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MotX and MotY, specific components of the sodium-driven flagellar motor, colocalize to the outer membrane in *Vibrio alginolyticus*

Mayuko Okabe, Toshiharu Yakushi,* Masaru Kojima and Michio Homma
Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-Ku, Nagoya, Japan.

Summary

Rotation of the sodium-driven polar flagella of *Vibrio alginolyticus* requires four motor proteins: PomA, PomB, MotX and MotY. MotX and MotY, which are unique components of the sodium-driven motor of *Vibrio*, have been believed to be localized in the inner (cytoplasmic) membrane via their N-terminal hydrophobic segments. Here we show that MotX and MotY colocalize to the outer membrane. Both proteins, when expressed together, were detected in the outer membrane fraction separated by sucrose density gradient centrifugation. As mature MotX and MotY proteins do not have N-terminal hydrophobic segments, the N-termini of the primary translation products must have signal sequences that are removed upon translocation across the inner membrane. Moreover, MotX and MotY require each other for efficient localization to the outer membrane. Based on these lines of evidence, we propose that MotX and MotY form a complex in the outer membrane. This is the first case that describes motor proteins function in the outer membrane for flagellar rotation.

Introduction

Bacterial flagellar motors are the locomotive organelles that drive the cell by rotating their helical flagellar filament (Berg, 1995; Blair, 1995; Macnab, 1996). In Gram-negative bacteria, the rotor portion of the flagellar basal body consists of an axial rod and four rings: L, P, MS and C rings. The L, P and MS rings are thought to be embedded in the outer membrane, the peptidoglycan layer, and the inner (cytoplasmic) membrane respectively (Derosier, 1998). It is speculated that the cytoplasmic C ring is surrounded by 6–16 stator particles (force-generating units) in the inner membrane (Blair and Berg, 1988; Khan *et al*., 1988; Muramoto *et al*., 1994). The motor is energized by a transmembrane electrochemical potential using either protons or sodium as the coupling ion (Manson *et al*., 1977; Hirota *et al*., 1981).

Motility (*mot*) mutants of bacteria have paralysed flagella and are unable to generate the torque required to rotate the filament. Two motor genes, *motA* and *motB*, have been identified in *Escherichia coli* and *Salmonella*, whose motors use protons as the coupling ion for the rotation of flagella (Dean *et al*., 1984; Stader *et al*., 1986). MotA and MotB are inner membrane proteins and are believed to convert the electrochemical potential to mechanical energy in concert with the rotor component. MotA has four transmembrane segments and one large cytoplasmic segment, which has been proposed to electrostatically interact with FliG (Zhou *et al*., 1995, 1998a; Lloyd *et al*., 1999). MotB has a single N-terminal transmembrane segment where an Asp residue is essential for proton flow as a part of the torque-generation process (Zhou *et al*., 1998b). MotB also has a peptidoglycan-binding motif at its C-terminus (Chun and Parkinson, 1988; De Mot and Vanderleyden, 1994). The transmembrane segments of MotA and MotB are thought to form a proton channel (Blair and Berg, 1990; Sharp *et al*., 1995a, b). MotA is thought to undergo a conformational change that is coupled to proton flow to alter its interaction with FliG, thereby driving rotation (Kojima and Blair, 2001).

Alkalophilic *Bacillus firmus* and *Vibrio* species use sodium as the coupling ion for flagellar rotation (Imae and Atsumi, 1989; Yorimitsu and Homma, 2001). *Vibrio alginolyticus* and *Vibrio parahaemolyticus* have sodium-driven polar flagella and proton-driven lateral flagella, which are used for movement on a surface (Atsumi *et al*., 1992; Kawagishi *et al*., 1995). The sodium-driven motor of *V. alginolyticus* rotates stably and remarkably fast, up to 1700 r.p.s., compared with rotation of the proton-driven motors of *E. coli* and *Salmonella*, which has a speed of around 300 and 200 r.p.s. respectively (Lowe *et al*., 1987; Kudo *et al*., 1990; Magariyama *et al*., 1994; Muramoto *et al*., 1995). Lateral flagellar motor genes, *lafT* and *lafU*

Accepted 5 July, 2002. *For correspondence. E-mail 4juji@cc.nagoya-u.ac.jp; Tel. (+81) 52 789 2992; Fax (+81) 52 789 3001.

of *V. parahaemolyticus*, are quite similar to their equivalents *motA* and *motB* in other proton motors (McCarter and Wright, 1993). In contrast, four motor genes (*pomA*, *pomB*, *motX* and *motY*) have been identified as essential for rotation of the sodium-driven polar flagellum in *Vibrio* (Asai *et al*., 1997; Gosink and Häse, 2000; McCarter, 2001; Okabe *et al*., 2001).

