A conserved residue, PomB-F22, in the transmembrane segment of the flagellar stator complex, has a critical role in conducting ions and generating torque

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Bacterial flagellar motors exploit the electrochemical potential gradient of a coupling ion (H^+ or Na^+) as their energy source, and are composed of stator and rotor proteins. Sodium-driven and proton-driven motors have the stator proteins PomA and PomB or MotA and MotB, respectively, which interact with each other in their transmembrane (TM) regions to form an ion channel. The single TM region of PomB or MotB, which forms the ion-conduction pathway together with TM3 and TM4 of PomA or MotA, respectively, has a highly conserved aspartate residue that is the ion binding site and is essential for rotation. To investigate the ion conductivity and selectivity of the Na^+ -driven PomA/PomB stator complex, we replaced conserved residues predicted to be near the conserved aspartate with H^+ -type residues, PomA-N194Y, PomB-F22Y and/or PomB-S27T. Motility analysis revealed that the ion specificity was not changed by either of the PomB mutations. PomB-F22Y required a higher concentration of Na^+ to exhibit swimming, but this effect was suppressed by additional mutations, PomA-N194Y or PomB-S27T. Moreover, the motility of the PomB-F22Y mutant was resistant to phenamil, a specific inhibitor for the Na^+ channel. When PomB-F22 was changed to other amino acids and the effects on swimming ability were investigated, replacement with a hydrophilic residue decreased the maximum swimming speed and conferred strong resistance to phenamil. From these results, we speculate that the Na^+ flux is reduced by the PomB-F22Y mutation, and that PomB-F22 is important for the effective release of Na^+ from PomB-D24.

Received 21 January 2011

Revised 9 May 2011

Accepted 26 May 2011

INTRODUCTION

Some ion channels and transporters that exist in cell membranes can selectively translocate only a particular ion. The high ion specificity of an ion channel or transporter is extremely important for signal transduction, membrane excitability and the homeostasis of organisms. Each selective ion transporter, which is coupled to a sodium ion, such as LeuT, NtpK or NhaA, possesses an ion binding pocket. A particular ion and/or a transport substrate interacts with the ion binding pocket, induces a conformational change, and is then translocated to the opposite side across the membrane (Gouaux & Mackinnon, 2005; Yamashita *et al.*, 2005; Hunte *et al.*, 2005; Murata *et al.*, 2005, 2008). For example, in the Na^+ -driven V-type ATPase of *Enterococcus hirae*, the transmembrane complex

V_o , which conducts Na^+ (or Li^+), is composed of a membrane rotor ring (K-ring) comprising oligomers of NtpK and a single copy of the NtpI subunit. It has been suggested that the cavity size of the K-ring binding pocket contributes to the ion specificity, that five residues (L^{61} , T^{64} , Q^{65} , Q^{110} , E^{139}) of NtpK are involved in the Na^+ binding to the K-ring, and that the essential glutamate E^{139} is conserved in the homologous subunits of H^+ -driven V-ATPase (Murata *et al.*, 2005, 2008). Bacterial flagellar motors are rotary motors which convert the electrochemical potential difference of a coupling ion (H^+ or Na^+) across the cytoplasmic membrane into torque, which rotates the flagellum. *Escherichia coli* and *Salmonella* use H^+ as the coupling ion, whereas *Vibrio* and alkaliphilic *Bacillus* use Na^+ (Kojima & Blair, 2004; Terashima *et al.*, 2008).

Each flagellar motor is composed of a stator and a rotor. The stator serves as a torque-generating unit and is thought to work by interacting with the rotor. MotA and MotB are stator proteins in the H^+ -driven motor of *E. coli* and *Salmonella*, while PomA and PomB, which are orthologues

Abbreviation: TM, transmembrane.

