Characterization of the periplasmic region of PomB, a Na\textsuperscript{+}-driven flagellar stator protein in \textit{Vibrio alginolyticus}

Na Li\textsuperscript{1,2}, Seiji Kojima\textsuperscript{2*} and Michio Homma\textsuperscript{2}

\textsuperscript{1}Division of Microbiology, Graduate School of Life Science, Northwest A&F University, Yanglin, Shaanxi, Yanglin 712100, China; \textsuperscript{2}Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

Running title: The periplasmic region of PomB

Keywords: flagellar motor, PomB, stator complex, assembly, motility

*Corresponding author:
Division of Biological Science, Graduate School of Science
Nagoya University, Furo-cho, Chikusa-ku
Nagoya 464-8602, Japan
Tel: +81 52 789 2992; Fax: +81 52 789 3001
E-mail: z47616a@cc.nagoya-u.ac.jp
ABSTRACT

The stator proteins PomA and PomB form a complex that couples Na\(^+\) influx to torque generation in the polar flagellar motor of *Vibrio alginolyticus*. This stator complex is anchored to an appropriate place around the rotor through a putative peptidoglycan-binding (PGB) domain in the periplasmic region of PomB (PomB\(_C\)).

To investigate the function of PomB\(_C\), a series of N-terminally truncated and in-frame deletions between the transmembrane (TM) segment and the PGB domain of PomB was constructed. A PomB\(_C\) fragment consisting of residues 135 to 315 (PomB\(_{C5}\)) formed a stable homodimer and significantly inhibited the motility of wild-type cells when over-expressed in the periplasm. An in-frame deletion (PomB\(_{AL}\)) of up to 80 residues retained function and its over-expression with PomA impaired cell growth. This inhibitory effect was suppressed by a mutation at the functionally critical Asp (D24N) in the TM segment of PomB, suggesting that a high Na\(^+\) influx through the mutant stator causes the growth impairment. The over-production of functional PomA/PomB\(_{AL}\) stators also reduced the motile fractions of the cells. That effect could be slightly relieved by a mutation (L168P) in the putative N-terminal \(\alpha\)-helix connecting to the PGB domain without affecting the growth inhibition, suggesting that a conformational change of the region including the PGB domain affects stator assembly. Our results reveal common features of the periplasmic region of PomB/MotB, and demonstrate that a flexible linker that contains a ‘plug’ segment is important for the control of Na\(^+\) influx through the stator.
complex as well as the stator assembly.

INTRODUCTION

The flagellum is used by most bacteria to swim in a liquid environment and to swarm on a surface. It consists of a flagellar filament, a hook and a basal body surrounded by multiple stator complexes (3, 22). The filament is a helical structure that serves as a screw propeller to change the rotary motion of the motor into thrust. The hook is a short tubular structure which serves as a universal joint to smoothly transmit the torque produced by the motor to the filament. The basal body of Gram-negative bacteria is a supramolecular complex composed of the rod, MS, P, L and C-ring (23). The fuel for motor rotation is the transmembrane (TM) gradient of ions, H\(^+\) or Na\(^+\), which go through the stator complex and are coupled to the torque generation to rotate the flagellum (37). However, the mechanism by which the electrochemical potential energy is converted into mechanical work is still not well understood.

Genetic, biochemical and biophysical studies to characterize the mechanism of rotation of the flagellar motor in *Escherichia coli* and in *Salmonella enterica* serovar Typhimurium have been actively carried out. In those motors, only 5 proteins, MotA, MotB, FliG, FliM and FliN, are in charge of the torque generation of the motor (4). The soluble rotor proteins FliG, FliM and FliN, which form the C-ring structure in the rotor (9, 39), are also responsible for switching the direction
of motor rotation (43, 44). The two membrane proteins, MotA and MotB, which
form the stator complex in an A₄B₂ stoichiometry, serve as a proton channel and they
couple proton flux to torque generation (6, 8, 17, 18, 34). In the fully functioning
motor, about a dozen stator complexes are assembled around the rotor to generate
torque (31).

The marine bacterium *Vibrio alginolyticus* has a Na⁺-driven single polar
flagellum and its motor requires PomA, PomB, MotX and MotY for torque
generation (1, 27, 30). The stator proteins PomA and PomB are homologs of MotA
and MotB respectively, and form the PomA₄PomB₂ complex that serves as a Na⁺
channel (32, 33, 45). PomB contains 315 amino acids and has a predicted single TM
segment (residues 15-39) at its N-terminus and a large C-terminal periplasmic
region (Fig. 1). Asp24 in the TM segment (Asp32 in MotB of *E. coli*) is absolutely
conserved in all PomB/MotB orthologs and is thought to be the Na⁺-binding site
which is critical for motor function (38). Recently, Na⁺ binding to the Asp24 residue
was directly shown by ATR-FTIR spectroscopy, providing the first evidence for the
interaction between the stator and its coupling ion (35). The point mutations D24N
and D24C do not confer any motility and reduce the polar localization of the
PomA/PomB stator complex, suggesting that Na⁺ binding at this critical aspartate
residue is required for stator incorporation into the motor (10). Our further analyses
revealed that the PomA/PomB stator assembly is a dynamic and reversible process
which depends on the Na⁺ flux through the motor (10) and also requires the T ring
structure of the basal body which is composed of MotX and MotY (36). Thus the assembly mechanism of the stator in Vibrio should be considered in those contexts, but our knowledge is still limited. The membrane localization of the PomA/PomB complex and of PomB alone was changed from the inner membrane to the intermediate fraction between the inner and outer membrane when co-expressed with MotX, suggesting an interaction between MotX and PomB (28).

When assembled, the stator is anchored at the PG layer near the rotor at the periplasmic region of the B subunit (PomB or MotB) (42). Residues 182-300 in the periplasmic region of PomB show sequence similarity to the OmpA-like proteins and includes a putative peptidoglycan-binding (PGB) motif, which is well conserved among proteins like OmpA and Pal (Fig. 1) (1). These kinds of proteins are outer membrane proteins that interact with the PG layer non-covalently (7, 15). The PGB motif of PomB (MotB) is believed to associate with the PG layer to anchor the PomA/PomB (MotA/MotB) complex around the rotor. Many mutations in this region of E. coli or Salmonella MotB completely abolish motility (5, 40). Recently, the crystal structure of the entire periplasmic region essential for motility (called the PEM) of Salmonella MotB was reported, and it appears to be a single-domain structure with a long N-terminal α-helix that protrudes from the domain (20). The core of the domain has a typical OmpA-like structure and shows considerable structural similarities to other PGB domains. Functional analyses based on the structural information revealed that the PGB domains must dimerize in order to form
a proton-conducting channel, and drastic conformational changes in the N-terminal portion of the PEM region are required for both the PG binding and the proton channel activation.

