the PomB-F33C mutation still localized at the cell poles. This residual localization is consistent with the fact that PomB-F33C confers very weak motility. The assembly of stator complexes into the flagellar motor does not seem to be severely affected by the mutation of PomB-F33C, but the motility was significantly reduced. So, we speculate that this mutation more severely affects energy transduction than stator assembly around the rotor. The Ser-248 residue is located in the peptidoglycan-binding motif of PomB and both the function and polar localization of PomB were lost in the S248F mutant. The S248F mutation may abolish binding of PomB to peptidoglycan or the T-ring, and consequently the stator complex could not assemble around the flagellar motor.

Next, we found that the polar localization of stator proteins depends on the extracellular Na^+ concentration. We could easily and reversibly regulate the polar localization of the stator complex by changing the concentration of Na^+ in the buffer. In addition, the polar localization of stator proteins was drastically reduced under conditions that collapsed the smf even when Na^+ was present. We examined whether the stator complex, 4PomA:2PomB, was dissociated in the absence of Na^+. We isolated stator complexes with similar molecular size both in the presence or absence of Na^+, indicating that the complex is retained without Na^+. In the presence of phenamil, which is a specific non-competitive inhibitor of the Na^+ channel or the Na^+-driven flagellar motor, the polar localization of GFP-PomB was reduced. These results indicate that the assembly of the stator complex into the flagellar motor requires not only Na^+ but also smf in the Na^+-driven motor of *V. alginolyticus*. Now we realize that motor rotation is regulated by the ion flux as well as by the association/dissociation of stator complexes via the coupling ion in the motor complex. Similar regulation was found in a polar flagellar motor of *Shewanella oneidensis* that has two potential stator systems, PomAB for Na^+-driven motor and MotAB for H^+-driven motor (Paulick *et al*., 2009). It has been shown that the polar flagellum is powered by both of the stator systems and their localizations are regulated in response to sodium concentrations.

In reconstituted Na^+-driven motors in *E. coli*, we observed the stepwise increase of rotational speed with time after the replacement of 0.1 μM Na^+ with 85 mM Na^+ in the medium (Sowa *et al*., 2005). This result is consistent with our interpretation that the assembly of stator complexes into the flagellar motor depends on their coupling ion, because if the stator complex is kept in the motor at low Na^+ concentrations, the rotational speed should be restored immediately to the original speed and a stepwise increase in the rotational speed should not be detected. The following physiological evidence also supports our results. The motility of *Rhodopseudomonas sphaeroides* is lost when the pmf is dissipated by the dark with addition of a H^+ uncoupler. Cell motility can be recovered upon regeneration of the pmf following illumination. However, cell motility did not immediately restart upon redevelopment of pmf (Armitage and Evans, 1985; Evans and Armitage, 1985). In these reports, authors discussed the possibility that the delay in re-initiation of motility is caused by the dissociation of stator units from the motor under the 0 pmf conditions, or by the inhibition of H^+ flow by direct interaction between the H^+ uncoupler and the motor. In the H^+-driven motor of *E. coli*, the recover of motor rotation also appears to be delayed after the pmf is recharged (Fung and Berg, 1995). These observations may suggest that the membrane potential is required for the assembly of stator proteins into the flagellar motor in the H^+-driven motor as we have shown in the Na^+-driven motor.

Based on the present results and the evidence as described above, we propose a hypothetical model of stator assembly onto the flagellar motor (Fig. 8). At first, PomA and PomB form the stator complex in the cytoplasmic membrane, and Na^+ does not flux into the cytoplasm. Hosking *et al*. (2006) proposed a mechanism that prevents H^+ flow into the cytoplasm via unincorporated stator complexes. When the Na^+ concentration outside the cytoplasmic membrane is sufficient (in the presence of smf), the stator complex interacts with the rotor (flagellar basal body), for example, via interactions between PomB and the T-ring, or between PomA and FliG. As a result of this interaction, the sodium ion channel is opened and Na^+ binds to its binding site. The bound Na^+ is released from the complex and fluxed into the cytoplasm, and consequently, rotational force is generated. Subsequently, Na^+ immediately binds to its binding site (Asp-24), and then the Na^+-bound form of the stator complex again interacts with the rotor. So, the stator complex is retained in the motor by continuous Na^+ influx. In the absence of smf, the stator complex may not interact strongly with the rotor.

