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Assembly of Motor Proteins, PomA and PomB, in the Na^+-driven Stator of the Flagellar Motor

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PomA and PomB are transmembrane proteins that form the stator complex in the sodium-driven flagellar motor of *Vibrio alginolyticus* and are believed to surround the rotor part of the flagellar motor. We constructed and observed green fluorescent protein (GFP) fusions of the stator proteins PomA and PomB in living cells to clarify how stator proteins are assembled and installed into the flagellar motor. We were able to demonstrate that GFP-PomA and GFP-PomB localized to a cell pole dependent on the presence of the polar flagellum. Localization of the GFP-fused stator proteins required their partner subunit, PomA or PomB, and the C-terminal domain of PomB, which has a peptidoglycan-binding motif. Each of the GFP-fused stator proteins was co-isolated with its partner subunit from detergent-solubilized membrane. From these lines of evidence, we have demonstrated that the stator proteins are incorporated into the flagellar motor as a PomA/PomB complex and are fixed to the cell wall *via* the C-terminal domain of PomB.

Keywords: GFP; membrane protein; energy transduction; sodium motive force; *Vibrio*

Introduction

Many bacteria can swim by rotating their helical flagellar filament in a liquid environment. The bacterial flagellum consists of a flagellar filament, a hook, and a basal body. The helical filament acts as a propeller and is connected to the hook that serves as the universal joint connecting the filament and basal body. The basal body is a part of the flagellar motor embedded in the cytoplasmic membrane.[1] The torque of flagellar rotation is generated by the interaction of the rotor and stator parts of this motor. The energy source for flagellar motor rotation is the electrochemical potential of either protons or sodium ions across the cell membrane; these driving ions define the two major subtypes of flagellar motor.[2,3] The flagellar motor of *Escherichia coli* is proton-driven,[4] whereas the flagellar motor of alkalophillic *Bacillus* and the polar flagellar motor of *Vibrio* species are sodium-driven.[5–7] It was reported recently in *Bacillus subtilis* that the distinctive stators for the different ion-coupling, sodium ion or proton, are functional in a bacterial cell.[8]

In Gram-negative bacteria, the basal body of the flagellum consists of several rings, termed L-ring, P-ring, MS-ring, and C-ring, which are embedded in the outer membrane, the peptidoglycan layer, the cytoplasmic membrane, and attached on the cytoplasmic surface of the MS-ring, respectively. The rod structure pierces these rings.[1] During flagellar construction, FliF proteins first assemble to form the MS-ring in the cytoplasmic membrane.[9] The C-ring, which consists of FliG, FliM, and FliN, is constructed together with an export apparatus on the cytoplasmic surface of the MS-ring.[10–14] Components of the rod and hook are transported by this export apparatus and assembled onto the periplasmic side of the MS-ring. Finally, flagellin molecules are exported and self-assemble to make up the flagellar filament. These axial flagellar proteins are transported through the central channel of the flagellum.[15,16] FlgH and FlgI, which are L-ring and P-ring proteins, are transported into the periplasm by the Sec machinery.[17]

MotA and MotB, components of proton-driven flagellar motor of *E. coli*, or PomA and PomB, components of the sodium-driven flagellar motor of *Vibrio alginolyticus*, are referred to as stator

Abbreviation used: GFP, green fluorescent protein.

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proteins, because MotB and PomB have peptidoglycan-binding motifs in their C-terminal regions through which the motor is anchored to the cell wall.[18,19] MotA and MotB, or PomA and PomB, which are the four transmembrane and one transmembrane proteins, are predicted to form a complex with 4A:2B stoichiometry and to function as a specific ion channel.[18,20–23] The putative ion-binding site is located in the transmembrane segment of the B subunit.[18,24] The stator complex is thought to interact electrostatically with FliG, one of the rotor components, to generate torque for the flagellar motor.[25] Stator and rotor components of the sodium-driven and proton-driven types are thought to work in a similar way,[26] although charged residues critical for flagellar rotation appear to be different between the *Vibrio* motor and the *E. coli* motor, or additional charged residues may be required in the *Vibrio* motor.[27,28]

A circular array of particles surrounding a larger particle was observed in freeze-fracture images of cytoplasmic membrane in *E. coli*; these particles are thought to represent stator complexes incorporated into flagellar motor.[29] The stator proteins are incorporated into the motor in the last step of flagellar assembly. The system of flagellar gene expression is hierarchical and tightly regulated on the transcriptional, translational, and post-translational levels. This hierarchy was classified into three classes by the expression order, and the stator genes are encoded as the last class, and their protein products are produced after the completion of hook–basal body assembly or rotor construction.[30–34] The stator proteins can be installed and are functional even after the completion of the entire flagellar structure.[35,36] However, the knowledge on the process of incorporation of the stator proteins into the flagellar motor is very limited. In this study, we developed green fluorescent protein (GFP)-fusion constructs of the stator proteins PomA and PomB, and observed them to clarify the localization of stator proteins in the living cells and how stator proteins are assembled and incorporated into the flagellar motor. We demonstrated that PomA and PomB were localized to the cell pole that is on the same side as the polar flagella generation, and they must interact with each other to be localized properly. We showed that the flagellar structure and C-terminal domain of PomB are required for the localization.

Results

Subcellular localization of GFP-PomA and GFP-PomB

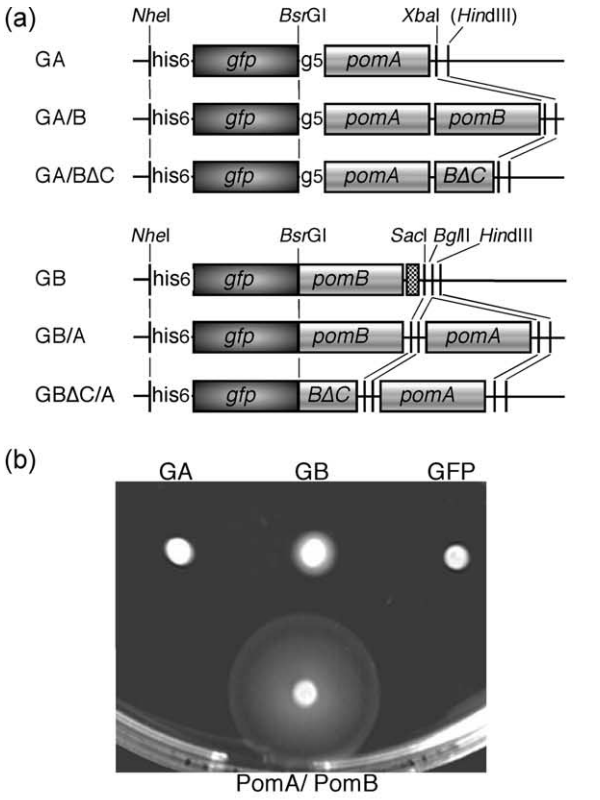
To investigate the localization of PomA and PomB proteins, which are the polar flagellar motor stator components of *V. alginolyticus*, we constructed expression systems for GFP-fused PomA and PomB proteins (Figure 1(a)). These N-terminal-fused constructs were designated GFP-PomA and GFP-PomB, respectively. The genes encoding the GFP-fused motor proteins were placed under the control of an arabinose-inducible promoter. They were expressed in NMB190 and NMB192 cells, which were isolated previously as *pomA* and *pomB* deletion strains, respectively. To examine the function of GFP-PomA and GFP-PomB proteins, we performed a swarm assay (Figure 1(b)). NMB190 cells producing GFP-PomA did not swarm at all. On the other hand, NMB192 cells producing GFP-PomB swarmed slightly. Under light microscopy, we could detect the swimming NMB192 cells producing GFP-PomB; however, their swimming ability was much lower than that of wild-type cells. The swimming speed and the fraction of the cells producing GFP-PomB were 17 μm/s and 26%, compared to 75 μm/s and 64% for cells producing wild-type PomB.