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A supplementary movie is available with the online version of this paper.

of MotA and MotB, are found in the Na⁺-driven motor of *Vibrio* spp. (Asai *et al.*, 1997; Dean *et al.*, 1984; Stader *et al.*, 1986). Only the Na⁺-driven motor of *Vibrio* spp. requires additional motor proteins, MotX and MotY, for torque generation (Okabe *et al.*, 2001; Okunishi *et al.*, 1996). PomA and PomB (MotA and MotB) are membrane proteins and form an A₄:B₂ heterohexamer (Kojima & Blair, 2004; Sato & Homma, 2000a, b; Yorimitsu *et al.*, 2004). PomA (MotA) has four transmembrane (TM) segments, whereas PomB (MotB) has only a single TM segment (Asai *et al.*, 1997; Chun & Parkinson, 1988; Zhou *et al.*, 1995). The C-terminal half of PomB (MotB) associates with the peptidoglycan layer and at least 11 PomA/B (MotA/B) complexes assemble around the rotor (Kojima *et al.*, 2009; Leake *et al.*, 2006; Reid *et al.*, 2006). The coupling ion is taken through an ion-conducting pathway formed in the PomA/B (MotA/B) complex (Blair & Berg, 1990; Sato & Homma, 2000b). The ion influx induces a conformational change of the stator complex, which causes an interaction between PomA (MotA) and the rotor component FliG, and it has been inferred that the torque is generated by that interaction (Kojima & Blair, 2001; Lloyd & Blair, 1997; Yorimitsu *et al.*, 2002; Zhou & Blair, 1997; Zhou *et al.*, 1998a). The ion-conducting pathway is thought to be formed mainly by the third and fourth TM segments of PomA (MotA) and the single TM segment of PomB (MotB) (Braun & Blair, 2001; Braun *et al.*, 2004; Sudo *et al.*, 2009b; Yakushi *et al.*, 2004). PomB (MotB) has a completely conserved aspartate residue (PomB-D24 for *Vibrio*, MotB-D32 for *E. coli*) which is essential for torque generation, and is a binding site of the coupling ion (Sudo *et al.*, 2009a; Zhou *et al.*, 1998b). Recently, it has been shown that this essential aspartate residue in PomB can be transferred to the TM segment of PomA. The transferred residue of PomA-N194D, which is a mutation in TM4, is able to partially rescue the motility defect in PomB-D24N, which suggests that PomA-N194 and PomB-D24 form an Na⁺ binding pocket for the coupling ion (Terashima *et al.*, 2010).

Studies using chimeric proteins between H⁺-type MotA/B of *Rhodobacter sphaeroides* or *E. coli* and Na⁺-type PomA/B of *Vibrio alginolyticus* suggest that the B subunit is important for determining the ion specificity of the stator complex (Asai *et al.*, 2000, 2003). In *Bacillus subtilis*, two types of stator complexes, MotA/B as the H⁺ type and MotP/S as the Na⁺ type, assemble around a single flagellar base, and the ion dependence of the functional hybrid stator complex (MotA/S or MotP/B) suggests the importance of the B subunit for determining the coupling ion (Ito *et al.*, 2005). It has been reported that exchanging only three amino acids between MotB and MotS in *B. subtilis* can convert the ion specificity from the H⁺ type to the Na⁺ type and vice versa (Terahara *et al.*, 2008). However, it remains unclear how the ion specificity is determined and how the coupling ion is coordinated by residues in the ion binding pocket, which then releases the ion from the binding pocket into the cytoplasm.

To investigate how the ion is selectively recognized in the stator complex and is released from it, we focused here on residues close to the essential aspartate residue of PomB. We constructed mutants in which the conserved residue in the Na⁺-type stator was changed to the corresponding residue in the H⁺-type stator, and the effects on motility were analysed. Thus we found a residue involved in determining the ion specificity and/or the ion release from PomB-D24.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Site-directed mutagenesis of *pomA* or *pomB* was done using the QuikChange procedure (Stratagene). *V. alginolyticus* was cultured in VC broth [0.5 % (w/v) Bacto tryptone, 0.5 % (w/v) yeast extract, 0.4 % (w/v) K₂HPO₄, 3 % (w/v) NaCl, 0.2 % (w/v) glucose] or in VPG500 medium [1 % (w/v) Bacto tryptone, 0.4 % (w/v) K₂HPO₄, 500 mM NaCl, 0.5 % (w/v) glycerol] at 30 °C. *E. coli* was cultured in LB broth [1 % (w/v) Bacto tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl]. Chloramphenicol was added to a final concentration of 2.5 µg ml⁻¹ for *V. alginolyticus* and 25 µg ml⁻¹ for *E. coli*.