PomA and PomB are orthologues of MotA and MotB respectively. Physical interaction between PomA and PomB has been demonstrated (Yorimitsu *et al*., 1999). A purified PomA/PomB complex was shown to have sodium-conducting activity *in vitro* (Sato and Homma, 2000a). It appears that PomA forms a stable homodimer and the two subunits function together (Sato and Homma, 2000b). A periplasmic loop region of PomA is considered important for dimer formation (Yorimitsu *et al*., 2000).

A hybrid motor that consists of PomB, MotX and MotY from *V. alginolyticus* and $MotA_{RS}$ from the proton-driven motor of *Rhodobacter spheroides* functions as a sodium-driven motor in *Vibrio* cells (Asai *et al*., 1999). In contrast, $MotB_{RS}$ functions with neither $MotA_{RS}$ nor PomA in *Vibrio*. Furthermore, the chimeric MomB protein, which contains the N-terminal transmembrane region of $MotB_{RS}$ and the C-terminus of PomB, serves in conjunction with $MotA_{RS}$ as a sodium-driven motor in *V. alginolyticus* (Asai *et al*., 2000).

MotX and MotY are unique to the sodium-driven flagellar motor of *Vibrio*. The deduced amino acid sequences of MotX and MotY show high identity among the *Vibrio* species. MotX and MotY have been characterized as membrane proteins because their sequences predict a single transmembrane segment. Both proteins are recovered from the membrane fraction when expressed in either *E. coli* or *Vibrio* (McCarter, 1994a; Okabe *et al*., 2001). The membrane localization of MotY in *E. coli* depends on MotX, and MotY remains in the soluble fraction unless MotX is coexpressed (McCarter, 1994a). MotX and MotY mutually stabilize by interaction within the membrane (Okabe *et al*., 2001). MotY also has a putative peptidoglycan-binding motif in its C-terminal region, as does MotB and PomB (McCarter, 1994b; Okunishi *et al*., 1996). MotX and MotY each have two cysteine residues, one of which is found in the conserved tetrapeptide CQLV that is in a similar position from the N-terminus.

In *E. coli*, there are no homologues of MotX and MotY. Overproduction of MotX in *E. coli* is lethal and its lethality is suppressed by amiloride, a sodium channel blocker (McCarter, 1994a). This harmful effect of MotX does not require MotY, nor is the production of MotY alone detrimental to *E. coli*. Therefore, it was suggested that MotX participates somehow in sodium translocation across the inner membrane. A corollary to this proposed function is that MotX and/or MotY may modulate the ion specificity of the PomA/PomB complex.

In this paper, we present evidence that MotX and MotY localize to the outer membrane. Our data require that an alternative model for the function of MotX and MotY be developed.

Results

Membrane localization of MotX and MotY

MotX and MotY were classified as membrane proteins because they both possess a single hydrophobic domain of sufficient length to span the inner membrane (McCarter, 1994a, b). Both proteins are predicted to have a large C-terminal periplasmic domain, topology that makes them type II membrane proteins (McCarter, 2001). It was also known that MotY can be recovered in the soluble fraction when expressed in *E. coli* in the absence of MotX (McCarter, 1994a). This result could be explained by the relatively low hydrophobicity of the MotY transmembrane region (Okabe *et al*., 2001). On the other hand, the sequence alignment of MotX and MotY of *Vibrio* species reveals that the putative transmembrane regions are not highly conserved (Okabe *et al*., 2001). That fact could indicate that the transmembrane domain of the proteins is not particularly important. A prediction of subcellular localization of MotX and MotY using an on-line programme (http://psort.ims.u-tokyo.ac.jp/) (Nakai and Kanehisa, 1991) showed that their most probable destination is the periplasmic space. Also, this predicted that their putative transmembrane segment may be cleaved during translocation across the inner membrane.