In immunoblot analysis of whole cell extracts, GFP-PomA and GFP-PomB proteins were detected at the expected molecular mass values of about 45 kDa and 66 kDa (estimated values from the gel, 50 kDa and 69 kDa, respectively) (Figure 1(c)). Some GFP-PomB degradation products with apparent sizes of 62 kDa and 32 kDa were detected by anti-GFP antibody (Figure 1(c), arrowheads marked as *1 and *2). On the other hand, the degraded products were not detected by anti-PomA or anti-PomB antibodies. GFP-PomB might be marginally functional in the flagellar motor of *V. alginolyticus*; however, we could not rule out the possibility that GFP-PomB proteins were cleaved into functional PomB proteins.

Cells harvested at mid-log phase were observed by fluorescence microscopy as described in Materials and Methods. In NMB190 cells producing GFP-PomA, a fluorescent dot was observed at the cell pole (Figure 2, GA) in medium containing 0.006% (w/v) arabinose. Similarly, a fluorescent dot at the cell pole was observed in NMB192 cells producing GFP-PomB (Figure 2, GB). Such fluorescent dots were observed in about 50% of cells expressing GFP-PomA or GFP-PomB at either of the poles. In a small number of cells, fluoresent dots were observed at both poles. When both strains were made to express GFP alone, which is a soluble protein, the fluorescence was diffuse throughout the cell (Figure 2, GFP).

Polar localization of the stator requires both PomA and PomB

It has been shown that PomA interacts with PomB to form a complex, and that the PomA/PomB complex functions as a sodium ion channel in the bacterial cytoplasmic membrane.[21,22] To examine whether the localization of GFP-fused stator proteins to a cell pole requires their partner subunit, this fusion protein was produced in NMB191, which is a *pomA/pomB* deletion strain. When GFP-PomA or GFP-PomB was produced in a *pomA* or a *pomB* deletion strain, fluorescent dots were detected at cell poles as shown above. On the other hand,



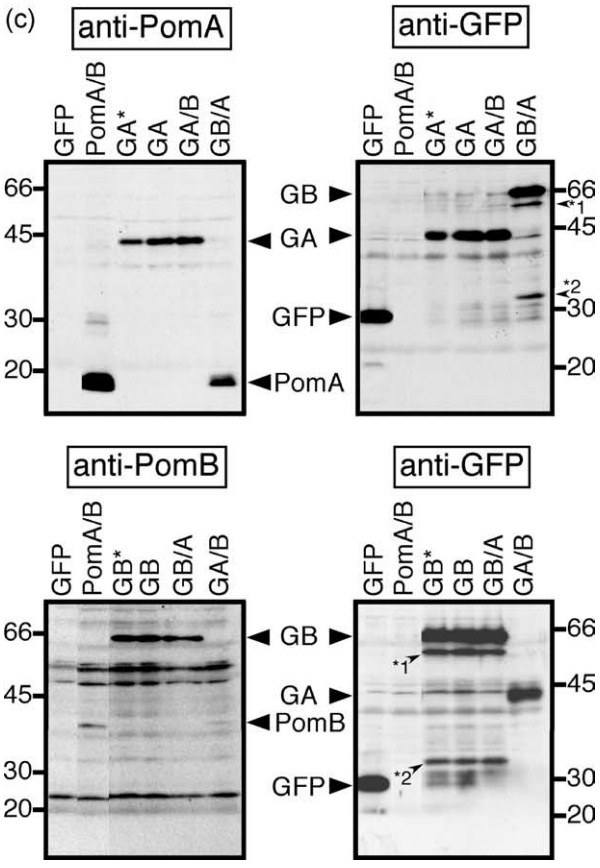


Figure 1. (a) Plasmid constructs for GFP-fused stator genes. All *gfp-stator* genes are under the control of the *araBAD* promoter in pBAD33. Dark gray and light gray boxes indicate the regions of *gfp* and *pomA* or *pomB*, respectively. *BΔC* indicates the *pomB* gene in which the C-terminal domain is deleted (PomBΔC). GFP and PomA are linked by five glycine residues (denoted as g5), and GFP and PomB are fused without a linker. A hexahistidine tag (his6) was fused to the N terminus of each GFP-fused protein. The checked box following the *gfp-pomB* gene indicates the transcriptional terminator. The plasmids were designed to encode GFP-PomA (GA), GFP-PomA and PomB (GA/B), GFP-PomA and PomBΔC (GA/BΔC), GFP-PomB (GB), GFP-PomB and PomA (GB/A), and GFP-PomBΔC and PomA (GBΔC/A). (b) Swarming behavior in cells expressing GFP-fused stator genes. NMB190 (Δ*pomA*) cells producing GFP-PomA (GA), NMB192 (Δ*pomB*) cells producing GFP-PomB (GB), and NMB191 (Δ*pomAB*) cells producing GFP or PomA and PomB (PomA/PomB) were grown in 0.25% (w/v) soft agar medium containing 0.01% (w/v) arabinose at 30 °C for seven hours. (c) Immunoblot of GFP-fused stator proteins in whole cell extracts. Proteins were detected with an anti-PomA1312 antibody, anti-PomB93 antibody, or anti-GFP antibody. GA* indicates GFP-PomA produced in NMB190 cells and GB* indicates GFP-PomB produced in NMB192 cells, and all other proteins (GFP, GFP-fused stator proteins, PomA, and PomB) were produced in NMB191 cells. Cells were grown at 30 °C for four hours in medium containing 0.01% arabinose. Arrowheads marked as *1 and *2 indicate degraded products detected by anti-GFP antibody. Molecular mass values (kDa) are shown on the left or right side of these panels.

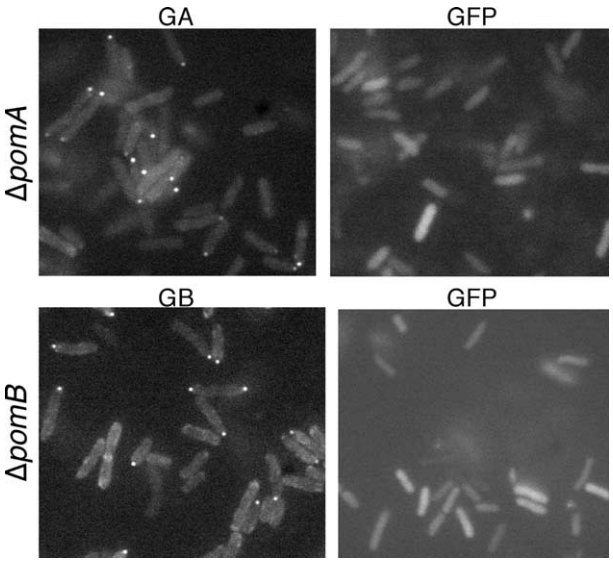


Figure 2. Subcellular localization of GFP-fused stator proteins. Cells producing GFP-fused stator proteins (abbreviated as in Figure 1) or GFP were grown at 30 °C for four hours in medium containing 0.006% (w/v) arabinose and were observed under a fluorescence microscope as described in Materials and Methods.