Swimming assay in semisolid agar. VPG500 semisolid agar [1 % (w/v) Bacto tryptone, 0.4 % (w/v) K₂HPO₄, 500 mM NaCl, 0.5 % (w/v) glycerol, 0.25 % (w/v) Bacto agar] containing 0.02 % (w/v) arabinose was used for motility assays of *V. alginolyticus*. A 1 µl aliquot of each overnight culture was spotted onto VPG500 semisolid agar containing 0.02 % (w/v) arabinose or VPG500 semisolid agar containing 0.02 % (w/v) arabinose and 200 µM phenamil, and was incubated at 30 °C for the desired time.

Introduction of plasmids into *V. alginolyticus*. Transformation of *Vibrio* cells was carried out using electroporation, as described previously (Kawagishi *et al.*, 1994).

Measurement of swimming speed for *Vibrio* cells. Overnight cultures were inoculated into VPG500 medium containing 0.02 % (w/v) arabinose at a 100-fold dilution. Cells were incubated at 30 °C for 4 h and were then collected by centrifugation at 2000 g for 5 min. Cells were resuspended in TMN (50 mM Tris/HCl, pH 7.0, 5 mM MgCl₂, 5 mM glucose, total ion strength 500 mM NaCl and KCl), TMN0 (Na⁺ 0 mM, K⁺ 500 mM), TMN5 (Na⁺ 5 mM, K⁺ 495 mM), TMN10 (Na⁺ 10 mM, K⁺ 490 mM), TMN20 (Na⁺ 20 mM, K⁺ 480 mM), TMN50 (Na⁺ 50 mM, K⁺ 450 mM), TMN100 (Na⁺ 100 mM, K⁺ 400 mM), TMN300 (Na⁺ 300 mM, K⁺ 200 mM) or TMN500 (Na⁺ 500 mM, K⁺ 0 mM) buffer. The suspensions were recentrifuged and then resuspended in the same TMN buffer, and cells were diluted to 1:50 in the same TMN buffer and kept at room temperature for 15 min. Swimming of the cells was observed using dark-field microscopy immediately after the addition of serine to a final concentration of 20 mM to make the cells swim smoothly. Swimming speeds were determined from analysis of at least 20 individual cells. To investigate the effect of phenamil on swimming, cells were suspended in TMN300 buffer and phenamil was added to a final concentration of 0, 2.5, 5, 10, 20, 50 or 100 µM. After the cells were exposed to phenamil for 15 s, swimming cells were recorded and then measured by using software for motion analysis (Move-tr/2D; Library Co.).

Immunoblotting. Cells were resuspended in SDS loading buffer and boiled at 95 °C for 5 min, and then were subjected to SDS-PAGE and immunoblotting as described previously (Yorimitsu *et al.*, 1999). The

Table 1. Bacterial strains and plasmids used in the studyAbbreviations: Mot⁻, non-motile; Cm^r, chloramphenicol resistant; P_{BAD}, *araBAD* promoter.

Strain or plasmid	Genotype or description	Reference
<i>V. alginolyticus</i> strains		
NMB191	VIO5 <i>pomAB</i> (Mot ⁻)	Yorimitsu <i>et al.</i> (1999)
sp2	NMB191, up-motile phenotype when PomAB is expressed	Terashima <i>et al.</i> (2010)
<i>E. coli</i> strain		
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB)</i> (F' <i>traD36 proAB lacI^r lacZΔM15</i>)	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pBAD33	Cm ^r P _{BAD}	Guzman <i>et al.</i> (1995)
pHFAB	<i>pomA</i> and <i>pomB</i> in pBAD33	Fukuoka <i>et al.</i> (2005)

