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Interactions of MotX with MotY and with the PomA/PomB Sodium Ion Channel Complex of the *Vibrio alginolyticus* Polar Flagellum*

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Rotation of the sodium ion-driven polar flagellum of *Vibrio alginolyticus* requires the inner membrane sodium ion channel complex PomA/PomB and the outer membrane components MotX and MotY. None of the detergents used in this study were able to solubilize MotX when it was expressed alone. However, when co-expressed with MotY, MotX was solubilized by some detergents. The change in the solubility of MotX suggests that MotY interacts with MotX. In agreement with this, a pull-down assay showed the association of MotY with MotX. Solubilized MotX and MotY eluted in the void volume from a gel-filtration column, suggesting that MotX and MotY form a large oligomeric structure(s). In the absence of MotY, MotX affected membrane localization of the PomA/PomB complex and of PomB alone but not of PomA alone, suggesting an interaction between MotX and PomB. We propose that MotX exhibits multiple interactions with the other motor components, first with MotY for its localization to the outer membrane and then with the PomA/PomB complex through PomB for the motor rotation.

The bacterial flagellum is driven by a specific ion flow depending on the electrochemical potential (1, 2). Two types of coupling ions, protons and sodium ions, are known. The motor is embedded in the cytoplasmic membrane at the base of the flagellar structure and is composed of the stator and rotor components. The rotor consists of a rod and two ring structures termed the MS and C rings. Two additional rings, the L and P rings that are embedded in the outer membrane and the peptidoglycan layer, respectively, surround the rod and act as a molecular bushing of the rotor in Gram-negative bacteria. It has been proposed that the C ring, which is mounted onto the cytoplasmic face of the MS ring, is surrounded by the stator particles (3, 4). In the proton-driven motors of *Escherichia coli* and *Salmonella*, the stator part of the force-generating unit is composed of MotA and MotB (5, 6). MotA has four transmembrane segments and one large cytoplasmic region (7). MotB has a single N-terminal transmembrane segment, in which an important Asp is present to convey protons (8), and a peptidoglycan-binding motif at the C terminus (9, 10). Several extensive lines of genetic and physiological studies have shown that the transmembrane segments of MotA and MotB form a H^+ channel complex (11–13).

The polar flagellum of *Vibrio alginolyticus* is driven by a sodium ion motor (14–16). In this motor, PomA, PomB, MotX, and MotY are required for force generation (17–20). PomA and PomB are orthologs of MotA and MotB, respectively. It has been shown that the PomA/PomB complex catalyzes sodium ion influx when the purified PomA/PomB complex is reconstituted into proteoliposomes (21). A complex formed from four PomA and two PomB molecules seems to act as a sodium ion channel.

MotX and MotY have been identified in *Vibrio* sp. as specific motor proteins for the sodium ion-driven motor (22, 23). No homologues have been reported in *E. coli* or *Salmonella*. Recently, MotY homologues were reported in the lateral flagellar system of *Vibrio* sp., in which the coupling ions are protons, and in the flagellum of *Pseudomonas aeruginosa* (24, 25). MotY has a peptidoglycan-binding motif in the C-terminal region, which is also found in MotB and PomB (20). MotX and MotY seem to interact with each other, although direct evidence for this interaction has not yet been obtained (19, 23). Recently we have shown that the N-terminal segments of MotX and MotY are cleaved, and the mature proteins are fractionated with the outer membrane, suggesting that they are outer membrane proteins (26).

The cytoplasmic region of MotA is postulated to interact with the rotor component FliG. Electrostatic interactions between conserved charges on FliG and those on MotA are important for the rotation of the proton-driven motor of *E. coli* (27). It is believed that both proton- and sodium ion-driven motors rotate by a similar mechanism because several chimeric motors composed of both types of components are functional (28–30). However, amino acid substitutions in the conserved charged residues of PomA or FliG in the sodium ion-driven motor of *V. alginolyticus* do not abolish motility (31, 32). These discrepancies between the proton- and sodium ion-driven motors suggest that other charged residues are required for the flagellar rotation in the sodium ion-driven motor or that MotX and MotY compensate for the effects of the amino acid substitutions in PomA and FliG. If the latter is true, there may be some interactions among the motor proteins, although there is no evidence for interactions between MotX and/or MotY and the PomA/PomB complex.