In the present study, we demonstrated that regulation of motor function occurs by the association/dissociation of stator complexes via their coupling ion. This type of regulation has been proposed for V-ATPase, which is a rotary motor that pumps out protons using the energy of ATP hydrolysis; the function of V-ATPase is regulated via reversible association/dissociation of the V_1 sector (soluble) and the V_o sector (membrane bound) (Kane and Smardon, 2003). V-ATPase is only active when

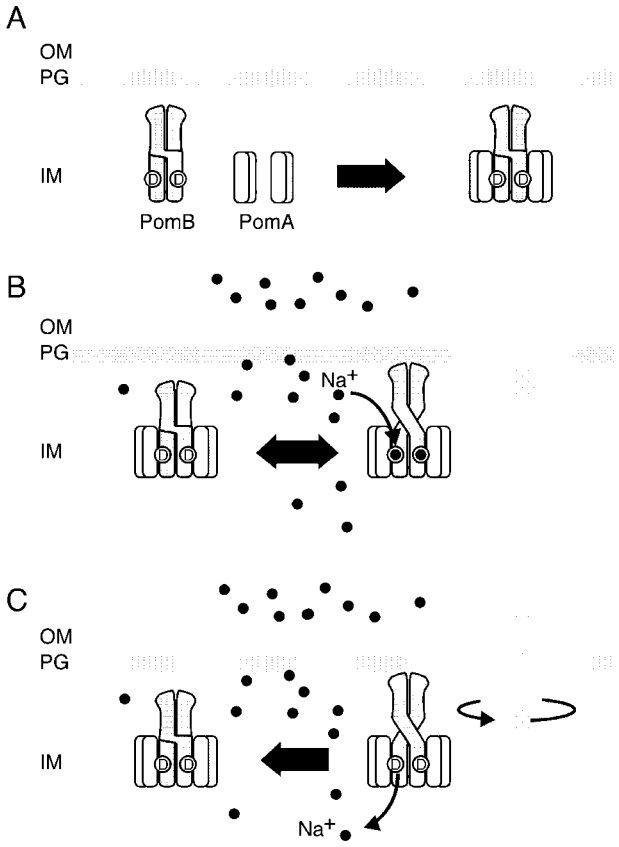

Fig. 8. Model of the stator assembly in the sodium-driven polar flagellar motor of *Vibrio*. PomA and PomB are coloured light grey and dark grey respectively. White circles with D in the PomB transmembrane region indicate the aspartic acid residue that binds Na^+ ions. Black circles indicate Na^+ ions.
A. PomA and PomB form a 4A:2B complex independent of Na^+.
B. The PomA/PomB complex diffuses in the inner membrane, and the PomA/PomB complex can locate around the rotor through the association with MotX in the T-ring or FliG. Then, Na^+ binds to its binding site (Asp-24).
C. The bound Na^+ is released from the complex and generates a rotational force. The stator complex is released from the rotor. Subsequently Na^+ immediately binds to its binding site (Asp-24), and the Na^+-bound form of the stator complex interacts with the rotor again. So, the stator complex is retained in the motor by the continuous Na^+ influx.

both sectors are assembled in the plasma membrane. Although the mechanism of association/dissociation is not well understood, their dissociation is known to be caused by glucose deprivation or a change in pH. Thus the activity of V-ATPase is regulated in response to environmental signals. Regulation of molecular machines by association/dissociation of relevant components may make it possible to rapidly respond to environmental changes and allow the recycling of the components without the requirement for *de novo* synthesis. The control of a molecular machine by association/dissociation of its components might be a strategy that evolved to allow organisms to live in varying environments. Our study provides clear and direct evidence that the function of the flagellar motor is regulated by dynamic coupling ion-dependent assembly of the structure. We predict that other supramolecules in the membrane, such as transporters or respiratory complexes, may be regulated in a similar way.