antibody to PomA (PomA1312) has been reported previously (Yorimitsu *et al.*, 1999) and the antibody to PomB (PomB_{C2}B0455) was prepared as follows: an N-terminally truncated PomB variant consisting of residues 59–315 (PomB_{C2}) with a His₆-tag fused at its C terminus was constructed, and was overexpressed in BL21(DE3) cells from plasmid pTSK36, a derivative of pET22b. Cells were disrupted and a soluble fraction was isolated by ultracentrifugation. His-tagged PomB_{C2} was purified from this soluble fraction by using a HisTrap column followed by a HiTrap Q column (GE Healthcare). Purified PomB_{C2} was separated by SDS-PAGE, stained with Coomassie blue R250 and excised for immunization. The rabbit anti-PomB_{C2} antibody (PomB_{C2}B0455) was produced by Biogate Co. Horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz) was used as the secondary antibody.

RESULTS

Motility of cells producing PomB-L35V/S38A/E41S or PomB-L35V in Na⁺-free buffer

MotA/B and MotP/S of *B. subtilis* are the stators for the H⁺- and Na⁺-driven motors, respectively. When conserved residues in the H⁺- or Na⁺-type motors were exchanged between MotB and MotS (MotB-V35L + A38S + S41Q or MotS-L41V + S44A + Q47S), MotA/B was converted to the Na⁺-driven type, whereas MotP/S was converted to the H⁺-driven type (Terahara *et al.*, 2008). Therefore, we substituted the corresponding residues of the Na⁺-type *Vibrio* PomB with those of the H⁺-type *E. coli* MotB (PomB-L35V + S38A + E41S or PomB-L35V) (Fig. 1). The swimming speeds of cells producing PomB-L35V or PomB-L35V + S38A + E41S were measured in buffer containing 0–300 mM NaCl (Fig. 2). The maximum swimming speed of cells producing PomB-L35V decreased to approximately 85 % of the wild-type value. The maximum swimming speed of cells producing PomB-L35V + S38A + E41S decreased to approximately 50 % of the wild-type value. However, in sodium-free buffer, cells producing PomB-L35V + S38A + E41S or PomB-L35V were not able to swim, and the swimming speed profiles were dependent on Na⁺ concentration (Fig. 2).

Motility of mutants converted from an Na⁺- to an H⁺-type residue

To investigate further the mechanism of the ion translocation and specificity in the stator complex, we focused on the residues that are conserved in the TM segments of the Na⁺-type stator proteins but not in those of the H⁺-type proteins, or on some residues around the boundary of the TM segments, because they may function as the entrance for ion conductance. We targeted the residues PomA-N194, PomB-F22 and PomB-S27, and replaced them with the corresponding amino acid of the H⁺-type protein (PomA-N194Y, PomB-F22Y and PomB-S27T) (Fig. 1). The swimming speeds of cells expressing PomA-N194Y, PomB-F22Y and PomB-S27T were measured in buffer containing 0–500 mM NaCl (Fig. 3). All mutants were able to swim in the presence of NaCl but not in Na⁺-free buffer, suggesting that the ion specificity was not converted. The maximum swimming speed of cells producing PomA-N194Y decreased to approximately 50 % of the wild-type value. The maximum swimming speed of cells producing PomB-F22Y was almost the same as that of the wild-type; however, these cells were unable to swim in the presence of 5 mM NaCl and appeared to have a swimming threshold between 5 and 10 mM NaCl (Fig. 3b). The swimming speed of cells expressing PomB-S27T decreased to approximately 60 % of the wild-type value. The amount of each mutant protein expressed from the plasmid was not affected by any of the mutations (data not shown).

To investigate whether the phenotype is affected by combinations of the mutations, mutants producing PomA-N194Y/PomB-F22Y, PomB-F22Y + S27T and PomB-F22Y + L35V were constructed, and their swimming speeds were measured at 0–50 mM NaCl (Fig. 4). These combination mutants were not able to swim in Na⁺-free buffer, suggesting that their ion specificity was not converted. Production of PomA-N194Y/PomB-F22Y and PomB-F22Y + S27T conferred swimming ability in buffer containing 5 mM Na⁺, but PomB-F22Y + L35V did not, indicating that PomA-N194Y and PomB-S27T suppressed the threshold phenotype of PomB-F22Y. Cells producing PomB-F22Y + L35V