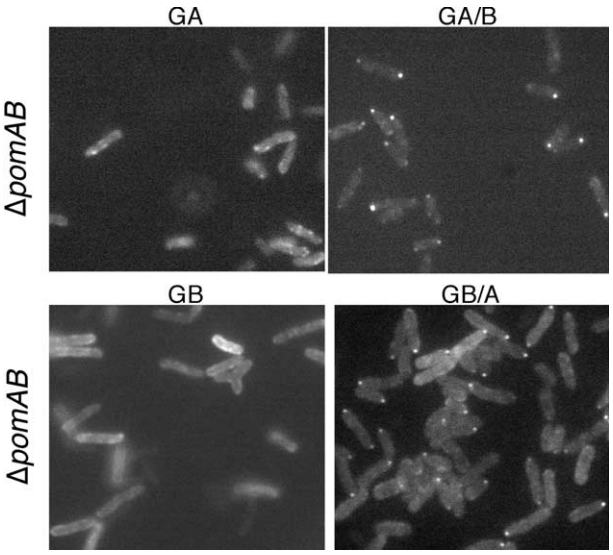


Figure 3. Subcellular localization of GFP-fused stator proteins (abbreviated as in Figure 1) in a *pomA/pomB* deletion strain. Cells were grown at 30 °C for four hours in medium containing 0.006% (w/v) arabinose.

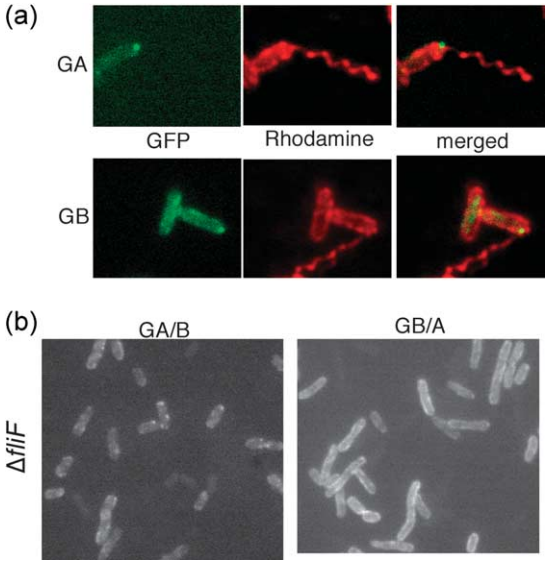


Figure 4. Polar localization of stator proteins dependent on flagellar assembly. (a) Co-localization of the polar flagellum and GFP-fused stator proteins. GFP-PomA was produced in NMB190 (Δ*pomA*) cells (upper panels), and GFP-PomB was produced in NMB192 (Δ*pomB*) cells (lower panels). Cells were treated with anti-polar flagellar antibody and rhodamine-conjugated anti-rabbit IgG antibody and the fluorescence of rhodamine and GFP was observed as described in Materials and Methods. (b) Subcellular localization of GFP-fused stator proteins in a non-flagellated strain. NMB196 (Δ*fliF*) cells producing GFP-PomA and wild-type PomB (left), and those producing GFP-PomB and wild-type PomA (right). Cells were grown at 30 °C for four hours in medium containing 0.006% (w/v) arabinose.

when GFP-PomA was produced alone in the *pomA/pomB* double mutant, fluorescent dots were not detected at the cell poles, but some fluorescent clusters were observed throughout the cell (Figure 3, GA). Similarly, GFP-PomB was produced alone in the *pomA/pomB* double mutant, fluorescent dots were not detected at the cell poles and fluorescence was diffused all over the cell (Figure 3, GB). When GFP-PomA or GFP-PomB was produced together with their wild-type partner subunit from the same plasmid in the *pomA/pomB* deletion strain, the fluorescent dots were localized at the cell poles (Figure 3, GA/B and GB/A).

By immunoblot analysis, we detected a similar amount of GFP-PomA protein in the *pomA* and *pomA/pomB* deletion strains (Figure 1(c), lanes GA* and GA). A similar amount of GFP-PomB protein was detected in the *pomB* and *pomA/pomB* deletion strains (Figure 1(c), lanes GB* and GB). These results suggest that the polar localization of PomA and PomB requires their partner subunit.

Polar localization of stator depends on flagellar assembly

To investigate whether the fluorescent dot colocalizes with flagellar structures, the polar flagellum was observed using a primary antibody against the polar flagellum and a secondary anti-rabbit IgG conjugated with rhodamine. As shown in Figure 4(a), the fluorescent dots from GFP-fused stator proteins and the polar flagellum were observed at the same pole. This was true for 84% (GFP-PomA) and 77% (GFP-PomB), respectively, of all cells that exhibited a fluorescent spot. Only a small percentage possessed either a fluorescent dot at a pole without a polar flagellum, or a fluorescent dot localized opposite to the flagellar pole (Table 1).

To investigate whether localization of GFP-PomA or GFP-PomB depends on the presence of the polar flagellum, each of the GFP-fused stator proteins was produced in a non-flagellated strain, NMB196, which is the *fliF* deletion mutant.[37] FliF is a component of the MS-ring, which is thought to be the first assembled component in the flagellar construction.[9] It has been shown that flagellar genes are regulated hierarchically, and that the late products of flagellar synthesis are not produced in basal body mutants.[30–34] To rule out the

Table 1. Cells containing fluorescent dots at the flagellated poles (%)

	Flagellated pole	Non-flagellated pole	Non-flagellated cells
GA(Δ*pomA*)	84	0	16
GB(Δ*pomB*)	77	5	18

The percentage of cells containing a fluorescent dot co-localizing with the polar flagellum, localizing to the opposite pole, or either pole of non-flagellated cells is given as a fraction of all cells with a fluorescent dot.

possibility that PomA and PomB proteins (encoded as part of the late class of genes) were not produced in the *fliF* mutant, either GFP-PomA and wild-type PomB, or GFP-PomB and wild-type PomA were produced from a single plasmid under control of the arabinose promoter. In this non-flagellated strain (the *fliF* mutant), GFP-PomA was localized diffusely and appeared to form some clusters throughout the cell, and GFP-PomB was diffuse all over the cell. Neither GFP-PomA nor GFP-PomB localized at cell poles (Figure 4(b)). Immunoblot analysis revealed similar amounts of GFP-PomA or GFP-PomB protein as well as their wild-type partners in the *pomA/pomB* deletion and non-flagellated strains (data not shown). These results indicate that PomA and PomB are being produced in a *fliF* mutant, and their polar localization of the stator proteins requires the presence of the polar flagellum or at least some of its components.