In this study, we have shown that MotX interacts with MotY and affects membrane localization of the PomA/PomB complex when MotY is absent. This is the first direct evidence for interaction of the inner membrane ion channel complex with the outer membrane component in the flagellar motor.

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TABLE I
Bacterial strains and plasmids
Rif^r, rifampicin-resistant; Laf$^-$, defective in lateral flagellar formation; Km^r, kanamycin-resistant

Strain or plasmid	Genotype or description	Reference
V. alginolyticus strains		
VIO542	Rif^r Laf$^-$ *motY542*	20
NMB94	Laf$^-$ *motX94*	19
NMB191	Rif^r Laf$^-$ Δ*pomAB*	34
Plasmids		
pSU41	*kan* (Km^r) P_{lac} *lacZα*	36
pIO6	pSU38, 1.0-kb HindIII-XbaI fragment (*motY*$^+$)	20
pKJ401	pSU41, 700-bp XbaI-BamHI fragment (*motX*$^+$)	26
pKJ502	pSU41, 1.0-kb SalI-XbaI fragment (*motY*$^+$)	26
pKJ503	pSU41, 1.0-kb SalI-XbaI fragment (*motY-his*$_6^+$)	26
pKJ601	pKJ502 (*motY*$^+$), 700-bp XbaI-BamHI fragment (*motX*$^+$)	26
pKJ602	pKJ502 (*motY*$^+$), 700-bp XbaI-BamHI fragment (*motX-his*$_6^+$)	This work
pKJ701	pKJ601 (*motY*$^+$ *motX*$^+$), 1.9-kb BamHI-SacI fragment (*pomAB*$^+$)	26
pMO401	pSU41, 700-bp ClaI-BamHI fragment (*motX*$^+$)	19
pMO402	pSU41, 700-bp ClaI-BamHI fragment (*motX-his*$_6^+$)	This work
pMO711	pKJ401 (*motX*$^+$), 1.9-kb BamHI-SacI fragment (*pomAB*$^+$)	This work
pMO712	pKJ502 (*motY*$^+$), 1.9-kb BamHI-SacI fragment (*pomAB*$^+$)	This work
pMO721	pKJ401 (*motX*$^+$), 800-bp BamHI fragment (*pomA*$^+$)	This work
pMO722	pKJ401 (*motX*$^+$), 1.1-kb BamHI-SacI fragment (*pomB*$^+$)	This work
pSK603	pSU41, 1.1-kb BamHI-SacI fragment (*pomB*$^+$)	37
pYA301	pSU41, 800-bp BamHI fragment (*pomA*$^+$)	37
pYA303	pSU41, 1.9-kb BamHI-SacI fragment (*pomAB*$^+$)	37

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The *E. coli* strain JM109 was used for DNA manipulations (33). The *V. alginolyticus* strains used in this study are listed in Table I. *V. alginolyticus* cells were cultured at 30 °C in VC medium (0.5% Tryptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.2% glucose) or in VPG medium (1% Tryptone, 0.4% K_2HPO_4, 3% NaCl, 0.5% glycerol). The *E. coli* strains used for DNA manipulations and membrane localization experiments were cultured at 37 °C in LB medium (1% Tryptone, 0.5% yeast extract, 0.5% NaCl). When necessary, kanamycin was added to a final concentration of 100 μg/ml (for *V. alginolyticus*) or 50 μg/ml (for *E. coli*).

Plasmid Construction—DNA manipulations were carried out according to standard procedures (35). Plasmids used in this study are listed in Table I. Plasmid pKJ402, which was constructed by PCR using synthetic oligonucleotide primers, encodes the C-terminal hexahistidine-tagged MotX (MotX-His_6). The nucleotide sequence of 5′-CATCACCATCACCATCACTAAGGATCC-3′ (the BamHI site is underlined) was inserted between the 3′ end of the *motX* open reading frame and the BamHI site of plasmid pKJ401. Because this plasmid complemented a *motX* mutant of *V. alginolyticus*, the C-terminal His_6 tag does not appear to perturb MotX function (data not shown). The 700-bp XbaI-BamHI fragment (containing *motX-his*$_6$) of pKJ402 was inserted into pKJ502 (carrying *motY*) to construct a plasmid, pKJ602, that carries an artificial *motY-motX-his*$_6$ operon. The 1.9-kb BamHI-SacI fragment (containing *pomAB*) of pYA303 was inserted into pKJ401 (carrying *motX*), constructing a plasmid, pMO711, that carries an artificial *motX-pomAB* operon. This BamHI-SacI fragment was inserted into pKJ502 (carrying *motY*) to construct a plasmid, pMO712, which carries an artificial *motY-pomAB* operon. The 0.8-bp BamHI fragment (containing *pomA*) of pYA301 was inserted into pKJ401 to construct a plasmid, pMO721, which carries an artificial *motX-pomA* operon. The 1.1-kb BamHI-SacI fragment (containing *pomB*) of pSK603 was inserted into pKJ401; the resultant plasmid, pMO722, carries an artificial *motX-pomB* operon. The motor genes of the plasmids constructed here do not have their native promoters and are under the control of the *lac* promoter operator. Transformation of *Vibrio* cells by electroporation was carried out as described previously (38).