Experimental procedures

Vibrio *strains, plasmids and cell preparation*

Bacterial strains and plasmids used in this study are shown in Table 2. *Vibrio* strains were derived from strain VIO5, which has wild-type polar flagella (Okunishi *et al*., 1996). NMB190 was transformed with pHFGA, NMB191 was transformed with pHFGAB or pHFGBA2, NMB192 was transformed with pJN227, and NMB198 was transformed with pTY201 respectively (Yorimitsu *et al*., 2003; Fukuoka *et al*., 2005). To investigate the function of PomB mutants, NMB191 was transformed with derivatives of plasmid pJN152 (Fukuoka *et al*., 2005). All point mutations were introduced by PCR using *pfu* DNA polymerase (Stratagene). Cells were precultured in VC medium and then grown in VPG500 medium. Chloramphenicol and kanamycin were added to final concentrations of 2.5 μg ml^{-1} and 100 μg ml^{-1} respectively. For swarming assays, overnight cultures were spotted onto VPG500 plates containing 0.25% agar.

Observation of localization with fluorescence microscopy

Overnight cultures were inoculated into VPG500 medium (1/100 volume) containing 0.006% arabinose for production of GFP-fused stator proteins and 0.01% arabinose for production of GFP-FliG, and cells were grown for 4 h at 30°C. To analyse the recovery of polar localization, 6 ml of cultures was harvested by centrifugation and suspended in 1.2 ml of TMN500 medium [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose, 500 mM NaCl], and incubated for 15 min. One hundred microlitres of cell suspension was retained for observation. The remaining 1.1 ml was centrifuged and cells were suspended in 1 ml of TMN0 medium [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose, 500 mM KCl]. Cells were harvested by centrifugation and suspended in 360 μl of TMN0 and incubated for 15 min. One hundred microlitres of this cell suspension was retained for observation. The remaining 260 μl was centrifuged and cells were suspended in TMN500 medium. One milligram per millilitre of kanamycin was added to motility media to prevent synthesis of GFP-fusion proteins.

To investigate the effect of various concentrations of Na^+, 1 ml aliquots of cell cultures were centrifuged and cell pellets were suspended in 1 ml of TMN0 (500 mM KCl), TMN10 (10 mM NaCl, 490 mM KCl), TMN50 (50 mM NaCl, 450 mM KCl), TMN100 (100 mM NaCl, 400 mM KCl), TMN300 (300 mM NaCl, 200 mM KCl) and TMN500 (500 mM NaCl) respectively. Cells were harvested by centrifugation and suspended in 200 μl of the same medium.

To investigate the effect of phenamil, 1 ml aliquots of cell culture were centrifuged and cell pellets were suspended in

Table 2. Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
V. alginolyticus		
VIO5	Rif^r, Pof$^+$, Laf$^-$	Okunishi *et al*. (1996)
NMB190	Rif^r, Pof$^+$, Laf$^-$ Δ*pomA*	Asai *et al*. (1999)
NMB191	Rif^r, Pof$^+$, Laf$^-$ Δ*pomAB*	Yorimitsu *et al*. (1999)
NMB192	Rif^r, Pof$^+$, Laf$^-$ Δ*pomB*	Asai *et al*. (2003)
NMB198	Rif^r, Pof$^+$, Laf$^-$ Δ*fliG*	Yorimitsu *et al*. (2003)
Plasmid		
pSU41	Km^r, P*lac*, *lacZα*	Bartolome *et al*. (1991)
pBAD33	Cm^r, P*BAD*	Guzman *et al*. (1995)
pHFGA	*his$_6$-gfp-pomA* in pBAD33	Fukuoka *et al*. (2005)
pJN227	*his$_6$-gfp-pomB* in pBAD33	Fukuoka *et al*. (2005)
pHFGBA2	*his$_6$-gfp-pomB and pomA* in pBAD33	Fukuoka *et al*. (2005)
pHFAB	*pomA* and *pomB* in pBAD33	Fukuoka *et al*. (2005)
pJN152	*his$_6$ -pomA* and *pomB* in pSU41	Fukuoka *et al*. (2005)