Inhibition of the motor rotation by the GFP-fused stator proteins

GFP-fused stator proteins were produced with their partner subunit in VIO5 cells, which have a wild-type polar flagellum. In a soft agar plate containing no arabinose, strains harboring a plasmid encoding either GFP-PomA and PomB, or GFP-PomB and PomA swarmed as well as the cells harboring GFP alone (Figure 5(a), left). On the other hand, in a soft agar plate containing 0.02% arabinose, the swarming ability of the cells producing GFP-fused stator proteins was reduced in comparison with that of the cells producing GFP alone (Figure 5(a), right). The motile fraction of VIO5 cells producing GFP-PomA and PomB grown in liquid medium containing 0.02% arabinose was 39% *versus* 66% without arabinose. The reduced swarming ability, which probably derived from the defect of motor rotation, suggests that a fraction of GFP-fused proteins had been incorporated into the stator complex.

In most VIO5 cells producing GFP-PomA and PomB with 0.006% arabinose induction, the fluorescent signal was diffuse throughout the cell, although a small number of cells had fluorescent dots at the cell poles (Figure 5(b), GA/B). Similarly, in VIO5 cells producing GFP-PomB and PomA, the fluorescence was diffuse in the entire cell and polar dots were observed rarely (Figure 5(b), GB/A). In medium containing 0.02% arabinose, fluorescent dots were not observed in VIO5 cells producing GFP-fused stator proteins; rather, strong fluorescence was observed all over the cell (data not shown). This high background expression may have obscured the fluorescent dots at the cell pole.

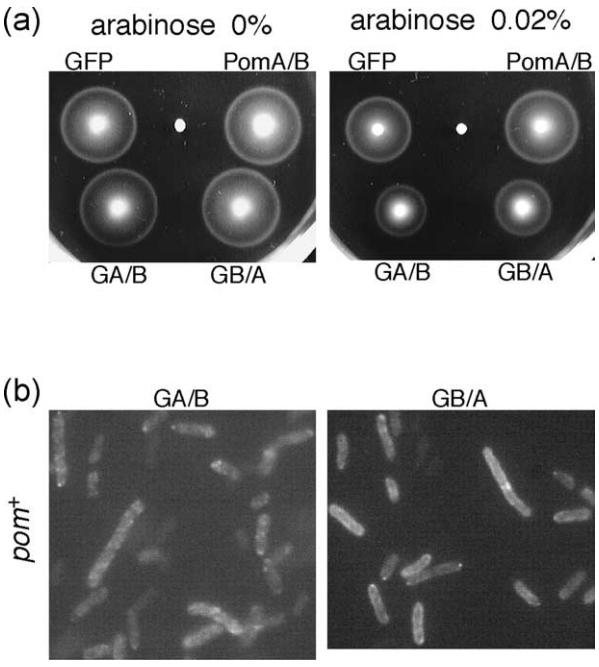


Figure 5. Effects of GFP-PomA and GFP-PomB on swarming and subcellular localization in wild-type cells. (a) VIO5 cells (pom^+) harboring the plasmid encoding GFP-fused stator proteins (abbreviated as in Figure 1) were swarmed at 30 °C for six hours on soft agar plates containing 0% or 0.02% (w/v) arabinose. Nonmotile NMB191 cells producing GFP were inoculated in the center of the plates. (b) Subcellular localization of GFP-fused stator proteins (abbreviated as in Figure 1) in wild-type cells. Cells were grown at 30 °C for four hours in medium containing 0.006% (w/v) arabinose.

Polar localization of stator proteins requires the C-terminal domain of PomB

MotB of *E. coli* and PomB of *V. alginolyticus* have peptidoglycan-binding motifs in their C-terminal domains, and it has been proposed that the stator is fixed to the cell wall *via* this motif.[18,19] It has been shown that a PomB protein missing this C-terminal domain, designated PomBΔC, can form a complex with PomA, but it is not functional and does not have a dominant effect on the wild-type strain.[38] In order to investigate the effect of the C-terminal domain of PomB on the polar localization of stator proteins, either GFP-PomA and PomBΔC, or

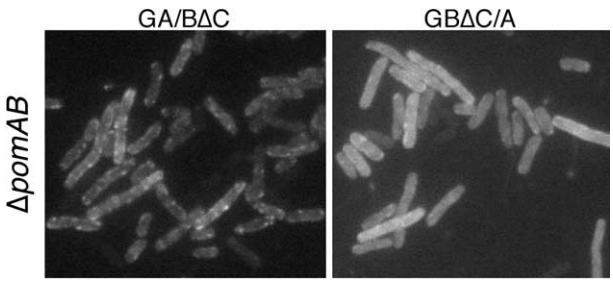


Figure 6. Effect of the C-terminal deletion of PomB on the polar localization of GFP-fused stator proteins. Cells producing GFP-PomA together with PomBΔC (left), and wild-type PomA together with GFP-PomBΔC (right) were grown at 30 °C for four hours in medium containing (GA/BΔC) 0.01% (w/v) arabinose or(GBΔC/A) 0.006% (w/v) arabinose.

GFP-PomBΔC and PomA were produced in the *pomA/pomB* deletion strain. In both cases, the GFP signals were diffuse and appeared to form some clusters in the entire cell region, without localizing to the cell poles (Figure 6). This suggests that the C-terminal region of PomB is necessary for the polar localization of the stator complex.

Next, we confirmed the interaction between GFP-PomA and wild-type PomB or PomBΔC, and between wild-type PomA and GFP-PomB or GFP-PomBΔC. Six histidine residues were added to the N termini of each GFP-fused stator protein. After isolation of the membrane fraction and solubilization of the GFP-fused stator protein with Chaps {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, the His-tagged GFP-fused stator proteins were bound to Ni-NTA agarose and eluted. Both wild-type PomB and PomBΔC co-eluted with GFP-PomA (Figure 7, left panels). The signal intensities of wild-type and deletion PomB were similar. Wild-type PomA co-eluted with GFP-PomBΔC as well as GFP-PomB (Figure 7, right panels). These results indicate that GFP-PomA and PomB or PomBΔC, and PomA and GFP-PomB or GFP-PomBΔC interact with each other and probably can form complexes in the cytoplasmic membrane. Some degraded products were detected by anti-PomA and anti-PomB antibodies (arrowheads marked as *1 and *2), and degradation products corresponding to monomeric PomA and PomBΔC were detected (arrowheads marked as *3 and *4).

Discussion

The bacterial flagella contain a rotary motor driven by the electrochemical potential of ions. Motor rotation is generated by the interaction of the stator part with the rotor part, thus converting the energy of ion flux into mechanical power. A circular array of particles surrounding a larger particle was observed in freeze-fracture images of cytoplasmic membrane in *E. coli*; these particles are thought to represent stator complexes composed of MotA and MotB.[29] However, the stator parts or Mot proteins were not associated with the rotor structure even under the mild condition used to isolate the C-ring with the rotor structure.[39] PomA and PomB, which are the stator components of the sodium-driven flagellar motor of *V. alginolyticus*, were likewise found not to be associated with the purified rotor structure (H. Terashima, T.Y. & M.H., unpublished results).