Based on the secondary structure prediction and the multiple-sequence alignment of PomB orthologs, it is plausible that the periplasmic region between residues 41 to 140 of PomB can serve as a tolerant flexible linker that connects the TM segment and the putative PEM region containing the PGB domain. Several in-frame deletions in the corresponding linker region (residues 51 to 110) of *E. coli* MotB showed that Δ51-60, Δ51-70, Δ51-80 and Δ51-90 caused growth inhibition but did not impair motility when over-produced. This indicated that deletion of the ‘plug’ segment (Fig. 1, residues P53 to P66 in *Salmonella* MotB, P52 to P65 in *E. coli* MotB), which consists of an amphipathic α-helix, induces a massive H⁺ influx through the stator channel that inhibits cell growth without affecting torque generation (13, 24). Δ51-100 of *Salmonella* MotB did not cause growth impairment and produced a functional and stable stator complex (20, 25). Interestingly, when mutations L119P or L119E in the N-terminal helix of the PEM region were introduced into the mutant MotB(Δ51-100), growth impairment was again observed without affecting motility, suggesting that the N-terminal region of PEM regulates the H⁺ translocation of the MotA/MotB stator complex (20). So far, the function and characteristics of the linker region and the PEM region of PomB are still unclear.

In this study, based on the sequence alignment of PomB with its orthologs and
the crystal structure of the PEM region of *Salmonella* MotB, we carried out mutational and biochemical studies on a series of N-terminally truncated and in-frame deletion mutants of PomB in *V. alginolyticus*. We found that the periplasmic PomB fragments form a homodimer, and that deletions in the PomB linker region cause growth impairment of the cells. We also investigated several mutations in the PEM region, measuring their effects on motility and growth.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and mutagenesis.** Bacterial strains and plasmids used in this study are listed in Table 1. The vector plasmid pTY57 was a kind gift from T. Yorimitsu, and the *pomB fliG* double deletion strain NMB200 was a kind gift from N. Nonoyama (Kojima, Nonoyama *et al.*, submitted). The design of the N-terminally truncated fragments of *V. alginolyticus* PomB and the site-directed mutagenesis were carried out according to a web-based secondary structure prediction program, PSIPRED (14), and the multiple-sequence alignment of PomB orthologs from various bacteria. Point mutations were generated using the QuikChange site-directed mutagenesis protocol (Stratagene). In-frame deletions of PomB were constructed according to the two-step PCR method reported previously (41) or the one-step PCR-based method. In the latter case, we designed a sense primer that anneals to the sequence encoding the downstream C-terminal ~7 residues at the
junction of the deletion, and an antisense primer that anneals to the sequence encoding the upstream N-terminal ~7 residues at the junction. The nicked circular strands with the desired deletions were generated by PCR using these two primers, and their 5’-end was phosphorylated by polynucleotide kinase (Takara), then ligated to produce the designed in-frame deletion constructs. The addition of the MotY leader sequence (residues 1-22) to the N-terminus of each PomB fragment was carried out by a two-step PCR method using the plasmid pAS101 (21) as a template of motY as described previously (16). Each mutation and plasmid construction was confirmed by DNA sequencing with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

**Culture of cells and motility assay.** *V. alginolyticus* cells were cultured at 30°C in VC medium (0.5% [wt/vol] polypeptone, 0.5% [wt/vol] yeast extract, 0.4% [wt/vol] K$_2$HPO$_4$, 3% [wt/vol] NaCl, 0.2% [wt/vol] glucose) or in VPG500 medium (1% [wt/vol] polypeptone, 0.4% [wt/vol] K$_2$HPO$_4$, 500 mM NaCl, 0.5% [wt/vol] glycerol). *E. coli* cells were cultured at 37°C in LB broth (1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] NaCl, 0.5% [wt/vol] Yeast extract) for DNA manipulations or in SB broth (1.2% [wt/vol] Bacto tryptone, 2.4% [wt/vol] Yeast extract, 0.5% [wt/vol] glycerol, 1.25% [wt/vol] K$_2$HPO$_4$, 0.38% [wt/vol] KH$_2$PO$_4$) for protein purification. For the motility assay of *Vibrio* cells on soft-agar plates, 2 µl of concentrated overnight cultures in VC medium with an optical density at 660 nm

---

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100
equal to 5 (OD$_{660}$=5) were spotted on VPG500 (or VPG, only for Fig. 4B) soft-agar plates (0.25% agar plates containing 2.5 µg/ml chloramphenicol, 0.2% or 0.02% arabinose). Measurement of the motile fraction of *Vibrio* cells was carried out as follows. Cells were cultured for 4 hr at 30ºC in VPG500 broth containing appropriate concentrations of arabinose and chloramphenicol. The cultures were then diluted 100-fold with VPG500 broth and motility was immediately observed and recorded using dark-field microscopy. Using this recorded data, we counted the numbers of motile cells in the observed fields, and the percentage among the cells observed is shown as the motile fraction. The recorded data were also used for the swimming speed measurement analyzed by software for motion analysis (Move-tr/2D, Library Co., Tokyo).

**Growth curves.** Growth of *Vibrio* cells was monitored as previously described (20). Overnight cultures of *Vibrio* cells in VC medium were diluted 1:100 in 3 ml VPG500 medium with (0.2% at final concentration) or without arabinose and were shaken at 30ºC. At every hr, the culture was diluted 1:10 in VPG500 broth to measure the value of OD$_{660}$.

**Preparation of whole cell extracts and the periplasmic fraction.** The periplasmic fraction of *Vibrio* cells was prepared using the method described by Nakamaru *et al.* (26). *Vibrio* cells were cultured at 30ºC for 4 hr in VPG500 or VPG broth containing
an appropriate concentration of arabinose. Cells were harvested and suspended in buffer A (1 M NaCl, 50 mM Tris-HCl, pH 7.5) at a cell concentration equivalent to an optical density at 660 nm of 5. This cell suspension was diluted 4 times with water to prepare the whole cell samples. The cells were collected again and resuspended in buffer B (50 mM Tris-HCl, pH 8.5, 2 mM EDTA, 0.95 M sucrose, 1 mg/ml lysozyme), and then were incubated at 30°C for 20 min. After centrifugation (17,000 × g, 10 min), the supernatants that contain the periplasmic proteins were collected as the periplasmic samples and the pellets were resuspended in water with the same volume of the periplasmic sample to prepare the spheroplast samples.