Membrane Preparation—*Vibrio* cells harboring plasmids were cultured at 30 °C in VPG medium. Cells were harvested, washed twice with V-buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 300 mM NaCl), and stored at −80 °C until use. The frozen cells were thawed and resuspended (0.2 g, wet weight/ml) in 20 mM Tris-HCl, pH 8.0, 5 mM $MgSO_4$, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml DNase I. Membrane vesicles were prepared by subjecting the suspension to a single passage through a French press (5501-M Ohtake Works) at 500 kg/cm^2 at 4 °C. Undisrupted cells were removed by low speed centrifugation (10,000 × *g* for 5 min at 4 °C), and the membrane fraction was recovered from the supernatant by ultracentrifugation at 100,000 × *g* for 2 h. The membrane pellet was suspended in 20 mM Tris-HCl, pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride, 20% (w/v) glycerol and then homogenized and stored at −80 °C until use.

Detection of Proteins—SDS-PAGE and immunoblotting were performed as described previously (34). The antibodies against MotX peptide and MotY peptide, which are referred to as MotX192 and MotY264, respectively, were prepared as described previously (19). Protein concentrations were determined with the BCA protein assay kit (Pierce).

Membrane Solubilization by Detergents—Membrane preparations were suspended in 20 mM Tris-HCl, pH 8.0, containing 0 or 450 mM NaCl to yield final concentrations of 10 mg of protein/ml. After 72 different kinds of detergents from Detergent Screens 1, 2, and 3 (Hampton Research) were added at 2-fold the critical micelle concentration, each suspension was mixed gently overnight at 4 °C and centrifuged for 10 min at 200,000 × *g* to separate the solubilized supernatant and the insoluble precipitate. For solubilization of MotX or MotY, five detergents were selected: *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-glucoside, *n*-decylsulfobetaine (ZWITTERGENT 3-10), octanoyl-*N*-methylglucamide (MEGA-8), and nonanoyl-*N*-methylglucamide (MEGA-9).

Pull-down Assay—Membranes from NMB94 harboring pKJ602 or pKJ601 were suspended in 20TPG (20 mM Tris-HCl, pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride, 10% (w/v) glycerol) containing 5 mM imidazole and 1.5% (w/v) *n*-octyl-β-D-glucoside to yield final concentrations of 10 mg of protein/ml. The suspension was stirred overnight at 4 °C and centrifuged for 10 min at 200,000 × *g*. The clarified supernatant was passed through a Ni^{2+}-NTA-agarose[1] column (Qiagen), and then the column was washed with the same buffer. Elution was conducted three times with 20TPG containing 800 mM imidazole and 1.5% (w/v) *n*-octyl-β-D-glucoside.

Gel-filtration Chromatography—The membrane fractions from NMB94 harboring pKJ601 were suspended in 20 mM Tris-HCl, pH 8.0, 1.5% (w/v) *n*-octyl-β-D-glucoside to yield final concentrations of 10 mg of protein/ml. The suspension was stirred overnight at 4 °C and centrifuged for 10 min at 200,000 × *g*. The clarified supernatant (200 μl) was separated on a Superdex™ 200HR 10/30 column (Amersham Biosciences) equilibrated with the same buffer at a flow rate of 0.5 ml/min. The eluates were collected in 500-μl fractions starting 14 min after sample injection.