In the present study, to investigate the assembly of the stator proteins PomA and PomB, we constructed GFP-fused PomA and PomB proteins to observe the proteins in living cells. GFP-PomB rescued the motility of the *pomB* deletion mutant, albeit weakly, whereas GFP-PomA did not rescue the motility of the *pomA* deletion mutant at all (Figure 1(b)). Stator proteins (PomA and PomB in *V. alginolyticus* or MotA and MotB in *E. coli*) are thought to form a complex containing four molecules of subunit A and two molecules of subunit B,[21–23] which then interacts electrostatically with FliG, one of the components of the rotor structure, *via* the cytoplasmic surface of subunit A.[25] Given this stoichiometry of the motor complex, the GFP-PomA/PomB complex and the PomA/GFP-PomB complex should contain four and two GFP molecules, respectively, in their cytoplasmic surfaces. These GFP-fused stator complexes probably have little or no ability to interact with the rotor

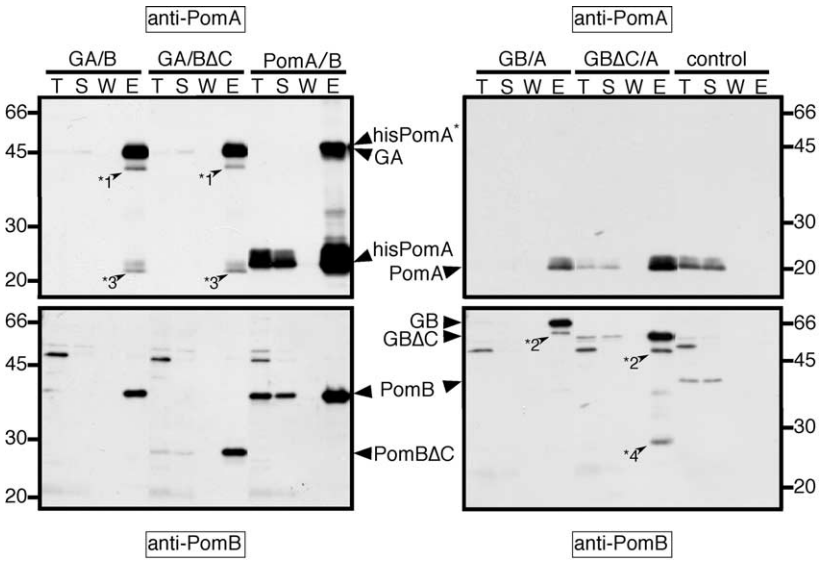


Figure 7. Western blots showing the interaction between PomA and PomB. The GFP-fused stator complex was prepared from NMB191 cells as described in Materials and Methods. PomA/B indicates his-tagged PomA and wild-type PomB, and control indicates wild-type PomA and PomB produced in NMB191 cells. PomA and PomB variants were detected with anti-PomA1312 and anti-PomB93 antibodies. The 45 kDa band marked by an arrowhead with his-PomA* in the lane of PomA/B is believed to be the dimer of his-PomA as observed previously.[50] The 45 kDa bands detected in the lanes of GA/B and GA/BΔC are GFP-PomA marked as GA with an arrowhead. The arrowheads marked as GB and GBΔC indicate GFP-PomB and GFP-PomBΔC, respectively. T, total membrane; S, solubilized fraction; W, washed fraction; E, eluted fraction. Arrowheads marked with *1 and *2 indicate degradation products that are 7 kDa shorter than the full-length GFP-fused stator proteins, and arrowheads marked with *3 and *4 indicate monomer-sized degradation products from GFP-PomA and GFP-PomBΔC.

because of steric interference by the GFP molecules on the cytoplasmic surface of the stator.

In the present study, we found that GFP-PomA and GFP-PomB proteins were localized at the cell poles (Figure 2). The fluorescent dot co-localized with the base of polar flagellum, and this localization was dependent on the presence of the polar flagellum. From these lines of evidence, we speculate that the fluorescent dots probably represent the stator complex surrounding the rotor complex. It has been postulated that eight to 12 stator units consisting of Pom or Mot complexes surround each rotor structure.[29,35,36] If this is true, the polar-localized fluorescent dot may correspond to the fluorescence from about 40 (GFP-PomA) or 20 (GFP-PomB) GFP molecules. These GFP-fused stator proteins conferred an inhibition for the motor function of wild-type cells when the expression levels were increased by an inducer. By inducing the expression of GFP-fused stator proteins with a low concentration of arabinose (0.006%), we could observe fluorescent dots localized at the poles of wild-type cells. However, the number of the wild-type cells that had polar-localized fluorescent dots was much smaller than the total number of *pomA/pomB* deletion cells that had polar-localized fluorescent dots, and the fluorescence was diffuse throughout the cell in the wild-type. The wild-type PomA/PomB complexes seemed to occupy most of the flagellar motors in wild-type cells. The GFP-fused stator complexes are probably much less able than the wild-type stator complexes to incorporate themselves into the motor. Polar localization of the GFP-fused stator proteins required the presence of their partner subunit. GFP-PomA or GFP-PomB without PomB or PomA, respectively, did not localize to the cell poles, and the resulting fluorescence was diffuse throughout the cell; GFP-PomA and GFP-PomB were likely spread out across the entire cell membrane. We speculate that stator proteins are incorporated into the flagellar motor as a unit of the stator complex.

Prior reports have suggested that MotA and MotB form a stable pre-assembly complex and incorporate themselves into the flagellar motor through electrostatic interactions between a rotor protein and MotA, fixing the stator complex to the cell wall through the C-terminal domain of MotB.[40] We demonstrated that the polar localization of GFP-fused stator proteins requires the C-terminal domain of PomB, which contains a putative peptidoglycan-binding motif. This domain is not necessary for association of PomA and PomB, but is essential for flagellar motor function, and the C-terminal-deleted PomB protein does not have a dominant effect on the wild-type strain.[38] Thus, it is likely that the stator complex lacking the C-terminal domain of PomB is not incorporated into the flagellar motor. The PomA/PomB complexes are probably fixed to the cell wall or the other components *via* the C-terminal domain of PomB and stably remain near the rotor structures. In *Vibrio* species, MotX and MotY are the essential components for the flagellar rotation. MotY has a peptidoglycan-binding motif similar to PomB,[41] and MotX is thought to form a complex in the outer membrane with MotY.[42,43] We showed recently that MotX interacts with the PomA/PomB complex.[44] In a *motX* or *motY* mutant strain of *V. alginolyticus*, the number of the cells that had polar localized GFP-fused stator proteins was reduced; furthermore, the GFP-fused stator proteins were little localized in the *pomA/pomB/motX/motY* deletion strain of *V. cholerae*, the motility of which is recovered by the genes of *V. alginolyticus* (unpublished results). MotX and MotY might be involved in the stabilization, localization, or incorporation of the PomA/PomB complex.

Several models for the polar localization of membrane proteins have been proposed.[45,46] We have shown that the chemoreceptors of *E. coli* form a cluster at the cell pole with some Che proteins.[47,48] The chemoreceptors are localized on the lateral cellular surface in a helical pattern similar to the Sec machinery (D. Shiomi, M. Yoshimoto & I. Kawagishi, personal communication). The chemoreceptors might be inserted into the cytoplasmic membrane by the Sec complexes and be directed to the cell pole. Alternatively, IcsA, an actin-nucleating protein of *Shigella*, is targeted directly to the membrane of the old pole, and laterally diffused IcsA molecules are digested by the action of a specific protease.[49] In this study, we found that localization of stator proteins to a cell pole in *V. alginolyticus* was dependent on the presence of the polar flagellum, but also that the GFP-fused stator proteins appeared to be inserted into the cytoplasmic membrane even in the flagella-defective strain. The stator proteins are produced after the completion of hook–basal body assembly or rotor construction,[31–34] and can be incorporated into the flagellar motor even after the whole flagellum is assembled.[35,36] This suggests that the stator proteins should be able to recognize the flagellar structure. The stator complex might be inserted into the cytoplasmic membrane at first and move through the membrane randomly. Finally, the stator complex might be incorporated into the flagellar motor through its interaction with the rotor.