Fig. 1. (a) Amino acid sequence alignments of TM3 and TM4 of PomA and the TM of PomB for various species. White letters on a black background show conserved residues which are predicted to be around the conserved aspartate and residues that were replaced in the triple mutant. The highly conserved Asp residue is marked with a filled circle. Ec, *E. coli*; Bs, *B. subtilis*; So, *Shewanella oneidensis*; Va, *V. alginolyticus*. (b) Putative membrane topology of TM3 and TM4 of PomA and the TM of PomB, and sites of residue substitutions made in this study. The bold circle shows the essential Asp of PomB (Asp24). White letters in filled squares show residues that were replaced in the triple mutant (PomB-L35 and S38, E41). White letters in filled circles show the conserved residues which are predicted to be around the conserved Asp and which replaced the H⁺-type residue in this study.

required an Na⁺ concentration between 10 and 20 mM NaCl to exhibit swimming.

Motility of various PomB-F22 mutants

The phenotype of the PomB-F22Y mutant suggested that the mutation may affect ion conduction or torque

Fig. 2. Swimming speeds of cells producing PomA/PomB (●), PomA/PomB-L35V+S38A+E41S (◆) or PomA/PomB-L35V (■). Overnight cultures of cells were inoculated in VPG500 medium and incubated at 30 °C for 4 h. Swimming speeds were measured in buffer containing 0–300 mM NaCl.

generation. We carried out site-directed mutagenesis to replace Phe22 of PomB with various amino acid residues, K, D, L, W, Y, A, S and N. The swimming speed for each mutant was then measured in buffer containing 0–500 mM Na⁺ (data not shown). Except for the F22K and F22D mutants, the other six mutants exhibited motility at 500 mM NaCl. F22L and F22W showed nearly the same Na⁺ dependence and swimming speed as the wild-type. On the other hand, F22A had an Na⁺ threshold to exhibit swimming between 5 and 10 mM NaCl, similar to F22Y (Fig. 5a), and F22S and F22N had a threshold between 10 and 20 mM NaCl (Fig. 5b, c). The maximum swimming speeds of the F22L, F22W and F22Y mutants were almost the same as the original F22, while that of the F22A, F22S and F22N mutants decreased by approximately 50 % compared with F22. We also checked the swimming ability of the PomB-F22 mutants in semisolid agar containing 500 mM NaCl (Fig. 6a). Except for the F22L mutant, the swimming abilities in liquid and in semisolid agar for each mutant were comparable. The swimming ring size of F22L decreased to approximately 50 % of the wild-type value. These results show that various mutations of the F22 residue of PomB seem to affect motility in an Na⁺ concentration-dependent manner. Small hydrophobic (F22A) or hydrophilic (F22S and F22N, F22Y) residues showed a threshold of Na⁺ concentration to exhibit swimming (Fig. 5). However, large hydrophobic residues (F22L and F22W) did not show such a threshold (data not shown). The side-chain volume of Leu and Asn is

Fig. 3. Swimming speeds of cells producing PomA/PomB (●), PomA-N194Y/PomB (▲), PomA/PomB-F22Y (■) or PomA/PomB-S27T (◆). (a) Swimming speeds were measured as described in the legend of Fig. 2. (b) Enlargement of the swimming speed data between 0 and 50 mM shown in (a).

almost the same; however, the substitutions conferred different phenotypes. Thus, the hydrophobicity of F22 seems to be important for stator function through ion conduction.

Phenamil resistance of various PomB-F22 mutants

If a mutation affects the Na^+ -dependent swimming property, it might also change the sensitivity to a Na^+ channel blocker or a specific inhibitor of Na^+ -driven motors