Membrane Localization of Motor Proteins—E. coli strain JM109 harboring plasmid pYA303, pMO711, pMO712, pSK603, pMO721, or pMO722 was grown on LB medium at 37 °C to mid-exponential phase, and expression of motor proteins was induced for 1 h by the addition of 1 mM isopropyl β-D-thiogalactopyranoside prior to harvest. The cells were harvested and converted to spheroplasts. The spheroplast suspension was sonicated, and the total membrane fraction was obtained by centrifugation (200,000 × *g*, 10 min) after the removal of unbroken spheroplasts. The total membranes were resuspended in 50 mM Tris-

[1] The abbreviation used is: Ni^{2+}-NTA, nickel-nitrilotriacetic acid.

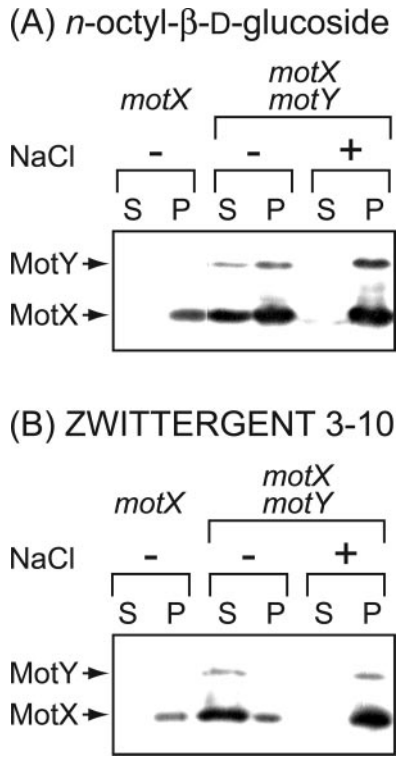

FIG. 1. **Solubilization of MotX and MotY by *n*-octyl-β-D-glucoside (*A*) or ZWITTERGENT 3-10 (*B*).** Membranes of NMB94 harboring pKJ402 (*motX*) or pKJ601 (*motYmotX*) were treated overnight with a 2-fold concentration of the critical micelle concentration of the detergent in the presence or absence of NaCl. The supernatant (*S*) and pellet (*P*) were separated by ultracentrifugation (200,000 × *g*, 10 min) and subjected to SDS-PAGE and immunoblotting using anti-MotX and anti-MotY antisera.

HCl, pH 7.5, containing 1 mM EDTA and then applied to a 35–55% (w/w) stepwise sucrose gradient containing 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA. After centrifugation at 80,000 × *g* for 12 h at 4 °C, the gradient was divided into 20–25 fractions from the bottom to the top. OmpA, SecG, MotX, MotY, PomA, and PomB proteins in each fraction were detected by SDS-PAGE followed by immunoblotting with the respective antibodies (19, 34, 39, 40).

RESULTS

Solubilization of MotX and MotY—Some detergents such as Triton X-100 solubilized MotY. However, MotX was not solubilized by any detergents used so far (26). To carry out biochemical characterization of these proteins in more detail, we examined 72 different kinds of detergents to determine whether they solubilize MotX and MotY. Because we have examined solubilization in the presence of 150 mM NaCl in a previous study (26), in this study we examined detergents in the presence or absence of 450 mM NaCl. We found that eight detergents, six nonionic (*n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-glucoside, CYMAL-3, MEGA-8, *n*-hexyl-β-D-glucoside, and MEGA-9) and two zwitterionic (ZWITTERGENT 3-10 and FOS-choline-8), solubilized MotX and MotY when co-expressed from a plasmid (Fig. 1 for the second and seventh detergents in the above list). MotX and MotY were solubilized by these detergents only in the absence, but not in the presence, of salt. From the detergents listed above, we used *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-glucoside, MEGA-8, MEGA-9, and ZWITTERGENT 3-10 to assess the solubility properties of MotX when it was expressed alone. None of the detergents solubilized MotX alone (Fig. 1 for the second and fifth detergents in the above list), suggesting that the solubility of MotX changed in the presence of co-expressed MotY, probably because of interactions between MotX and MotY.