Rif^r, rifampicin-resistant; Km^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant; P*lac*, *lac* promoter; P*BAD*, *araBAD* promoter; Pof$^+$, normal polar flagellar formation; Laf$^-$, defective in lateral flagellar formation.

1 ml of TMN500 respectively. Cells were harvested by centrifugation and resuspended in 100 μl of TMN500. One microlitre of DMSO solution containing 30 mM, 10 mM, or no phenamil was added to each cell suspension.

To investigate the effect of CCCP, 1 ml aliquots of cell culture were centrifuged and cell pellets were suspended in 1 ml of TMN50 (pH 7.0) respectively. Cells were harvested by centrifugation and resuspended in 100 μl of TMN50 (pH 7.0). One microlitre of DMSO containing 2 mM or no CCCP was added to each cell suspension.

For observation of polar localization, cell suspensions (20 μl each) were loaded into the space between a polylysine-coated coverslip and a microscope slide with a spacer. In this space, about 20 μl of solution can be retained. Additional motility medium was loaded into the space between the coverslip and the slide to remove the remaining unattached cells, and the samples were incubated for 15 min at room temperature. Cells were observed by normal epi-illumination fluorescence microscopy (B×50, Olympus). Images were recorded every 1 s (exposure time) and processed using a CCD camera (Orca-ER, Hamamatsu Photonics) and imaging software (IPLab, ver. 3.9.5r2, Scanalytics). For observation of the diffusion of GFP-PomB, we used Olympus IX71 based oblique-illumination fluorescence microscopy (Fukuoka *et al*., 2007) and images were recorded by using EMCCD camera by video rate (DV860-BV, Andor Technology).

Measurement of motility

Cells were harvested by centrifugation and were suspended TMN0, TMN10, TMN50, TMN100, TMN300 and TMN500. Cells were harvested by centrifugation and resuspended in the same medium. Cell suspensions were diluted 100-fold in the same motility medium and incubated for 15 min. Finally, a 1 M serine solution was added to a final concentration of 20 mM to suppress changes in swimming direction. Cell motility was observed by dark-field microscopy and recorded on a videotape. Swimming speed was determined as described (Atsumi *et al*., 1996).

Size exclusion chromatography

Membrane fractions were prepared as described (Fukuoka *et al*., 2005). Five milligram per millilitre of membrane proteins was solubilized in each condition. In the presence of Na^+, solubilization was carried out in 20TNPDG [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM PMSF, 1 mM DTT, 10% (w/v) glycerol] containing 5 mM imidazole and 2.5% (w/v) Chaps {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate}. Ni-NTA agarose with his-tagged proteins bound was washed with the same buffer containing 1% Chaps, and the associated proteins were eluted with 750 ml of 20TNPDG containing 200 mM imidazole and 1% Chaps. For the Na^+-free condition, stator proteins were isolated in the same way, but we used 20TKPDG [20 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.5 mM PMSF, 1 mM DTT, 10% (w/v) glycerol] instead. Five hundred millilitres of eluted proteins was loaded on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 20TNPDG or 20TKPDG containing 1% Chaps, and run at a flow rate of 0.5 ml min^{-1} with the appropriate buffer respectively. Eluted proteins in fractions (500 μl each) were precipitated with trichloroacetic acid, washed with acetone, and then analysed by SDS-PAGE followed by immunoblotting. Thyroglobulin (669 kDa), Ferritin (440 kDa), Aldolase (158 kDa) and Conalbumin (75 kDa) were used for size markers.