Materials and Methods

Bacterial strains, growth conditions, and media

Bacterial strains are listed in Table 2. *V. alginolyticus* was cultured at 30 °C in VC medium (0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.4% (w/v) K_2HPO_4, 3% (w/v) NaCl, 0.2% (w/v) glucose) or in VPG500 medium (1% (w/v) polypeptone, 0.4% (w/v) K_2HPO_4, 500 mM NaCl, 0.5% (w/v) glycerol). *E. coli* was cultured in LB broth (1% (w/v) tryptone peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl). Chloramphenicol

Table 2. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
V. alginolyticus		
VIO5	Rif^r, Pof$^+$, Laf$^-$	41
NMB190	Rif^r, Pof$^+$, Laf$^-$ Δ*pomA*	52
NMB191	Rif^r, Pof$^+$, Laf$^-$ Δ*pomAB*	50
NMB192	Rif^r, Pof$^+$, Laf$^-$ Δ*pomB*	26
NMB196	Rif^r, Pof$^-$, Laf$^-$ Δ*fliF*	37
Plasmid		
pSU21	Cm^r, P*lac*, *lacZα*	53
pSU41	Km^r, P*lac*, *lacZα*	53
pBAD24	Ap^r, P*BAD*	54
pBAD33	Cm^r, P*BAD*	54
pTrcHisB	Ap^r, P*trc*	Invitrogen
pYA301	*pomA* in pSU41	51
pMK101	*pomA* in pSU21	55
pYA303	*pomA* and *pomB* in pSU41	51
pSK603	*pomB* in pSU41	51
pTY200	*his$_6$-gfp* in pBAD33	37
pHFGA	*his$_6$-gfp-pomA* in pBAD33	This work
pJN227	*his$_6$-gfp-pomB* in pBAD33	This work
pJN152	*his$_6$-pomA and pomB* in pSU41	This work
pHFGAB	*his$_6$-gfp-pomA* and *pomB* in pBAD33	This work
pHFGAB2	*his$_6$-gfp-pomA* and *pomBΔC* in pBAD33	This work
pHFGBA2	*his$_6$-gfp-pomB* and *pomA* in pBAD33	This work
pHFGBA3	*his$_6$-gfp-pomBΔC* and *pomA* in pBAD33	This work
pHFA1	*pomA* in pBAD24	This work
pHFA2	*pomA* in pBAD33	This work
pHFAB	*pomA* and *pomB* in pBAD33	This work

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

was added to a final concentration of 2.5 μg/ml for *V. alginolyticus* and 25 μg/ml for *E. coli*. For swarming assay, overnight cultures were spotted onto 0.25% agar-VPG500 plates containing 2.5 μg/ml of chloramphenicol and appropriate concentrations of arabinose.

Plasmids

Plasmids and primers are listed in Tables 2 and 3, respectively, and the plasmid constructs are shown in Figure 1(a). Plasmids carrying motor genes under the control of the *araBAD* promoter are pBAD33-based plasmids. Fragments encoding *pomA* were amplified by PCR using the sense primer *Bsr*GI-5G-PomA and the antisense primer PomA-XbaI, and this fragment was inserted into the *Bsr*GI and XbaI sites of pTY200ΔH (the HindIII site in the multi-cloning site was deleted); this plasmid was designated pHFGA. pHFA1 was constructed by inserting the EcoRI/XbaI fragment from pMK101 (containing *pomA* gene) into the EcoRI/XbaI site of pBAD24. The EcoRV/SalI fragment from pHFA1-was inserted into the EcoRV/SalI sites of pBAD33ΔH (the HindIII site in the multi-cloning site was deleted); this plasmid was designated pHFA2. A PCR fragment of *pomB*, which was amplified using pYA303 as a template, a universal primer, and the antisense-primer PomB-*Xba*I, was inserted into the HindIII (located in the open reading frame of *pomA*) and XbaI sites of pHFGA and pHFA1, and the resulting plasmids were designated pHFGAB and pHFAB, respectively. The XbaI/SacI fragment from pSK603 was inserted into the NheI/SacI sites of pTrcHisB to create the plasmid pTricHisB-PomB. A DNA fragment containing the *pomB* open reading frame was prepared by PCR using pTricHisB-PomB as a template together with the sense primer BsrGI-PomB and the pTrc-reverse primer as the antisense primer. The resulting fragment was cloned into pTY200 digested with BsrGI and HindIII, and the resulting plasmid was designated pJN227 and carried *gfp-pomB*. The plasmid pHFGBA2 carrying *gfp-pomB* and *pomA* were constructed as follows. To remove the transcriptional terminator following the *pomB* coding region, a DNA fragment including the *pomB* was prepared by PCR using pJN227 as a template together with the sense primer *Bsr*GI-PomB and the antisense primer PomB-SacI; the resulting fragment was re-cloned into the *Bsr*GI and SacI sites of pJN227. The BamHI-digested fragment containing of *pomA* gene of pMK101

Table 3. Primers

Primer	Sequence
BsrGI-5G-PomA	5′-GCTGTACAAGGGAGG CGGAGGCGGA ATGGATTTAGCAACC CTATTAG-3′
PomA-XbaI	5′-GCTCTAGATTACTCG TCAATCTCAAGGGC-3′
PomB-XbaI	5′-GCTCTAGAGCCTAGT TATTGAATTACCGGC-3′
BsrGI-PomB	5′-GTCTGTACAAGGATG ATGAAGATAACAAAT GCG-3′
PomB-SacI	5′-AGCGAGCTCGCCTAG TTATTGAATTACCGG C-3′
PomBΔC-XbaI	5′-GCTCTAGATTATGCA CCTTTTTCACGCATA C-3′
PomBΔC-SacI	5′-AGCGAGCTCTTATGC ACCTTTTTCACGCAT AC-3′

was inserted into the BglII site of the resultant plasmid; this new plasmid was designated pHFGBA2. A *pomB* fragment missing the C-terminal domain was amplified using pYA303 as a template, the universal reverse primer, and the PomB-ΔC-XbaI primer, and was cloned into the HindIII and XbaI sites of pHFGAB; the resulting plasmid was designated pHFGAB2. A *pomB* fragment missing the C-terminal domain was amplified using pJN227 as the template together with the primers BsrGI-PomB and PomB-ΔC-SacI, and the resulting fragment was cloned into the BsrGI and SacI sites of pHFGBA2; this plasmid was designated pHFGBA3.