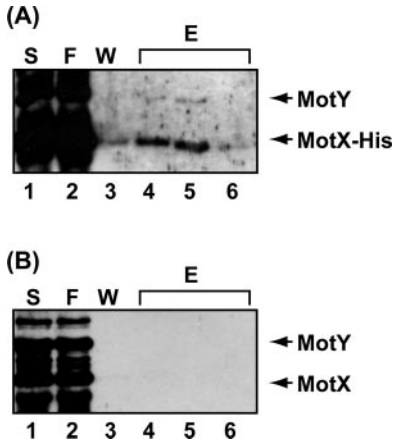

FIG. 2. **Pull-down assay of MotY with MotX-His$_6$.** Membranes of NMB94 (*motX*) harboring pKJ602 (*motYmotX-his$_6$*) (*A*) or pKJ601 (*motYmotX*) (*B*) were solubilized overnight with 1.5% *n*-octyl-β-D-glucoside in the absence of NaCl. The supernatant was passed through a Ni^{2+}-NTA column. The bound materials were eluted with high imidazole buffer after extensive washing of the column with low imidazole buffer as described under "Experimental Procedures." Solubilized membrane supernatant (*S*), column flow-through (*F*), column wash (*W*), and eluate (*E*) fractions were analyzed by SDS-PAGE and immunoblotting using anti-MotX and anti-MotY antisera.

Interaction between MotX and MotY—To investigate whether MotX actually interacts with MotY, we performed a pull-down assay with MotX-His$_6$. The binding efficiency of MotX-His$_6$ to a Ni^{2+}-NTA resin was very poor, and almost the same amount of MotX-His$_6$ was recovered in the flow-through fraction as was in the applied materials (Fig. 2*A*, compare *lanes 1* and *2*). This may be because of sequestration of the C-terminal His$_6$ tag by oligomerization of MotX (see below). However, a small but significant amount of MotX-His$_6$ appeared in the elution fractions. Untagged MotY was also found in the elution fractions (Fig. 2*A*). On the other hand, neither MotX nor MotY was detected when untagged MotX and MotY were used, confirming that no nonspecific binding to the column occurred. Therefore, we conclude that MotX binds to MotY.

Analytical Gel-filtration Chromatography of MotX and MotY—To estimate the molecular size of the MotX/MotY complex, the proteins solubilized by *n*-octyl-β-D-glucoside were analyzed by a Superdex™ 200 gel-filtration column. Both MotX and MotY eluted in the void volume (Fig. 3). Thus, it is likely that MotX and MotY form an oligomer(s) too large to analyze. In agreement with this result, such large oligomers were also detected in the high molecular weight fractions of a sucrose density gradient centrifugation experiment (data not shown). To rule out the possibility of artifactual oligomerization because of their overexpression, MotX solubilized from wild-type cells expressing only chromosomal levels of *motX* was also shown to elute in the void volume (data not shown).

The biochemical properties of MotY seemed to be drastically influenced by salt. Because most of MotY was present in the periplasmic space in soluble form in the absence of MotX (26), both membrane-bound (Fig. 4*A*) and soluble (Fig. 4*B*) MotY were analyzed by gel-filtration chromatography. Both forms eluted in the void volume in the absence of NaCl (Fig. 4, *upper panels*). On the other hand, in the presence of 300 mM NaCl, both membrane-bound and soluble MotY appeared in the fractions between albumin (67 kDa) and chymotrypsinogen A (25 kDa), which corresponds to the monomeric size of MotY (Fig. 4, *lower panels*). Similarly, the monomeric form of MotY was observed at the same concentration of KCl (data not shown).

Alteration in Membrane Localization of PomA and PomB by Co-expression with MotX—When co-expressed, MotX and MotY

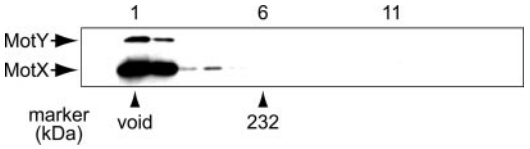

FIG. 3. **Analytical gel-filtration chromatography of MotX and MotY with a Superdex™ 200HR 10/30 column.** The membrane fractions of VIO542 (*motY*) harboring pKJ601 (*motYmotX*) were solubilized overnight with 1.5% *n*-octyl-β-D-glucoside, and the solubilized supernatant was separated and fractionated by the column as described under "Experimental Procedures." MotX and MotY in each elution fraction were detected by immunoblotting with anti-MotX and anti-MotY antisera after SDS-PAGE. The positions of the molecular mass markers, blue dextran (void volume) and catalase (232 kDa), are shown *below* the *panel*.