Immunoblotting whole cell extracts

Δ*pomAB* cells producing GFP-PomB variants and PomA were grown in VPG500 containing 0.006% arabinose at 30°C for 4 h. Cells were harvested by centrifugation. Cell pellets were suspended in SDS-loading buffer containing β-mercaptoethanol. Samples were separated by SDS-PAGE and proteins were detected with anti-PomB93 and anti-PomA1312 antibodies.

Acknowledgements

We thank Y. Sowa for useful comments and K.M. Thormann for exchanging unpublished results of a submitted paper. This

work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (to M.H. and S.K.); from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to A.I.); and from the Japan Science and Technology Agency (to M.H., S.K. and A.I.).

References

Armitage, J.P., and Evans, M.C.W. (1985) Control of the proton motive force in *Rhodopseudomonas sphaeroides* in the light and dark and its effect on the initiation of flagellar rotation. *Biochimica Biophysica Acta* **806:** 42–55.

Asai, Y., Kawagishi, I., Sockett, E., and Homma, M. (1999) Hybrid motor with the H^+-and Na^+-driven components can rotate *Vibrio* polar flagella by using sodium ions. *J Bacteriol* **181:** 6332–6338.

Asai, Y., Yakushi, T., Kawagishi, I., and Homma, M. (2003) Ion-coupling determinants of Na^+-driven and H^+-driven flagellar motors. *J Mol Biol* **327:** 453–463.

Atsumi, T., Sugiyama, S., Cragoe, E.J., Jr, and Imae, Y. (1990) Specific inhibition of the Na^+-driven flagellar motors of alkalophilic *Bacillus* strains by the amiloride analog phenamil. *J Bacteriol* **172:** 1634–1639.

Atsumi, T., Maekawa, Y., Yamada, T., Kawagishi, I., Imae, Y., and Homma, M. (1996) Effect of viscosity on swimming by the lateral and polar flagella of *Vibrio alginolyticus*. *J Bacteriol* **178:** 5024–5026.

Bartolome, B., Jubete, Y., Martínez, E., and de la Cruz, F. (1991) Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene* **102:** 75–78.

Blair, D.F. (2003) Flagellar movement driven by proton translocation. *FEBS Lett* **545:** 86–95.

Blair, D.F., Kim, D.Y., and Berg, H.C. (1991) Mutant MotB proteins in *Escherichia coli*. *J Bacteriol* **173:** 4049–4055.

Braun, T.F., Poulson, S., Gully, J.B., Empey, J.C., Van W.S., Putnam, A., and Blair, D.F. (1999) Function of proline residues of MotA in torque generation by the flagellar motor of *Escherichia coli*. *J Bacteriol* **181:** 3542–3551.

Evans, M.C.W., and Armitage, J. (1985) Initiation of flagellar rotation in *Rhodopseudomonas sphaeroides*. Evidence for the direct interaction of anionic uncouplers with the flagellar motor. *FEBS Lett* **186:** 93–97.

Fukuoka, H., Yakushi, T., Kusumoto, A., and Homma, M. (2005) Assembly of motor proteins, PomA and PomB, in the Na^+-driven stator of the flagellar motor. *J Mol Biol* **351:** 707–717.

Fukuoka, H., Sowa, Y., Kojima, S., Ishijima, A., and Homma, M. (2007) Visualization of functional rotor proteins of the bacterial flagellar motor in the cell membrane. *J Mol Biol* **367:** 692–701.

Fung, D.C., and Berg, H.C. (1995) Powering the flagellar motor of *Escherichia coli* with an external voltage source. *Nature* **375:** 809–812.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. *J Bacteriol* **177:** 4121–4130.

Hosking, E.R., Vogt, C., Bakker, E.P., and Manson, M.D. (2006) The *Escherichia coli* MotAB proton channel unplugged. *J Mol Biol* **364:** 921–937.

Kane, P.M., and Smardon, A.M. (2003) Assembly and regulation of the yeast vacuolar H^+-ATPase. *J Bioenerg Biomembr* **35:** 313–321.