Detection of PomA and PomB proteins in *Vibrio* cells

Vibrio cells harboring plasmids encoding the stator proteins were cultured at 30 °C for four hours in VPG500 medium in the presence of arabinose. The cells were harvested by centrifugation and suspended in distilled water to an A_{660} of 10. The same volume of SDS loading buffer was added to the cell suspensions and boiled at 100 °C for five minutes. The proteins were separated by SDS-PAGE and immunoblotting was performed using an anti-PomA antibody (PomA1312) and anti-PomB antibody (PomB93) as described.[50]

Detection of interaction between PomA and PomB

Vibrio cells harboring the plasmids coding the stator proteins were cultured at 30 °C for four hours in VC medium containing 0.02% arabinose. Six histidine residues were added to the N terminus of each GFP-fused stator protein. Cells were harvested by centrifugation, washed with V buffer (25 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10 mM $MgCl_2$), and resuspended in 20TMPD (20 mM Tris–HCl (pH 8.0), 5 mM $MgSO_4$, 0.5 mM PMSF, 1 mM DTT) containing 5 μg/ml of DNase I. The cells were disrupted by sonication. After unbroken cells were removed by low-speed centrifugation (10,000*g* for five minutes at 4 °C), the membrane was recovered by ultracentrifugation (150,000*g* for one hour at 4 °C). The membrane pellet was homogenized with 20TPD (20 mM Tris–HCl (pH 8.0), 0.5 mM PMSF, 1 mM DTT) containing 20% (w/v) glycerol, and stored at −80 °C. The membrane homogenate was mixed with 20TNPD (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5 mM PMSF, 1 mM DTT) containing 10% (w/v) glycerol, 5 mM imidazole, and 2.5% (w/v) Chaps to a final total protein concentration of 5 mg/ml and incubated for ten minutes on ice. The solubilized samples were centrifuged at 150,000*g* for 30 minutes at 4 °C. Ni-NTA agarose was added to the supernatant, and the mixture was incubated for one hour at 4 °C. The Ni-NTA agarose was washed by centrifugation three times with 20 TNPD containing 10% (w/v) glycerol, 5 mM imidazole, and 1% (w/v) Chaps at 4 °C. The associated proteins were eluted by 20TNPD containing 10% (w/v) glycerol, 200 mM imidazole, and 1% (w/v) Chaps at 4 °C. The same volume of SDS loading buffer was added to the eluted solutions and boiled at 100 °C for five minutes. The proteins were separated by SDS-PAGE and immunoblotting was performed using anti-PomA antibody (PomA1312) and anti-PomB antibody (PomB93) as above.

Observation of the subcellular localization of GFP-PomA and GFP-PomB

Vibrio cells harboring plasmids encoding GFP-fused proteins were grown at 30 °C for four hours in VPG500 medium containing arabinose. A sample (1 ml) of cultured cells were harvested by centrifugation and suspended in 200 μl of TMN500 (50 mM Tris–HCl (pH 7.5), 5 mM MgCl, 5 mM glucose, 500 mM NaCl). A small aliquot of the cell suspension was spotted onto a glass slide, and cells were fixed on the slide with an equal volume of 0.5% agarose dissolved in TMN500. Cells were observed by fluorescence microscopy (Olympus. BX50). The images were recorded and processed using a digital camera (Hamamatsu Photonics, C4742-95) and imaging software (Hamamatsu Photonics, AQUA-Lite, Ver. 1.0). For observation of the polar flagellum, the cell suspension was placed on the slide covered with a glass cover-slip with spacers, washed with TMN500 twice, and the anti-polar flagellum antibody and anti-rabbit IgG conjugated with rhodamine were applied. After washing with TMN500, cells attached to the cover-slip were observed.

Measurement of swimming speed

Cells grown as described above were harvested and suspended in TMN500. The cell suspensions were diluted 100-fold into TMN500 containing 20 mM serine, which was used to suppress changes in swimming direction. Cell motility was observed under a dark-field microscope and recorded on videotape. Swimming speed was determined as described.[51] The average swimming speed was obtained by measuring at least 20 swimming tracks. The swimming fraction was measured from the same videotape.

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References

1. Macnab, R. M. (2003). How bacteria assemble flagella. *Annu. Rev. Microbiol.* **57**, 77–100.
2. McCarter, L. L. (2001). Polar flagellar motility of the *Vibrionaceae*. *Microbiol. Mol. Biol. Rev.* **65**, 445–462.
3. Yorimitsu, T. & Homma, M. (2001). Na^+-driven flagellar motor of *Vibrio*. *Biochim. Biophys. Acta*, **1505**, 82–93.
4. Manson, M. D., Tedesco, P., Berg, H. C., Harold, F. M. & van der Drift, C. (1977). A proton motive force drives bacterial flagella. *Proc. Natl Acad. Sci. USA*, **74**, 3060–3064.
5. Hirota, N., Kitada, M. & Imae, Y. (1981). Flagellar motors of alkalophilic *Bacillus* are powered by an electrochemical potential gradient of Na^+. *FEBS Letters*, **132**, 278–280.
6. Chernyak, B. V., Dibrov, P. A., Glagolev, A. N., Sherman, M. Y. & Skulachev, V. P. (1984). A novel