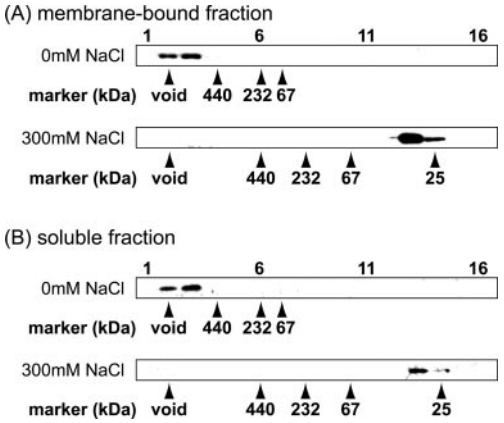

FIG. 4. **Analytical gel-filtration chromatography of MotY with or without salt.** After VIO542 (*motY*) cells harboring pIO6 (*motY*) were disrupted by sonication, the soluble and membrane fractions were prepared in the presence or absence of NaCl. *A*, the membrane fractions of VIO542 (*motY*) cells harboring pIO6 (*motY*) were solubilized with 1.5% *n*-octyl-β-D-glucoside overnight without NaCl or with 300 mM NaCl. The detergent-solubilized fractions were separated and fractionated by gel-filtration chromatography with a Superdex™ 200HR 10/30 column as described under "Experimental Procedures." *B*, the soluble fractions were analyzed. MotY in each of the elution fractions was detected by immunoblotting with anti-MotY antiserum after SDS-PAGE. The positions of the molecular mass markers, blue dextran (void volume), ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), and chymotrypsinogen A (25 kDa), are shown *below* the *panels*.

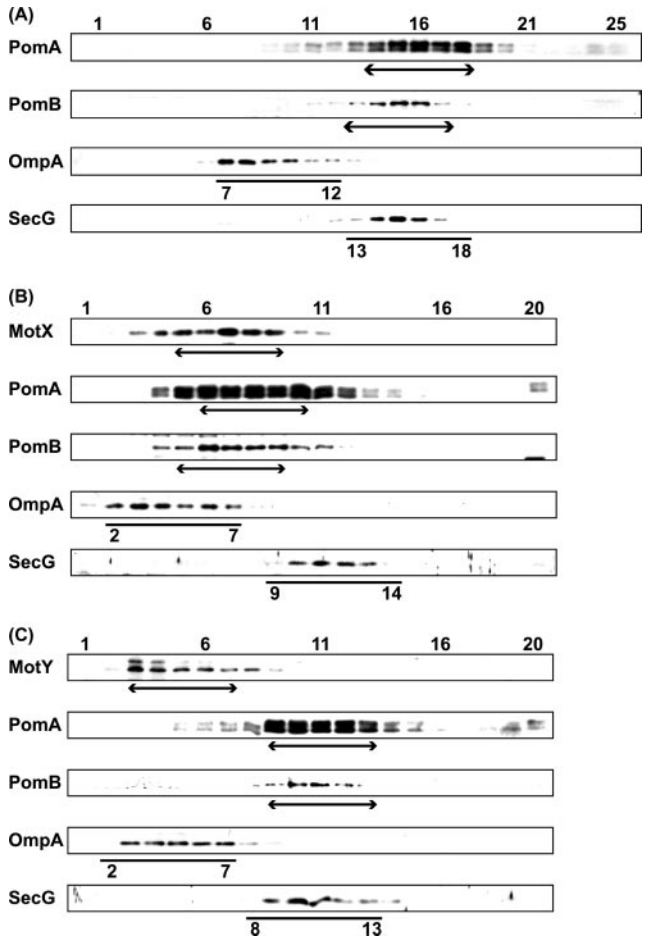

FIG. 5. **Membrane localization of PomA, PomB, MotX, and MotY in *E. coli* JM109 harboring pYA303 (*pomAB*) (*A*), pMO711 (*motXpomAB*) (*B*), or pMO712 (*motYpomAB*) (*C*).** OmpA and SecG were used as the diagnostic marker proteins for the outer and inner membranes, respectively. Membrane preparations were analyzed by sucrose density gradient centrifugation followed by fractionation into 25 or 20 fractions. PomA, PomB, MotX, MotY, OmpA, and SecG in each fraction were analyzed by immunoblotting after SDS-PAGE. OmpA and SecG were detected in fractions 7–12 and 13–18 (*A*), 2–7 and 9–14 (*B*), and 2–7 and 8–13 (*C*), respectively. PomA, PomB, MotX, and MotY are demarcated in the fractions with *double-headed arrows*.

are detected in the outer membrane fraction after sucrose density gradient ultracentrifugation. In contrast, in the absence of MotY, MotX is detected in intermediate fractions between the inner and outer membrane fractions (26). When PomA, PomB, MotX, and MotY are expressed, MotX and MotY are mainly found in the outer membrane fractions, whereas PomA and PomB are present in the inner membrane fractions (26).