**Detection of Proteins.** Protein samples were suspended in sodium dodecyl sulfate (SDS) loading buffer containing 5% (vol/vol) β-mercaptoethanol and were boiled for 5 min. Proteins were separated by SDS-PAGE and immunoblotting was performed using an anti-PomB antibody (PomB_{C2B0455}) (Terauchi *et al*., in preparation).

**Purification of PomB fragments.** PomB_{C} fragments were over-produced from the plasmids pTSK32 (PomB_{C1}), pTSK33 (PomB_{C2}), pTSK34 (PomB_{C3}), pTSK35 (PomB_{C4}) and pTSK36 (PomB_{C5}) in *E. coli* BL21(DE3). Fresh colonies were inoculated into 1 liter SB medium containing 100 µg/ml ampicillin and cells were grown at 37°C to an OD_{660} of ~0.2, then cooled to room temperature and induced
with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures were continued for about 4 more hr at 24°C. Cells were collected by centrifugation (7000 × g), and then were resuspended in 50 ml buffer C (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing one tablet of Complete protease inhibitor cocktail (Roche Diagnostics). Cells were then disrupted using a French Press (Ohtake Works), centrifuged at 100,000 × g for 30 min, and the supernatant (soluble fraction) was loaded onto a HisTrap (GE Healthcare) column. The bound proteins were eluted with a linear 0-500 mM gradient of imidazole in buffer C and the peak fractions were collected.

To purify the PomB<sub>Cs</sub> protein from the periplasm of Vibrio cells, overnight cultures of VIO5 cells harboring the plasmid pLSK7 were diluted 1:100 into 1 liter VPG500 broth containing 0.2% arabinose and were grown at 30°C for 4 hr. Cells were collected by centrifugation (7000 × g), washed once with buffer A, and then resuspended in buffer B. After incubation at 30°C for 20 min, the supernatants were collected by centrifugation (7000 × g), after which imidazole and MgCl<sub>2</sub> were added at final concentrations of 5 mM and 2 mM, respectively. After ultracentrifugation (186,000 × g, 30 min), the supernatants were loaded onto a HisTrap column and proteins were purified as described above.

**Analytical size exclusion column chromatography.** Analytical size exclusion column chromatography of purified PomB<sub>C</sub> fragments was performed with a
Superdex 75 10/300 GL column (GE Healthcare) using an AKTA system (GE Healthcare). The column was equilibrated with buffer C and was eluted at a flow rate of 0.6 ml/min. Conalbumin (75 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (29 kDa), Ribonuclease A (13.7 kDa) and Aprotinin (6.5 kDa) were used as size markers (GE healthcare).

RESULTS

Dimerization of the periplasmic fragments of PomB. It is presumed that the periplasmic region of PomB has functional importance with respect to stator assembly and activation and that its dimer formation is crucial to form the Na⁺-conducting channel. To characterize the periplasmic region of Vibrio alginolyticus PomB, we constructed several N-terminally truncated variants of PomB (PomB₁) that lack the TM and the plug segments based on the multiple-sequence alignment and secondary structure prediction of the PomB orthologs. (Fig. 1B). We purified those His-tagged fragments and analyzed them by analytical size exclusion chromatography to evaluate their oligomerization. PomB₁ did not behave well and degraded during the purification, so we did not further study that fragment. All other fragments were stable and well behaved, having single discrete peaks in their chromatograms (Fig. 2). The estimated molecular weights from the elution profiles were 68 kDa, 58 kDa, 49 kDa and 41
kDa, for PomB\textsubscript{C2}, PomB\textsubscript{C3}, PomB\textsubscript{C4} and PomB\textsubscript{C5}, respectively, indicating that the sizes of those fragments are nearly twice the deduced molecular masses of the PomB\textsubscript{C} monomers (29 kDa, 27 kDa, 23 kDa and 22 kDa, respectively), suggesting that those PomB\textsubscript{C} fragments also formed dimers in solution.

**Multicopy effects of PomB\textsubscript{C} fragments on motility.** A previous study had shown that a *Salmonella* MotB\textsubscript{C} fragment significantly inhibits the motility of wild-type cells when over-produced in the periplasm (19). To test whether PomB\textsubscript{C} fragments exhibit a similar effect on motility, two smaller PomB\textsubscript{C} fragments (PomB\textsubscript{C4} and PomB\textsubscript{C5}), which include the corresponding region of MotB\textsubscript{C6}, were fused to a leader sequence of MotY at their N terminus to direct their location to the periplasmic space (Fig. 3A). We introduced the plasmids pLSK5 (MotY leader::PomB\textsubscript{C4}-His\textsubscript{6}) and pLSK7 (MotY leader::PomB\textsubscript{C5}-His\textsubscript{6}) in VIO5, the wild-type strain for the Na\textsuperscript{+}-driven polar flagellar motility, and their motilities were examined on soft-agar plates including 0.2% arabinose. VIO5 cells harboring pLSK5 or pLSK7 showed significantly smaller motility rings, compared to VIO5 cells harboring the vector, the plasmid pLSK6 or pLSK8, which expressed leader-less PomB\textsubscript{C} fragments (Fig. 3B). The effect of PomB\textsubscript{C5} on motility was slightly larger than PomB\textsubscript{C4}. Motility inhibition was not observed on plates without arabinose (data not shown). We detected the PomB\textsubscript{C} fragments in whole-cell lysates, as well as in spheroplast and in periplasmic fractions from these strains...
(Fig. 3C). In whole-cell lysates, PomB\(_C^4\) and PomB\(_C^5\) fragments fused to the MotY leader sequence were more abundant than those without the MotY leader sequence. In the spheroplast fraction, PomB\(_C\) fragments were detected at a similar level regardless of the presence or absence of the leader sequence. In the periplasmic fraction, only PomB\(_C\) fragments with the leader sequence were detected with a higher level for PomB\(_C^5\) than PomB\(_C^4\), which is consistent with the slightly stronger motility inhibition of PomB\(_C^5\) than PomB\(_C^4\). To further confirm the periplasmic location of PomB\(_C\), we purified PomB\(_C^5\) protein from the periplasmic fraction and analyzed it using mass spectroscopy. The mass of purified PomB\(_C^5\) was almost the same as the leader-less fragment, confirming the cleavage of the MotY signal peptide during the export into the periplasm. These results indicate that although the effects of PomB\(_C\) are weaker than those of MotB\(_C\), the periplasmic over-production of PomB\(_C\) still significantly interferes with the motility of wild-type cells. We speculate that PomB\(_C\) fragments in the periplasm are folded and can associate with endogenous motor proteins to interfere with motor rotation.