such as phenamil. We checked the motilities of various PomB-F22 mutants in the presence of 200 μM phenamil by using semisolid agar containing 500 mM NaCl (Fig. 6b). The F22K and F22D mutants did not show swimming expansion even in the absence of phenamil. F22A showed a similar swimming expansion profile to that of the wild-type, while F22L and F22Y showed a slightly larger swimming expansion than the wild-type. F22W, F22S and F22N showed an even larger swimming expansion than F22L and F22Y. We next measured the swimming speeds of cells producing PomB-F22W, F22S, F22N and F22Y in buffer containing 300 mM Na^+ with a final concentration of 0–100 μM phenamil (Fig. 7). Mutants producing F22W or F22Y were able to swim in the presence of 100 μM phenamil, but the swimming speed of cells decreased with increasing phenamil concentration. On the other hand, the motility of cells producing F22S or F22N remained at the same level, even at higher concentrations of phenamil. F22S and F22N showed strong resistance to phenamil. These results suggest that replacement of Phe22 with a large hydrophobic (W) or hydrophilic residues (S and N, Y) weakens the interaction with phenamil.

Conversion of the ion specificity in the up-motile strain

When PomA-N194Y, PomB-F22Y or PomB-S27T was produced in the sp2 strain, which gives an increased motility phenotype (Terashima *et al.*, 2010), PomA-N194Y conferred slight swimming ability (mean $\pm$ SD $1.8 \pm 0.76 \mu\text{m s}^{-1}$) even in Na^+ -free buffer (see Supplementary Movie), but the wild-type and PomB-F22Y and PomB-S27T mutants did not (data not shown). Because we suspected possible contamination with Na^+ from the VPG500 broth, we washed the cells twice with Na^+ -free buffer. Cells producing PomA-N194Y conferred only slight swimming ability, even after the additional washes. In 0 mM Na^+ buffer with phenamil or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), this mutant was no longer able to confer motility. From these results, we were not able to determine which ion was used to rotate the flagellar motor by PomA-N194Y. However, the wild-type PomA/B was not able to confer motility at all by using the standard protocol with two washes. Therefore, we speculate that PomA-N194Y may use a different ion, such as H^+ , to rotate the flagellar motor; alternatively, the PomA-N194 stator may be driven by an extremely low Na^+ concentration.

DISCUSSION

It has been proposed that the highly conserved aspartate residue (PomB-D24) binds the coupling ion and forms an ion binding pocket with PomA-N194 (Fig. 8) (Sudo *et al.*, 2009a; Terashima *et al.*, 2010). If the ion binding pocket is not the correct size for the coupling ion or if residues of the ion binding pocket are not correctly aligned to interact with the coupling ion, the conformational change of the

Fig. 4. Swimming speeds of the mutant stators combined with PomB-F22Y. Swimming speeds of cells producing PomA/PomB (a), PomA/PomB-L35V (b), PomA-N194Y/PomB-F22Y (c), PomA/PomB-F22Y+S27T (d) or PomA/PomB-F22Y+L35V (e) were measured as described in the legend of Fig. 2 in buffer containing 0–50 mM NaCl.

stator complex followed by the binding and release of the coupling ion will probably not occur. In other words, if the Na^+ binding pocket of PomA/B becomes properly coordinated to H^+ by mutagenesis, we should be able to produce an H^+ -driven PomA/B complex. It has been shown clearly that the ion specificity can be changed by replacing some residues of MotB around the putative periplasmic entrance of the ion (Terahara *et al.*, 2008).

Therefore, we changed the corresponding residues of PomB, but this had no effect on ion selectivity. Other amino acid residues of the periplasmic surface may be important for determining ion binding and specificity. Unfortunately, we could not identify the periplasmic surface residues which are involved in ion binding and specificity. However, we found that PomA-N194 may be important for ion specificity and may create an ion binding

Fig. 5. Swimming speeds of various PomB-F22 mutants. Swimming speeds of cells producing F22A (a), F22S (b) or F22N (c) were measured as described in the legend of Fig. 2 in buffer containing 0–50 mM NaCl.

site with PomB-D24 at the central TM region. Some periplasmic surface residues may also contribute to the ion specificity. This means that the recognition mechanism for

Fig. 6. Effects of phenamil on the swimming abilities of the various PomB-F22 mutants in semisolid agar. (a) Cells were inoculated on 0.25 % agar VPG plates and were incubated at 30 °C for 5 h. (b) Cells were inoculated on 0.25 % agar VPG plates containing 200 μM phenamil and were incubated at 30 °C for 24 h.

an ion is not simple and that multiple sites are necessary to determine the specificity, as discussed below.