type of energetics in a marine alkali-tolerant bacterium: Na^+-driven motility and sodium cycle. *FEBS Letters*, **164**, 38–42.
7. Atsumi, T., McCarter, L. & Imae, Y. (1992). Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. *Nature*, **355**, 182–184.
8. Ito, M., Hicks, D. B., Henkin, T. M., Guffanti, A. A., Powers, B. D., Zvi, L. *et al*. (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.
9. Kubori, T., Shimamoto, N., Yamaguchi, S., Namba, K. & Aizawa, S. (1992). Morphological pathway of flagellar assembly in *Salmonella typhimurium*. *J. Mol. Biol.* **226**, 433–446.
10. Fan, F., Ohnishi, K., Francis, N. R. & Macnab, R. M. (1997). The FliP and FliR proteins of *Salmonella typhimurium*, putative components of the type III flagellar export apparatus, are located in the flagellar basal body. *Mol. Microbiol.* **26**, 1035–1046.
11. Francis, N. R., Sosinsky, G. E., Thomas, D. & Derosier, D. J. (1994). Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. *J. Mol. Biol.* **235**, 1261–1270.
12. Katayama, E., Shiraishi, T., Oosawa, K., Baba, N. & Aizawa, S. (1996). Geometry of the flagellar motor in the cytoplasmic membrane of *Salmonella typhimurium* as determined by stereo-photogrammetry of quick-freeze deep-etch replica images. *J. Mol. Biol.* **255**, 458–475.
13. Kubori, T., Okumura, M., Kobayashi, N., Nakamura, D., Iwakura, M. & Aizawa, S. I. (1997). Purification and characterization of the flagellar hook-basal body complex of *Bacillus subtilis*. *Mol. Microbiol.* **24**, 399–410.
14. Minamino, T. & Macnab, R. M. (1999). Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *J. Bacteriol.* **181**, 1388–1394.
15. Macnab, R. (1996). Flagella and motility. In *Escherichia coli* and Salmonella (Neidhardt, F. C., ed.), pp. 123–145, American Society for Microbiology, Washington, DC.
16. Namba, K., Yamashita, I. & Vonderviszt, F. (1989). Structure of the core and central channel of bacterial flagella. *Nature*, **342**, 648–654.
17. Homma, M., Komeda, Y., Iino, T. & Macnab, R. M. (1987). The *flaFIX* gene product of *Salmonella typhimurium* is a flagellar basal body component with a signal peptide for export. *J. Bacteriol.* **169**, 1493–1498.
18. Asai, Y., Kojima, S., Kato, H., Nishioka, N., Kawagishi, I. & Homma, M. (1997). Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium. *J. Bacteriol.* **179**, 5104–5110.
19. De Mot, R. & Vanderleyden, J. (1994). The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both gram-positive and gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. *Mol. Microbiol.* **12**, 333–334.
20. Blair, D. F. & Berg, H. C. (1990). The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor. *Cell*, **60**, 439–449.
21. Sato, K. & Homma, M. (2000). Functional reconstitution of the Na^+-driven polar flagellar motor component of *Vibrio alginolyticus*. *J. Biol. Chem.* **275**, 5718–5722.
22. Yorimitsu, T., Kojima, M., Yakushi, T. & Homma, M. (2004). Multimeric structure of the PomA/PomB channel complex in the Na^+-driven flagellar motor of *Vibrio alginolyticus*. *J. Biochem.* **135**, 43–51.
23. Kojima, S. & Blair, D. F. (2004). Solubilization and purification of the MotA/MotB complex of *Escherichia coli*. *Biochemistry*, **43**, 26–34.
24. Kojima, S. & Blair, D. F. (2001). Conformational change in the stator of the bacterial flagellar motor. *Biochemistry*, **40**, 13041–13050.
25. Zhou, J. D., Lloyd, S. A. & Blair, D. F. (1998). Electrostatic interactions between rotor and stator in the bacterial flagellar motor. *Proc. Natl Acad. Sci. USA*, **95**, 6436–6441.
26. Asai, Y., Yakushi, T., Kawagishi, I. & Homma, M. (2003). Ion-coupling determinants of Na^+-driven and H^+-driven flagellar motors. *J. Mol. Biol.* **327**, 453–463.
27. Yorimitsu, T., Sowa, Y., Ishijima, A., Yakushi, T. & Homma, M. (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. Fukuoka, H., Yakushi, T. & Homma, M. (2004). Concerted effects of amino acid substitutions 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.
29. Khan, S., Dapice, M. & Reese, T. S. (1988). Effects of *mot* gene expression on the structure of the flagellar motor. *J. Mol. Biol.* **202**, 575–584.
30. Prouty, M. G., Correa, N. E. & Klose, K. E. (2001). The novel σ^{54}- and σ^{28}-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. *Mol. Microbiol.* **39**, 1595–1609.
31. Kim, Y. K. & McCarter, L. L. (2000). Analysis of the polar flagellar gene system of *Vibrio parahaemolyticus*. *J. Bacteriol.* **182**, 3693–3704.
32. Komeda, Y., Kutsukake, K. & Iino, T. (1980). Definition of additional flagellar genes in *Escherichia coli* K12. *Genetics*, **94**, 277–290.
33. Komeda, Y. (1986). Transcriptional control of flagellar genes in *Escherichia coli* K-12. *J. Bacteriol.* **168**, 1315–1318.
34. Kutsukake, K., Ohya, Y. & Iino, T. (1990). Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. *J. Bacteriol.* **172**, 741–747.
35. Blair, D. F. & Berg, H. C. (1988). Restoration of torque in defective flagellar motors. *Science*, **242**, 1678–1681.
36. Block, S. M. & Berg, H. C. (1984). Successive incorporation of force-generating units in the bacterial rotary motor. *Nature*, **309**, 470–472.
37. Yorimitsu, T., Mimaki, A., Yakushi, T. & Homma, M. (2003). The conserved charged residues of the C-terminal region of FliG, a rotor component of the Na^+-driven flagellar motor. *J. Mol. Biol.* **334**, 567–583.
38. Yakushi, T., Hattori, N. & Homma, M. (2005). Deletion analysis of the carboxyl-terminal region of the PomB component of the *Vibrio alginolyticus* polar flagellar motor. *J. Bacteriol.* **187**, 778–784.
39. Driks, A. & DeRosier, D. J. (1990). Additional structures associated with bacterial flagellar basal body. *J. Mol. Biol.* **211**, 669–672.
40. VanWay, S. M., Hosking, E. R., Braun, T. F. & Manson, M. D. (2000). Mot protein assembly into the bacterial flagellum: a model based on mutational analysis of the *motB* gene. *J. Mol. Biol.* **297**, 7–24.
41. Okunishi, I., Kawagishi, I. & 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.

42. Okabe, M., Yakushi, T., Asai, Y. & Homma, M. (2001). Cloning and characterization of *motX*, a *Vibrio alginolyticus* sodium-driven flagellar motor gene. *J. Biochem.* **130**, 879–884.
43. Okabe, M., Yakushi, T., Kojima, M. & Homma, M. (2002). MotX and MotY, specific components of the sodium-driven flagellar motor, colocalize to the outer membrane in *Vibrio alginolyticus*. *Mol. Microbiol.* **46**, 125–134.
44. Okabe, M., Yakushi, T. & 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.
45. Shapiro, L., McAdams, H. H. & Losick, R. (2002). Generating and exploiting polarity in bacteria. *Science*, **298**, 1942–1946.
46. Lybarger, S. R. & Maddock, J. R. (2001). Polarity in action: asymmetric protein localization in bacteria. *J. Bacteriol.* **183**, 3261–3267.
47. Homma, M., Shiomi, D., Homma, M. & Kawagishi, I. (2004). Attractant binding alters arrangement of chemoreceptor dimers within its cluster at a cell pole. *Proc. Natl Acad. Sci. USA*, **101**, 3462–3467.
48. Banno, S., Shiomi, D., Homma, M. & Kawagishi, I. (2004). Targeting of the chemotaxis methylesterase/deamidase CheB to the polar receptor-kinase cluster in an *Escherichia coli* cell. *Mol. Microbiol.* **53**, 1051–1063.
49. Robbins, J. R., Monack, D., McCallum, S. J., Vegas, A., Pham, E., Goldberg, M. B. & Theriot, J. A. (2001). The making of a gradient: IcsA (VirG) polarity in *Shigella flexneri*. *Mol. Microbiol.* **41**, 861–872.
50. Yorimitsu, T., Sato, K., Asai, Y., Kawagishi, I. & Homma, M. (1999). Functional interaction between PomA and PomB, the Na^+-driven flagellar motor components of *Vibrio alginolyticus*. *J. Bacteriol.* **181**, 5103–5106.
51. Kojima, S., Asai, Y., Atsumi, T., Kawagishi, I. & Homma, M. (1999). Na^+-driven flagellar motor resistant to phenamil, an amiloride analog, caused by mutations of putative channel components. *J. Mol. Biol.* **285**, 1537–1547.
52. Asai, Y., Kawagishi, I., Sockett, E. & Homma, M. (1999). Hybrid motor with the H^+-and Na^+-driven components can rotate *Vibrio* polar flagella by using sodium ions. *J. Bacteriol.* **181**, 6322–6338.
53. Bartolome, B., Jubete, Y., Martínez, E. & 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.
54. Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. *J. Bacteriol.* **177**, 4121–4130.
55. Kojima, S., Kuroda, M., Kawagishi, I. & Homma, M. (1999). Random mutagenesis of the pomA gene encoding the putative channel component of the Na^+-driven polar flagellar motor of *Vibrio alginolyticus*. *Microbiology*, **145**, 1759–1767.

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