**Function of in-frame deletion constructs of PomB (PomB\(_L\)).** To test whether the linker region of *Vibrio alginolyticus* PomB (residues 41 to 140) contains amino acids that are important for function, we also constructed a series of in-frame deletion constructs of PomB (hereafter called PomB\(_L\)) that lack residues 41-60,
41-70, 41-80, 41-90, 41-100, 41-110, 41-120, 41-130 or 41-140 (Fig. 1B). These constructs were co-expressed with PomA in the pomAB null strain NMB191 from the arabinose inducible plasmids and the motilities of those cells were assayed on soft-agar plates containing 0.02% arabinose (Fig. 4A). The two largest deletions (Δ41-130 and Δ41-140) completely impaired motility, but all others retained function, forming motility rings with slightly reduced sizes compared to the wild-type. Therefore, the PEM for PomB is defined for the region containing residues 121 to 315. When PomB∆L proteins were co-expressed with PomA at a higher level (induced by 0.2% arabinose, Fig. 4B), ring sizes of Δ41-60, Δ41-100 and Δ41-120 were significantly smaller, compared to Δ41-70, Δ41-80, Δ41-90 and Δ41-110, whose ring sizes were slightly smaller than the wild-type. Therefore, some of the deletion constructs show a multicopy effect on motility. Expression and stability of the deletion constructs were analyzed by immunoblot analysis of whole-cell extracts (Fig. 4C). Only faint or weak bands were detected for the nonfunctional derivatives (Δ41-130 and Δ41-140). These deletions seem to affect protein stability or expression, so that mutant stators may not support motility. All other proteins were detected at a level comparable to the wild-type. We next investigated the free-swimming motility of cells expressing each PomA/PomB∆L stator using dark-field microscopy. When stators were induced by 0.02% arabinose, all in-frame deletion strains swam at reduced and somewhat similar speeds, less than one-half the wild-type (Fig. 4D). There seems to be no significant correlation
between the swimming speed and length of the deletion, and that trend was also observed for the motile fraction of the swimming cells (Table 2, left column). Motile fractions of the deletion constructs were within 23–34%, about one-half the wild-type (47%). When the stator expression was induced at a high level (by 0.2% arabinose), motile fractions of all deletion strains were reduced (Table 2, right column), especially ones that form smaller motility rings at 0.2% arabinose (Δ41-60, Δ41-100, Δ41-120; Fig. 4B, only 2–6%), further showing that these in-frame deletions in PomB cause a multicopy effect on motility.

The effect of PomBΔL over-production on cell growth. In *V. alginolyticus* PomB, residues V44 to F58 (corresponding to P52 to P65 in *E. coli* MotB) have been predicted to be a ‘plug’ segment for the Na⁺ channel in the PomA/PomB stator complex. We speculated that the multicopy effect on motility observed for some in-frame deletion constructs (Fig. 4B and Table 2) might be caused by the growth impairment. To test this possibility, we monitored cell growth in VPG500 broth containing 0.2% arabinose. When PomBΔL was co-over-expressed with PomA in NMB191 from the plasmid, a dramatic decrease in cell growth was observed in all deletion constructs. Fig. 5A shows typical growth curves observed for cells expressing Δ41-60 (strong multicopy effect), Δ41-110 (did not show a multicopy effect), and the wild-type stator, which demonstrates the greatly reduced cell growth for deletion constructs compared to the wild-type, especially seen at the log
phase. Similar growth impairment was observed for all other deletion constructs (data not shown). These results indicate that the predicted segment 44 to 58 can function as the ‘Plug’ in PomB, and if the deleted region(s) contains this segment, over-production of the PomA/PomB\(_{\Delta L}\) stator will impair cell growth. Since all deletion constructs caused similar growth impairment, hereafter we focused on the shortest functional construct, \(\Delta 41-120\), in this study.

If this growth impairment is caused by the massive Na\(^+\) influx through the PomA/PomB\(_{\Delta L}\) stator complex as seen in the MotA/MotB H\(^+\)-driven stator (13, 24), a mutation that disrupts the Na\(^+\)-binding site in the stator (PomB D24N) should eliminate such a deleterious effect. Therefore, we introduced the mutation D24N into PomB\(_{\Delta L}\) (\(\Delta 41-120\)) and cell growth was monitored (Fig. 5B). As expected, the D24N mutation eliminated the growth inhibition, but did not affect protein stability or expression (data not shown). Moreover, as the Na\(^+\) concentration was decreased in the growth medium, the inhibitory effect became weaker (data not shown). This suggests that the growth impairment can be mainly attributed to the Na\(^+\) influx through the PomA/PomB\(_{\Delta L}\) (\(\Delta 41-120\)) complex rather than being a general effect due to the over-production of membrane protein. Thus this region, especially the ‘plug’ segment, appears to be important for the regulation of Na\(^+\) influx. A similar growth inhibition was observed when the PomA/PomB\(_{\Delta L}\) (\(\Delta 41-120\)) complex was over-expressed in NMB200 (\(\Delta pomB\Delta fliG\)), which does not have the hook-basal body structure (Fig. 5C), and in the wild-type strain VIO5 (data not shown),
showing that this inhibitory effect does not require the flagellar basal body.