In this study, we found that PomA-N194Y confers the ability to swim in an Na^+ -free buffer in the increased motility strain sp2. However, we could not determine what the coupling ion is or whether the Na^+ -type PomA/B has been converted to the H^+ type. Attenuated total reflection Fourier transform infrared spectroscopy has shown that the PomA/B complex has at least three Na^+ binding carboxyl residues. One of the Na^+ binding carboxyl residues is the essential aspartate, PomB-D24, and the others are as yet unidentified (Sudo *et al.*, 2009a). We suggest that other as-yet-unidentified Na^+ binding sites may contribute to determining the ion specificity and/or to promoting ion flow by efficient binding of the ions. Because PomA-N194 is thought to be the residue that forms the ion binding pocket with PomB-D24, the dramatic decrease of the swimming speed of cells expressing PomA-N194Y may derive from a slow flux of Na^+ because of inefficient recognition or binding of ions. The corresponding residue

Fig. 7. Effects of phenamil on the swimming abilities of the various PomB-F22 mutants. Swimming speeds of cells producing PomB (a), PomB-F22Y (b), F22W (c), F22S (d) or F22N (e) were measured as described in the legend of Fig. 2 in buffer containing 300 mM NaCl and various concentrations of phenamil.

of the H⁺-type MotA is conserved as tyrosine. This may imply that the tyrosine and/or asparagine contributes to the ion specificity (H⁺ or Na⁺) of the flagellar motor. We think that PomA-N194 might be one of the most important residues for ion specificity, although there are probably other residues that contribute. NtpK, which is a V-ATPase rotor ring component, coordinates Na⁺ with the essential carboxyl group of E139, the carbonyl group of Q65 and Q110, the hydroxyl group of T64 and the main chain carbonyl group of L61 (Murata *et al.*, 2005).

Therefore, we speculate that the ion binding pocket of PomA/B is composed of residues other than PomB-D24 and PomA-N194 that coordinate Na⁺ ions. So, to generate an H⁺-driven PomA/B complex, the introduction of two or more mutations with PomA-N194Y in TM4 may be required in other TM regions (TM1–3) of PomA.

It has been reported that the PomA-D31C mutant cells require a higher Na⁺ concentration (greater than 19 mM NaCl) to exhibit swimming motility (Kojima *et al.*, 2000).

Fig. 8. Model of the ion flux pathway. Asp24 of PomB forms an Na^+ binding site which is suggested to make an ion binding pocket with Asn194 of PomA. Cys31 of PomB and Ala39 of MotB form a pair of amino acids for the H^+ and Na^+ channels with Leu183 of PomA and Met206 of MotA, respectively (Sudo *et al.*, 2009a, b; Terashima *et al.*, 2010). Phe22 of PomB is near an ion flow pathway and is close to the Na^+ binding site of Asp24, and might be involved in the release of Na^+ from PomB-D24. It has been suggested that PomA-D148 and PomB-P16 contribute to forming a high-affinity phenamil binding site at the inner face of the PomA/PomB complex.

When PomA-D31 was substituted to charge-inverted or large side-chain amino acids, the Na^+ thresholds of those mutants were even higher, above 50 mM NaCl. It has been speculated that the efficiency of Na^+ flow is increased by the negative charge at D31 and that Na^+ ions may be recruited by this negative charge. The motility of cells producing PomB-F22Y was decreased at low Na^+ concentration but did not change at high Na^+ concentration compared with the wild-type. We thus hypothesized that the F22Y mutation affects ion flux or torque generation. F22L, F22W and F22Y conferred almost the same maximal swimming speed as the wild-type, while the maximal swimming speeds of the F22A, F22S and F22N mutants were decreased to approximately 50 % of the wild-type value. The F22A, F22S and F22N mutants also showed a threshold of Na^+ concentration for swimming motility. On semisolid agar containing 500 mM NaCl, except for the F22L mutant, all swimming ring sizes correlated with the swimming speeds in the liquid. The size of the swimming ring of the F22L mutant was decreased to approximately 50 % of the wild-type value on semisolid agar, suggesting that torque might be reduced. The