To test whether MotX and/or MotY affect membrane localization of the PomA/PomB complex, we carried out sucrose density gradient centrifugation experiments. The model organism *E. coli* was used because methods for the separation of the inner and outer membranes of *V. alginolyticus* are not as well established. OmpA and SecG were used as the diagnostic marker proteins for the outer and inner membranes, respectively. PomA and PomB were detected in the inner membrane fractions in the absence of both MotX and MotY (Fig. 5*A*).

Next, *E. coli* cells expressing PomA, PomB, and either MotX or MotY were fractionated (Fig. 5, *B* and *C*). When PomA, PomB, and MotX were co-expressed, they were all detected in intermediate fractions between the inner and outer membrane fractions. On the other hand, when PomA, PomB, and MotY were co-expressed, MotY was found in the outer membrane, whereas PomA and PomB were detected in the inner membrane fractions. These results suggest that MotX, but not MotY, affects membrane localization of PomA and PomB. PomB was detected in the inner membrane fractions when it was expressed by itself (Fig. 6*A*). When PomB was co-expressed with MotX, PomB was detected in the same intermediate fractions as MotX, even in the absence of PomA (Fig. 6*B*). Co-expressed MotX did not affect membrane localization of PomA in the absence of PomB (data not shown), suggesting that in the absence of MotY, MotX binds to PomB but not to PomA.

DISCUSSION

Several observations have suggested that MotX interacts with MotY, although no direct evidence for such an interaction has been obtained (19, 23, 26). In this study, we attempted to characterize the biochemical properties of MotX and MotY in more detail. We searched for conditions under which detergents can solubilize these proteins. We tried 72 different kinds of detergents and found that co-expressed MotX and MotY were solubilized by eight different detergents in the absence of salt. In contrast, MotX alone was not solubilized by these detergents at all, indicating that MotY strongly affects the solubility of MotX. Pull-down assay showed that untagged MotY co-eluted with MotX-His_6, indicating a direct interaction between these proteins. Because overexpression of MotX can suppress a series

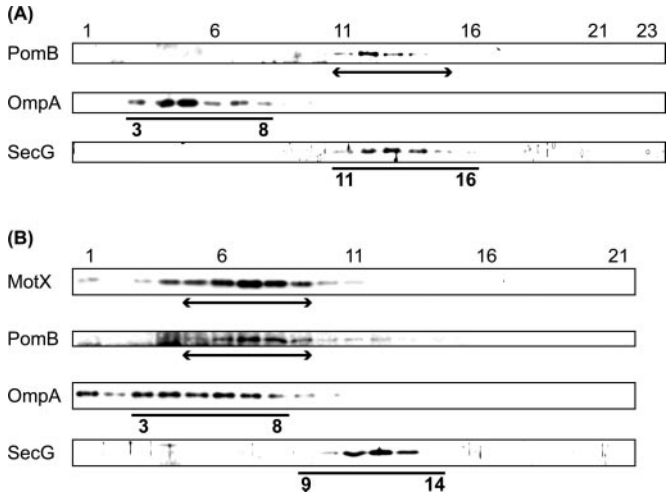

FIG. 6. **Membrane localization of PomB and MotX in *E. coli* JM109 harboring pSK603 (*pomB*) (*A*) or pMO722 (*motXpomB*) (*B*).** OmpA and SecG were used as the diagnostic marker proteins for the outer and inner membranes, respectively. Membrane preparations were analyzed by sucrose density gradient centrifugation followed by fractionation into 23 or 21 fractions. PomB, MotX, OmpA, and SecG in each fraction were analyzed by immunoblotting after SDS-PAGE. OmpA and SecG were detected in fractions 3–8 and 11–16 (*A*) and 3–8 and 9–14 (*B*), respectively. PomB and MotX are demarcated in the fractions with *double-headed arrows*.

of *motY* mutations (19), it is suggested that MotY is involved in the proper targeting of MotX to the flagellar motor rather than in torque generation. In fact, MotX fails to localize to the outer membrane in the absence of MotY (26).