Effects of mutations in the putative helix of the N-terminal PEM region in PomB_{\text{AL}}(\Delta 41-120) on motility. The crystal structure of Salmonella MotB_{C} and subsequent functional analyses suggested that large conformational changes in the PEM region are required to anchor the stator to the PG layer and to activate its H^+-conducting activity (20). To investigate the function of the PEM region in PomB, we again aligned PomB orthologs and predicted the secondary structure at the N-terminal PEM region (Fig. 6A). Since mutations L119P or L119E in the amphipathic helix 1 of Salmonella MotB_{C} would be predicted to disrupt hydrophobic interactions between this helix and the PGB core domain, we mutated the semi-conserved hydrophobic residues predicted to be on helix 1 of PomB_{C}. Point mutations M153P, M157P, L160P, I164P and L168P were introduced into PomB_{\text{AL}}(\Delta 41-120) (Fig. 6A). When mutant PomB_{\text{AL}}(\Delta 41-120) was co-expressed with PomA in NMB191 in the presence of 0.02% arabinose, all mutants formed the same size motility ring as wild-type PomB_{\text{AL}}(\Delta 41-120) (data not shown). In the presence of 0.2% arabinose, a slightly larger motility ring was formed by the L168P mutant, after 7 hr of incubation at 30°C (Fig. 6B). We then investigated the motile fraction of the cells under these conditions (Table 3). The motile fraction of cells that over-produced the L168P mutant was higher than any other mutants as
well as the wild-type PomB\(_{\Delta L}(\Delta 41-120)\) (Table 3). This indicates that the higher motile fraction of the L168P mutant contributes to the slightly larger motility ring formation, as seen in some of the deletion constructs (Fig. 4B and Table 2). Immunoblot analysis of the whole-cell extracts of NMB191 cells expressing the mutant PomA/PomB\(_{\Delta L}(\Delta 41-120)\) complex induced by 0.2% arabinose showed that the amount of L168P protein decreased greatly (Fig. 6C) and the growth inhibition by wild-type PomB\(_{\Delta L}(\Delta 41-120)\) was not rescued by this mutation (Fig. 5D). These results suggest that the conformation of the PomA/PomB\(_{\Delta L}(\Delta 41-120)\) complex might be altered into a more favorable state for stator assembly and anchoring to the PG layer by the L168P mutation.

**DISCUSSION**

Although the basic mechanism of torque generation is shared with the H\(^+\)-driven motor, there are some distinct characteristics for the Na\(^+\)-driven polar flagellar motor of *Vibrio alginolyticus*. The PomA/PomB stator assembly depends on Na\(^+\) (10), and also requires the T ring structure that consists of MotX and MotY (36). Such distinct characteristics might be assigned to the specific functional roles of the periplasmic region of PomB, since the structure-based functional analysis of the corresponding region of *Salmonella* MotB revealed its functional importance especially for stator assembly and activation (20). In this study, we investigated the
role of the periplasmic region of PomB using mutational and biochemical approaches.

We constructed 5 PomB fragments (PomB₇) with N-terminal truncations of 37, 58, 89, 120 or 134 residues. Analytical size exclusion chromatography showed that those soluble PomB₇ fragments form dimers, and the two smaller constructs, PomB₄ and PomB₅, impaired the motility of wild-type cells when over-produced in the periplasm. This suggests that these PomB₇ fragments are properly folded so that they can associate with motor proteins to interfere with rotation, as has been reported for *Salmonella* MotB (19). It has been shown that MotX is likely to interact with PomB (28). Thus it is also possible that the inhibition of motility might be caused by the titration of MotX from the motor by the over-production of PomB₇ in the periplasm. However, we could not detect MotX in the periplasmic fraction when PomB₅ was over-produced in the periplasm, and it was not co-purified with PomB₅ by affinity chromatography (data not shown). This indicates that MotX, which is not a membrane protein but has a strong insoluble nature (28, 29), is not solubilized by the periplasmic PomB₅. Therefore, the MotX-PomB interaction may be weak outside of the motor and may require conformational changes in PomB or MotX that are induced to strengthen the interactions only when the stator is located around the rotor. For further investigation of the MotX-PomB interaction, more mutational analyses will be required for both proteins. For example, the mutations in PomB or MotX that make
the stator constitutively localize around the rotor despite the \( \text{Na}^+ \) concentration in the medium could cause stronger interactions between them.

The region that connects the TM segment (15 to 39) and the putative PEM region (141 to the C-terminus) is slightly longer than the *Salmonella* or *E. coli* MotB, and is predicted to be a tolerant flexible linker, with the putative ‘plug’ segment at its N-terminus. We constructed 9 in-frame deletion mutants of PomB at this region (PomB\(_{\Delta} \) proteins), and found that most PomB\(_{\Delta} \) proteins can form a functional stator, except for the two largest deletion constructs (\( \Delta 41-130 \) and \( \Delta 41-140 \)). Those two proteins were detected in low amounts in whole cell extracts, indicating that reduced protein stability or expression might be the reason for the loss of function. However, our attempt to isolate motile revertants from the strains expressing these mutant stators has not been successful so far, so it is also possible that the critical length or region of PomB exists between residues 121 to 131. A comparative study for *Salmonella* MotB showed that deletion of the corresponding linker region did not affect their function up to a deletion of 40 residues (25). On the other hand, 7 functional deletion constructs of PomB (up to a deletion of 80 residues) retain reduced but similar levels of function, showing swimming speeds and motile fractions at about one-half the wild-type level when induced by 0.02% arabinose (Fig. 4 and Table 2). All these constructs lack the plug segment, so somehow deletion of the plug caused the reduction of PomB function to a certain extent. Lower motile fractions may be attributed to inefficient stator assembly.
around the rotor that could cause a decreased swimming speed, so we speculate that deletion of the plug may affect the stator assembly.

Over-production of all functional PomA/PomB_{\Delta L} stators impaired cell growth in the presence of 0.2% arabinose (Fig. 5). A similar inhibition was observed when the stator complex with the shortest functional PomB, PomB_{\Delta L}(\Delta 41-120), was over-expressed in the pomB fliG double deletion mutant or in wild-type cells, indicating that this growth impairment occurs without the flagellar basal-body structure. This inhibitory effect was eliminated by the introduction of the D24N mutation. Moreover, when the concentration of Na^{+} was reduced in the medium, the growth inhibition was weakened (data not shown), which suggests that the growth impairment is related to the Na^{+} influx through the PomA/PomB_{\Delta L} stator. This implies that the growth inhibition may be caused by the lack of the ‘Plug’, which regulates the ion flow of the stator complex composed of PomA and PomB and prevents the premature ion translocation across the cell membrane when the stator is not incorporated into the motor and freely diffuses in the membrane. Our results suggest that the deletion of 80 residues in PomB is sufficient to alter the conformation of the PomA/PomB complex to allow active Na^{+} translocation. The plug function may be commonly conferred in the stator complex of either the Na^{+}-driven type or the H^{+}-driven type.