aromatic ring at position F22 might be important to generate maximal torque. In the F22Y mutant, the hydroxyl group of Tyr might lead to the motility defect under low Na^+ conditions. We speculate that the Na^+ flux might be reduced by the replacement of F22 in PomB with hydrophilic residues. In other words, the hydrophobicity at PomB-F22 is important to effectively release Na^+ from PomB-D24. On the other hand, in the H^+ -driven motor, the residue corresponding to PomB-F22 is a conserved Tyr of MotB, implying that a hydroxyl group in this position is important for H^+ flux. We speculate that the aromatic ring at this position is important to generate a strong torque.

Aromatic amino acids are often found in ion channels and are important to regulate ion flow. It has been reported that the highly conserved Phe of voltage sensor proteins is important for channel function. For example, Phe233 of the Shaker K^+ channel might provide the rigid side chain on the structure and is important for its function. Among the residues replaced with various amino acids at Phe233, only two substitutions (Tyr and Trp) produce currents near wild-type levels with a negative V_m (midpoint voltage) (Tao *et al.*, 2010). We do not know whether the function of the Phe residue of Shaker is similar to that of Phe22 of PomB; however, aromatic amino acids may be used to maintain the size of the pore and to regulate ion flow in channels.

Cells expressing PomB-F22Y had a phenamil-resistant phenotype. It has been reported that the PomA-D148Y, PomB-P16S and PomB-A23S mutations, which are located on the cytoplasmic side of the TM segments, confer phenamil resistance upon the PomA/B complex, and it has been proposed that phenamil interacts with the cytoplasmic side of the ion-conducting pathway (Jaques *et al.*, 1999; Kojima *et al.*, 1999). Replacement of Phe22 in PomB with large hydrophobic (W) or hydrophilic residues (S and N) also conferred phenamil resistance upon the motor. Among these mutants, F22S and F22N had strong phenamil resistance. These results are consistent with the idea that Phe22 is involved in an ion-conducting pathway and is close to the Na^+ binding site.

PomB-F22Y would be on nearly the opposite helix face from PomB-D24 according to helical wheel modelling. However, a cross-linking study of MotA/B proposed that one MotA and one MotB subunit form an ion binding pocket and that the carboxyl group of MotB-D32 (corresponding to PomB-D24) directs ions to the pocket, although MotB-Y30 (corresponding to PomB-F22) is thought to face the other MotA subunit (Braun *et al.*, 2004). In the cross-linking study of TolQR, which are inner-membrane proteins of the Tol-Pal system that show homology with MotA/B and PomA/B, it has been proposed that TolR rotates between two TolQ subunits (Zhang *et al.*, 2009). If the TM region of PomB is also assumed to rotate among the TM regions between the two PomA subunits, even residues on the helix face opposite the PomB TM can orient to the helix face of the PomA TM segments.

On the basis of our results, combined with previous studies, we propose a model for the ion flux pathway through MotA/B and PomA/B ion channels (Fig. 8). First, the ion passes through the periplasmic side of a channel pore, a part of which is formed by Leu183 of PomA and Cys31 of PomB, or Met206 of MotA and Ala39 of MotB (Sudo *et al.*, 2009b). Next, the ion binds to the pocket that is formed mainly by Asn194 of PomA and Asp24 of PomB, or by Tyr217 of MotA and Asp32 of MotB (Sudo *et al.*, 2009b; Terashima *et al.*, 2010). Finally, an event to generate torque starts, and the ion is released from the ion binding pocket to the cytoplasm, and at this stage, Phe22 of PomB or Tyr30 of MotB might be important. In this study, we introduced mutations into TM4 of PomA or into the TM of PomB. To convert from the Na⁺ to the H⁺ type, mutations need to be introduced in residues of PomA TM1, 2 or 3, and the mutations need to be combined.

ACKNOWLEDGEMENTS

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan, the Japan Science and Technology Corporation (to M.H. and to S.K.), the Soft Nano-Machine Project of the Japan Science and Technology Agency (to M.H.) and the Japan Society for the Promotion of Science (to H.T.).

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Edited by: P. W. O'Toole