Analytical gel-filtration chromatography showed that MotX and MotY, either expressed from a plasmid or from the chromosome, eluted in the void volume, suggesting that MotX and MotY together form a large oligomeric structure(s). At the moment, it is not clear whether the MotX/MotY oligomer(s) correspond to a homogeneous complex or just an aggregate(s). However, it is interesting to note that the stator protein complex also exists as a large oligomer consisting of at least four PomA (MotA) and two PomB (MotB) proteins (21, 41, 42).

It has been reported that overproduction of MotX is lethal to *E. coli* cells and that cell death caused by overproduction of MotX was dependent on the external NaCl or KCl concentration (23). The oligomeric state of MotY is also strongly influenced by salt concentration; it is monomeric in the presence of 300 mM NaCl or KCl, but it forms a large oligomeric structure(s) in the absence of salt and elutes in the void volume by gel-filtration chromatography (Fig. 4). Detergents that were able to solubilize the MotX/MotY complex worked only in the absence of salt (Fig. 1). Thus, salt concentration appears to be a key factor for the conformation and/or the oligomeric state of MotX, MotY, and the MotX/MotY complex.

We have shown previously that when expressed alone, MotX is not fractionated with the inner or the outer membrane (26). Thus, MotX exhibits different cellular locations depending on whether or not MotY is present. In this study, we found that in the absence of MotY, MotX altered the membrane localization of the PomA/PomB complex and PomB, but not PomA, to fractions between the inner and outer membrane in sucrose density gradient centrifugation (Figs. 5*B* and 6*B*). This result suggests strongly that MotX binds to PomB. It is not clear whether the PomA/PomB complex and PomB are detached from the membrane vesicles or whether membrane vesicles containing PomA/PomB and PomB are isolated from those containing SecG (used as an inner membrane marker protein) and those containing OmpA (used as an outer membrane marker protein). It is possible, however, that MotX binds tightly to PomA/PomB or

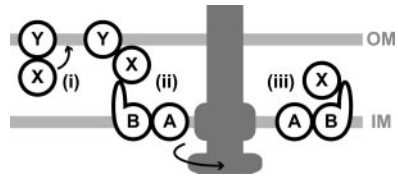

FIG. 7. **Schematic illustration of motor assembly and rotation.** *i*, MotY binds to and delivers MotX to the outer membrane. *ii*, after MotX localizes to the outer membrane, the stator complex can rotate the rotor. *iii*, in the absence of MotY, MotX does not localize to the outer membrane but can still interact with PomB. The MotX/PomA/PomB complex cannot efficiently assemble to the motor. *A*, PomA; *B*, PomB; *X*, MotX; *Y*, MotY; *OM*, outer membrane; *IM*, inner membrane.

PomB, thereby pulling them toward the intermediate fraction when they are separated by sucrose density gradient centrifugation. In our previous studies of the chimeric flagellar motor, MotX and MotY have been shown to be required for the motor function if the periplasmic region of the chimeric B subunits derive from PomB (28–30). Therefore, we suggest that MotX probably binds to the periplasmic region of PomB to make a functional stator. Thus, the interaction between PomB and MotX is physiologically relevant to the torque generation process, and therefore, MotX probably forms a part of the stator of the sodium ion-driven flagellar motor in *Vibrio* sp.

Based on the available information, we propose a mechanism of assembly and a functional state of the stator complex of sodium ion-driven motor (Fig. 7). (i) MotX forms a complex with MotY, which is then targeted to the outer membrane. (ii) MotX associated with the outer membrane interacts with the PomA/PomB complex in the inner membrane through the periplasmic region of PomB to produce torque. At the moment, we cannot rule out the possibility that MotY is dissociated from the complex because even in the absence of MotY, a less functional stator is formed by overproduction of MotX (19). It is likely, however, that MotY remains with MotX in the complex because MotX and MotY are fractionated together with the outer membrane when all of the four components are expressed (26). (iii) If MotY is absent, the MotX/PomA/PomB complex does not efficiently localize to an appropriate position in relation to the motor; therefore, the motor does not rotate fully.

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