Motile fractions of cells over-expressing PomA/PomB_{\Delta L} stators (induced by 0.2% arabinose) correlated with the level of motility reduction, showing that all
mutant strains exhibit reduced motile fractions compared to the wild-type, especially for the 3 mutants that formed smaller motility rings (Fig. 4B and Table 2). Since over-production of all functional PomA/PomB_{AL} stators caused growth inhibition, this multicopy effect on motility may be caused both by growth impairment and by reduced motile fractions. The reason why the higher level of expression caused a reduced motile fraction is not clear. It is possible that these mutant stators may conduct higher amounts of \text{Na}^{+} than others, whose activity could not be distinguished by the simple growth inhibition assay performed here. If so, the higher \text{Na}^{+} influx may cause a slight reduction of the \text{Na}^{+}-motive force that may affect \text{Na}^{+}-dependent stator assembly, and thereby cause the reduced motile fraction. To test this possibility, we need more direct measurements of the \text{Na}^{+}-conducting activity of mutant stators. Some of the PomB_{AL} proteins did not show a multicopy effect on motility, so we speculated that proper anchoring to the PG layer and assembly around the rotor depend on the conformation of the PEM region of PomB, which can be varied by the deletions. Therefore, we investigated mutations at the putative helix in the N-terminal PEM region. In \textit{Salmonella} MotB, a large conformational change in this region has been proposed for the assembly and activation of the MotA/B_{AL} complex, and the mutations L119P or L119E in this region affect cell growth without impairing motility (20). We mutated the corresponding hydrophobic residues of PomB_{AL}(\Delta 41-120) to tryptophan and expected to observe effects similar to those seen in MotB_{AL}. However, a significant
effect on motility was only seen in the highly over-produced L168P mutant (induced by 0.2% arabinose) (Fig. 6B). That mutation did not affect growth (Fig. 5D), but showed a significantly higher motile fraction of cells compared to the other mutations in this region (Table 3). That could be the main reason for the slightly improved motility on soft-agar plates (Fig. 6B), which suggests that the stator assembly was improved. A similar phenotype to Δ41-120/L168P was observed for the deletion constructs (Δ41-70, Δ41-80, Δ41-90 and Δ41-110), which formed significantly larger motility rings and showed higher motile fractions than Δ41-120 at 0.2% arabinose (Fig. 4B). We speculate that the L168P mutation alters the conformation of the PEM region to allow more efficient PG anchoring, so that this mutant stator complex could be more efficiently incorporated into the motor. Similarly, the deletion constructs described above may have conformations that allow them to be anchored around the rotor. Therefore, the putative helix of the N-terminal PEM region of PomB and its connected linker region play key roles in stator assembly and activation.

In summary, our results show that the plug is a common feature for PomB/MotB proteins which is important for stator function, and that the flexible linker which connects the TM segment and the PEM region is dispensable but its appropriate length or conformation is required for proper function. The basic properties and roles of the PEM region of PomB are similar to those of MotB, but there are distinct features, such as the interaction with the T ring (presumably with
MotX). The region in PomB responsible for interactions was not identified in this study and should be unveiled in the future. We emphasize that the in-frame deletion construct $\text{PomB}_{\Delta L}(\Delta 41-120)$, which is the shortest functional PomB protein that lacks the periplasmic linker region (including the ‘plug’ segment), shows an inhibitory effect on growth when over-produced. That property is quite useful for evaluating the $\text{Na}^+$-conducting activity of the stator $\text{in vivo}$ as shown in this study, as well as the $\text{in vitro}$ reconstitution system using the purified stator complex.

ACKNOWLEDGEMENTS

We thank David Blair for the careful reading of the manuscript and comments, Tomohiro Yorimitsu and Natsumi Nonoyama for kindly providing the plasmid pTY57 and the strain NMB200, respectively, and Sachi Tatematsu and Mayumi Taniguchi for technical assistance. This work was supported by Grants-in-Aid for Scientific Research [to S.K. (20051009), to M.H. (18074003)] from the Ministry of Education, Culture, Sports, Science and Technology of Japan.
REFERENCES


interaction between PomA and PomB, the Na\(^+\)-driven flagellar motor components of
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V. alginolyticus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIO5</td>
<td>Rif^r, Pof^r, Laf^-</td>
<td>(30)</td>
</tr>
<tr>
<td>NMB191</td>
<td>Rif^r, Pof^r, Laf^-ΔpomAB</td>
<td>(46)</td>
</tr>
<tr>
<td>NMB200</td>
<td>Rif^r, Pof^r, Laf^-ΔpomBΔfliG</td>
<td>N. Nonoyama</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Host for over-expression from the T7 promoter</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Recipient for cloning experiments</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET19b</td>
<td>T7 expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pBAD33</td>
<td>Cm^r, P_BAD</td>
<td>(12)</td>
</tr>
<tr>
<td>pTY57</td>
<td>Cm^r, P_BAD, with a multicloning site of pBAD24</td>
<td>T. Yorimitsu</td>
</tr>
<tr>
<td>pSU41</td>
<td>Km^r, P_lac lacZα</td>
<td>(2)</td>
</tr>
<tr>
<td>pAS101</td>
<td>pSU41/MotY</td>
<td>(21)</td>
</tr>
<tr>
<td>pHFAB</td>
<td>pBAD33/PomA+PomB</td>
<td>(11)</td>
</tr>
<tr>
<td>pTSK32</td>
<td>pET19b/PomBC1-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK33</td>
<td>pET19b/PomBC2-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK34</td>
<td>pET19b/PomBC3-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK35</td>
<td>pET19b/PomBC4-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK36</td>
<td>pET19b/PomBC5-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK5</td>
<td>pTY57/MotY_L::PomBC4-His_6^a</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK6</td>
<td>pTY57/MotY_L::PomBC5-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK7</td>
<td>pTY57/MotY_L::PomBC6-His_6^a</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK8</td>
<td>pTY57/MotY_L::PomBC7-His_6</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK9</td>
<td>pBAD33/PomA+PomB_Δ(41-60)</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK10</td>
<td>pBAD33/PomA+PomB_Δ(41-70)</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK11</td>
<td>pBAD33/PomA+PomB_Δ(41-80)</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK12</td>
<td>pBAD33/PomA+PomB_Δ(41-90)</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK13</td>
<td>pBAD33/PomA+PomB_Δ(41-100)</td>
<td>This study</td>
</tr>
<tr>
<td>pLSK14</td>
<td>pBAD33/PomA+PomB_Δ(41-110)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK37</td>
<td>pBAD33/PomA+PomB_Δ(41-120)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK38</td>
<td>pBAD33/PomA+PomB_Δ(41-130)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK39</td>
<td>pBAD33/PomA+PomB_Δ(41-140)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK37-D24N</td>
<td>pBAD33/PomA+PomB_Δ(41-120, D24N)</td>
<td>This study</td>
</tr>
<tr>
<td>PTK37-M153P</td>
<td>pBAD33/PomA+PomB(Δ41-120, M153P)</td>
<td>This study</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>pTSK37-M157P</td>
<td>pBAD33/PomA+PomB(Δ41-120, M157P)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK37-L160P</td>
<td>pBAD33/PomA+PomB(Δ41-120, L160P)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK37-I164P</td>
<td>pBAD33/PomA+PomB(Δ41-120, I164P)</td>
<td>This study</td>
</tr>
<tr>
<td>pTSK37-L168P</td>
<td>pBAD33/PomA+PomB(Δ41-120, L168P)</td>
<td>This study</td>
</tr>
</tbody>
</table>