Kojima, S., and Blair, D.F. (2001) Conformational change in the stator of the bacterial flagellar motor. *Biochemistry* **40:** 13041–13050.

Kojima, S., and Blair, D.F. (2004) Solubilization and purification of the MotA/MotB complex of *Escherichia coli*. *Biochemistry* **43:** 26–34.

Kojima, S., Asai, Y., Atsumi, T., Kawagishi, I., and Homma, M. (1999) Na^+-driven flagellar motor resistant to phenamil, an amiloride analog, caused by mutations in putative channel components. *J Mol Biol* **285:** 1537–1547.

Kojima, S., Shinohara, A., Terashima, H., Yakushi, T., Sakuma, M., Homma, M., *et al.* (2008) Structural insight into the stator assembly revealed by the crystal structure of MotY. *Proc Natl Acad Sci USA* **105:** 7696–7701.

Leake, M.C., Chandler, J.H., Wadhams, G.H., Bai, F., Berry, R.M., and Armitage, J.P. (2006) Stoichiometry and turnover in single, functioning membrane protein complexes. *Nature* **443:** 355–358.

Macnab, R. (1996) Flagela and motility. In Escherichia coli *and* Salmonella. Neidhardt, F.C. (chief-ed.). Washington, DC: American Society for Microbiology, pp. 123–145.

Okabe, M., Yakushi, T., and Homma, M. (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.

Okunishi, I., Kawagishi, I., and Homma, M. (1996) Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*. *J Bacteriol* **178:** 2409–2415.

Paulick, A., Koerdt, A., Lassak, J., Huntley, S., Wilms, I., Narberhaus, F., and Thormann, K.M. (2009) Two different stator systems drive a single polar flagellum in*Shewanella oneidensis* MR-1. *Mol Microbiol* **71:** 836–850.

Reid, S.W., Leake, M.C., Chandler, J.H., Lo, C.J., Armitage, J.P., and Berry, R.M. (2006) The maximum number of torque-generating units in the flagellar motor of *Escherichia coli* is at least 11. *Proc Natl Acad Sci USA* **103:** 8066–8071.

Sato, K., and Homma, M. (2000) Multimeric structure of PomA, the Na^+-driven polar flagellar motor component of *Vibrio alginolyticus*. *J Biol Chem* **275:** 20223–20228.

Sowa, Y., Rowe, A.D., Leake, M.C., Yakushi, T., Homma, M., Ishijima, A., and Berry, R.M. (2005) Direct observation of steps in rotation of the bacterial flagellar motor. *Nature* **437:** 916–919.

Terashima, H., Fukuoka, H., Yakushi, T., Kojima, S., and Homma, M. (2006) The *Vibrio* motor proteins, MotX and MotY, are associated with the basal body of Na^+-driven flagella and required for stator formation. *Mol Microbiol* **62:** 1170–1180.

Yakushi, T., Maki, S., and Homma, M. (2004) Interaction of PomB with the third transmembrane segment of PomA in the Na^+-driven polar flagellum of *Vibrio alginolyticus*. *J Bacteriol* **186:** 5281–5291.

Yorimitsu, T., and Homma, M. (2001) Na^+-driven flagellar motor of *Vibrio*. *Biochim Biophys Acta* **1505:** 82–93.

Yorimitsu, T., Sato, K., Asai, Y., Kawagishi, I., and Homma,

M. (1999) Functional interaction between PomA and PomB, the Na^+-driven flagellar motor components of *Vibrio alginolyticus*. *J Bacteriol* **181:** 5103–5106.

Yorimitsu, T., Mimaki, A., Yakushi, T., and Homma, M. (2003) The conserved charged residues of the C-terminal region of FliG, a rotor component of Na^+-driven flagellar motor. *J Mol Biol* **334:** 567–583.

Zhou, J., Sharp, L.L., Tang, H.L., Lloyd, S.A., Billings, S., Braun, T.F., and Blair, D.F. (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.

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