1 Each PomB fragment is fused to a MotY leader sequence (MotY\*). Rif\*, rifampicin resistant; Cm\*, chloramphenicol resistant; Pof\*, normal polar flagellar formation, Laf\*, defective in lateral flagellar formation. P\textsubscript{BAD}; arabinose promoter.
Table 2. Motile fraction of cells expressing PomB deletion variants

<table>
<thead>
<tr>
<th>Strain</th>
<th>0.02% arabinose</th>
<th>0.2% arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD33 (vector)</td>
<td>0 (n=213)</td>
<td>0 (n=241)</td>
</tr>
<tr>
<td>pHFAB (full length)</td>
<td>47 (n=275)</td>
<td>38 (n=294)</td>
</tr>
<tr>
<td>pLSK9 (∆41-60)</td>
<td>24 (n=207)</td>
<td>2 (n=370)</td>
</tr>
<tr>
<td>pLSK10 (∆41-70)</td>
<td>34 (n=340)</td>
<td>20 (n=333)</td>
</tr>
<tr>
<td>pLSK11 (∆41-80)</td>
<td>29 (n=207)</td>
<td>13 (n=245)</td>
</tr>
<tr>
<td>pLSK12 (∆41-90)</td>
<td>31 (n=270)</td>
<td>14 (n=220)</td>
</tr>
<tr>
<td>pLSK13 (∆41-100)</td>
<td>30 (n=227)</td>
<td>6 (n=311)</td>
</tr>
<tr>
<td>pLSK14 (∆41-110)</td>
<td>34 (n=311)</td>
<td>31 (n=279)</td>
</tr>
<tr>
<td>pTSK37 (∆41-120)</td>
<td>23 (n=250)</td>
<td>2 (n=415)</td>
</tr>
<tr>
<td>pTSK38 (∆41-130)</td>
<td>0 (n=202)</td>
<td>0 (n=210)</td>
</tr>
<tr>
<td>pTSK39 (∆41-140)</td>
<td>0 (n=217)</td>
<td>0 (n=245)</td>
</tr>
</tbody>
</table>

N is the total number of cells that were analyzed. The NMB191 (∆pomAB) strain containing each plasmid was cultured at 30°C in VPG500 broth for 4 hr with 0.02% or 0.2% arabinose, and their swimming motility was observed using dark-field microscopy. Motile fractions were measured as described in the Materials and Methods.
Table 3. Motile fraction of cells expressing PomBΔL(Δ41-120) with mutations at the putative PEM region

<table>
<thead>
<tr>
<th>Strain</th>
<th>Motile fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHFAB</td>
<td>26 (n=358)</td>
</tr>
<tr>
<td>pTSK37</td>
<td>1 (n=265)</td>
</tr>
<tr>
<td>pTSK37 M153P</td>
<td>2 (n=180)</td>
</tr>
<tr>
<td>pTSK37 M157P</td>
<td>0 (n=316)</td>
</tr>
<tr>
<td>pTSK37 L160P</td>
<td>4 (n=224)</td>
</tr>
<tr>
<td>pTSK37 I164P</td>
<td>4 (n=283)</td>
</tr>
<tr>
<td>pTSK37 L168P</td>
<td>10 (n=225)</td>
</tr>
</tbody>
</table>

N is the total number of cells that were analyzed. The NMB191 strain containing each plasmid was cultured at 30°C in VPG500 broth for 4 hr with 0.2% arabinose, and their swimming motility was observed using dark-field microscopy. Motile fractions were measured as described in the Materials and Methods.
Figure Legends

**FIG. 1.** The PomB topology model and constructs used in this study. (A) The putative topology of PomB. PomB contains 315 amino acids and has a single TM segment (residues 15 to 39) and a ‘Plug’ segment (residues 44 to 58, speculated to prevent premature Na\(^+\) translocation across the cell membrane) at its N-terminus. D24 is regarded as the critical Na\(^+\) binding site. Amino acids 143 to 169 are predicted to form a putative N-terminal helix of the PEM region. The large periplasmic region includes an ‘OmpA-like domain’ (residues 182-300) with a PGB motif (residues 231-259). The putative helix (residues 247-261) that may form the dimer interface in the PGB domain is also indicated. (B) Primary structure of PomB and schematic representation of N-terminally truncated PomB fragments (PomB\(_C\)) and in-frame deletions (PomB\(_\Delta\)). The in-frame deletion derivatives constructed in this study lack the putative ‘Plug’ segment and part of the periplasmic linker region.

**FIG. 2.** Elution profiles of purified PomB fragments by analytical size exclusion chromatography using a Superdex 75 10/300 GL column. PomB\(_{C2}\) (A), PomB\(_{C3}\) (B), PomB\(_{C4}\) (C), PomB\(_{C5}\) (D) and standard marker proteins (E) were analyzed. Arrows in (E) indicate the elution peaks of the PomB fragments with their numbers (e.g., “2” means PomB\(_{C2}\)). Closed symbols in (E) indicate the elution peaks of
marker proteins: Conalbumin (9.69 ml, 75 kDa), Ovalbumin (10.35 ml, 43 kDa), Carbonic Anhydrase (11.65 ml, 29 kDa), Ribonuclease A (13.39 ml, 13.7 kDa) and Aprotinin (15.75 ml, 6.5 kDa). OD\textsubscript{280}, optical density at 280 nm; mAU, milli absorbance units; M.W., Molecular weight.

FIG. 3. Over-expression of PomB\textsubscript{C} fragments in the periplasm interferes with motility. (A) Schematic representation of PomB\textsubscript{C} fragments designed for periplasmic localization. Plasmids pLSK5 and pLSK7 encode the PomB\textsubscript{C4} and PomB\textsubscript{C5} fragments (residues 121-315 and 131-315), respectively. Their N-termini were fused to a MotY signal peptide (residues 1-22 of MotY) and their C-termini were fused to a hexahistidine tag. The plasmids pLSK6 and pLSK8 do not contain the MotY signal peptide at their N-terminus. All constructs were placed under control of the arabinose promoter. (B) Motility assay of VIO5 harboring pTY57 (vector), pLSK5, pLSK6, pLSK7 or pLSK8 on VPG500 soft-agar plates containing 0.2% arabinose. Plates were incubated at 30°C for 4 hr. (C) Detection of PomB\textsubscript{C} fragments. Whole-cell proteins, spheroplast and periplasmic fractions were prepared from VIO5 cells that harbor pTY57 (vector control), pLSK5, pLSK6, pLSK7 or pLSK8 grown in VPG500 broth at 30°C for 4 hr in the presence of 0.2% arabinose; they were analyzed by SDS-PAGE followed by immunoblotting with the anti-PomB antibody.
**FIG. 4.** Effect of in-frame deletions in PomB on motility. (A and B) Motility of NMB191 (ΔpomAB) cells co-expressing PomA and PomB<sub>AL</sub> proteins. NMB191 cells harboring the respective plasmids (pHFAB, pLSK9, pLSK10, pLSK11, pLSK12, pLSK13, pLSK14, pTSK37, pTSK38 or pTSK39) were inoculated into VPG semi-solid agar containing 0.02% (A) or 0.2% (B) arabinose and were incubated at 30°C for 6 hr. For the vector control, we used pBAD33. (C) Immunoblot detection of PomB<sub>AL</sub> proteins. NMB191 cells harboring the respective plasmids as in (A) or (B) were grown in VPG broth containing 0.02% arabinose at 30°C for 4 hr, after which whole cell proteins were prepared and were analyzed by immunoblotting with the anti-PomB antibody. The asterisk indicates a non-specific band detected by the anti-PomB antibody. (D) Swimming speeds of cells expressing PomA/PomB<sub>AL</sub> stators. NMB191 cells harboring the respective plasmids as above were grown in VPG broth containing 0.02% arabinose at 30°C for 4 hr, then their swimming motilities were observed using dark-field microscopy. Swimming speeds were analyzed by motion analysis software.

**FIG. 5.** Over-production of PomB<sub>AL</sub> impairs cell growth. (A) NMB191 cells harboring the plasmids pHFAB (→), pLSK9 (■) or pLSK14 (○) were cultured in VPG500 broth at 30°C for 7 hr with 0.2% arabinose added at the beginning (at 0 hr). Cell growth was monitored at the absorbance of 660 nm every hr. (B) NMB191 cells harboring the plasmids pHFAB (→), pTSK37 (■) or pTSK37...
D24N (→) were cultured in VPG500 broth at 30°C for 7 hr with 0.2% arabinose added at the beginning (at 0 hr). Cell growth was monitored at the absorbance of 660 nm every hr. (C) NMB200 cells harboring the plasmids pTSK37 (←) or pTSK37 D24N (→) were cultured at 30°C for 7 hr with 0.2% arabinose added at the beginning. (D) Effect of mutations at the putative helix of the N-terminal PEM region on growth impairment. Strains with pHFAB (←), pTSK37 (←), or pTSK37 L168P (←) were cultured in VPG500 broth at 30°C for 7 hr with 0.2% arabinose added at the beginning.

FIG. 6. Effects of mutations in the putative helix of the N-terminal PEM region in PomBΔL41-120 on motility. (A) Multiple-sequence alignment of PomB orthologs at the putative helix of the N-terminal PEM region. The aligned sequences are: StMotB, *Salmonella enterica* serovar Typhimurium MotB; EcMotB, *Escherichia coli* MotB; HpMotB, *Helicobacter pylori* MotB; AaMotB, *Aquifex aeolicus* MotB; VaPomB, *Vibrio alginolyticus* PomB. Alignment was carried out using ClustalW software. Secondary structures were predicted by the web-based program PSIPRED (14), and the predicted α-helix and β-sheet are indicated as a line below each sequence except for *Salmonella* MotB, whose crystal structure (MotB_{C2}) has been determined and its structural elements are indicated above the sequence. Residues emphasized by the gray boxes are the semi-conserved hydrophobic ones. L119 of *Salmonella* MotB and the mutations generated are...
shown with triangles. (B) Motility of NMB191 cells expressing mutant PomB\textsubscript{AL}(41-120) proteins. Overnight cultures of NMB191 cells harboring the plasmid pTSK37 with each mutation were spotted on VPG500 soft-agar plates containing 0.2% arabinose at 30\degree C for 7 hr. For the vector control, we used pBAD33. (C) Detection of mutant PomB\textsubscript{AL}(41-120) proteins. Cells were cultured in VPG500 broth at 30\degree C for 4 hr with 0.2% arabinose. Whole cell proteins were prepared and were analyzed by immunoblotting. Vector, pBAD33 in NMB191.
Li et al, Fig. 1
Elution volume (ml)

Li et al, Fig. 2
Li et al, Fig. 3
Li et al, Fig. 4
Li et al, Fig. 5
<table>
<thead>
<tr>
<th>α1</th>
<th>α2</th>
<th>β1</th>
<th>β2</th>
</tr>
</thead>
<tbody>
<tr>
<td>StMotB 99</td>
<td>--EKQPNIDELKKRMEQSR</td>
<td>LNKLRGDLDQLIESDPKLRALRPHLKIDLVQEEGLRIQIIDS 156</td>
<td></td>
</tr>
<tr>
<td>EcMotB 98</td>
<td>--NKQPNIEELKKRMEQSR</td>
<td>LRGDLDQIIESDPKLRALRPHLKIDLVQEEGLRIQIIDS 155</td>
<td></td>
</tr>
<tr>
<td>HpMotB 77</td>
<td>---------------KEEOMAESKPACQNTKATTAKGKGSTLIDQG5ID--</td>
<td>--121</td>
<td></td>
</tr>
<tr>
<td>AaMotB 77</td>
<td>---------------MENFH</td>
<td></td>
<td>--121</td>
</tr>
<tr>
<td>VaPomB 135</td>
<td>-TQNNESSAPADMCQGS KS</td>
<td></td>
<td>--191</td>
</tr>
</tbody>
</table>

(A) 

(B) 

(C) 

Li et al, Fig. 6