To determine the subcellular localization of MotY, we carried out subcellular fractionation such as spheroplasting and membrane separation using sucrose density gradient centrifugation. *E. coli* cells expressing either or combinations of the motor proteins were converted into spheroplasts and then fractionated to periplasmic, cytoplasmic and membrane fractions. The *lac* operon repressor, LacI, maltose-binding protein, MBP, and OmpA were used as the diagnostic marker proteins for the cytoplasm, periplasm and membranes respectively. As shown in Fig. 1, we compared the lanes designated as W and others, and a significant portion of MotY seemed to be degraded during the fractionation, even if coexpressed with MotX from pKJ601 and pKJ701. When MotY was expressed by itself, however, a relative amount of MotY was highest in the periplasmic fraction (lanes 10–12). Even under these conditions, a little MotY could be detected in the membrane fraction if high amounts of the sample were loaded on SDS-PAGE (data not shown). When coexpressed with MotX, MotY was detected exclusively in the membrane fraction (Fig. 1, lanes 14–16). The plasmid pKJ601 ($motX^+$ $motY^+$) complemented *motX* and *motY* mutants of *Vibrio* (data not shown), which means

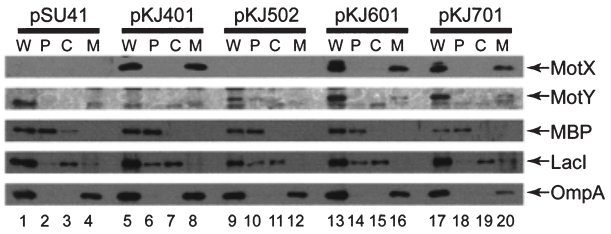

Fig. 1. Subcellular localization of MotX and MotY. *Escherichia coli* strain JM109 cells harbouring plasmid pSU41 (vector), pKJ401 (*motX*$^+$), pKJ502 (*motY*$^+$), pKJ601 (*motX*$^+$ *motY*$^+$) or pKJ701 (*motX*$^+$ *motY*$^+$ *pomAB*$^+$) were converted to spheroplasts. The periplasmic fraction was separated from spheroplasts by low speed centrifugation (10 000 *g*, 5 min). The spheroplasts were disrupted by sonication. Then, cytoplasmic and membrane fractions were prepared by ultracentrifugation. Equivalent amounts of the whole cell (W), periplasmic (P), cytoplasmic (C) and membrane (M) fractions were subjected to SDS-PAGE, followed by immunoblotting using anti-MotX, MotY, OmpA, MBP and LacI antisera.

MotX and MotY produced from the plasmid are functional. The localization of MotX and MotY were not changed upon coexpression with PomA/PomB (lanes18–20). Figure 1 suggests that the previously observed soluble form of MotY (McCarter, 1994a) resides in the periplasm, not in the cytoplasm of *E. coli*. MotX, on the other hand, is found in the membrane fraction despite the presence or absence of the other motor proteins.

As coexpressed MotX and MotY were both detected in the membrane fraction, the inner versus outer membrane localization of the two proteins was determined by density-gradient centrifugation (Fig. 2). *V. alginolyticus* NMB191 (Δ*pomAB*) harbouring plasmid pKJ701 (*motX*$^+$ *motY*$^+$ *pomAB*$^+$) was analysed for localization of MotX, MotY and PomA/PomB. The plasmid pKJ701 complemented the *motX*, *motY* or Δ*pomAB* mutation of *Vibrio*, indicating that functional MotX, MotY and PomA/PomB proteins are synthesized from the artificial *motY-motX-pomAB* operon (data not shown). As shown in Fig. 2A, MotX and MotY colocalized in the higher density fraction, in which an OmpA-like protein could also be detected. In contrast, PomA and PomB were detected in the lower density fraction. This result clearly indicates that MotX and MotY localize to a different membrane than PomA/PomB in *V. alginolyticus*.

The inner versus outer membrane localization of MotX and MotY was also determined in *E. coli* because methods for separation of the inner and outer membranes of *V. alginolyticus* are not as well established as in *E. coli*. Also, the antisera against diagnostic inner and outer membrane proteins are not available. Membranes of strain JM109 harbouring plasmid pKJ601 (*motX*$^+$ *motY*$^+$) were fractionated by sucrose density gradient centrifugation (Fig. 2B). The relative amounts of the inner membrane protein SecG (Nishiyama *et al*., 1993), the outer membrane protein OmpA (Klose *et al*., 1988), and MotX and MotY were determined by immunoblotting. MotX and MotY were contained in the same membrane fraction as OmpA (Fig. 2B). A negligible amount of MotX and MotY was detected in the inner membrane fraction, where SecG was present. When *E. coli* cells expressing PomA, PomB, MotX and MotY were fractionated, most of MotX and MotY were again found in the outer membrane fraction, whereas PomA and PomB were present in the inner membrane fraction (Fig. 2C). A very little part of MotX could be detected in the inner membrane fraction, indicating the

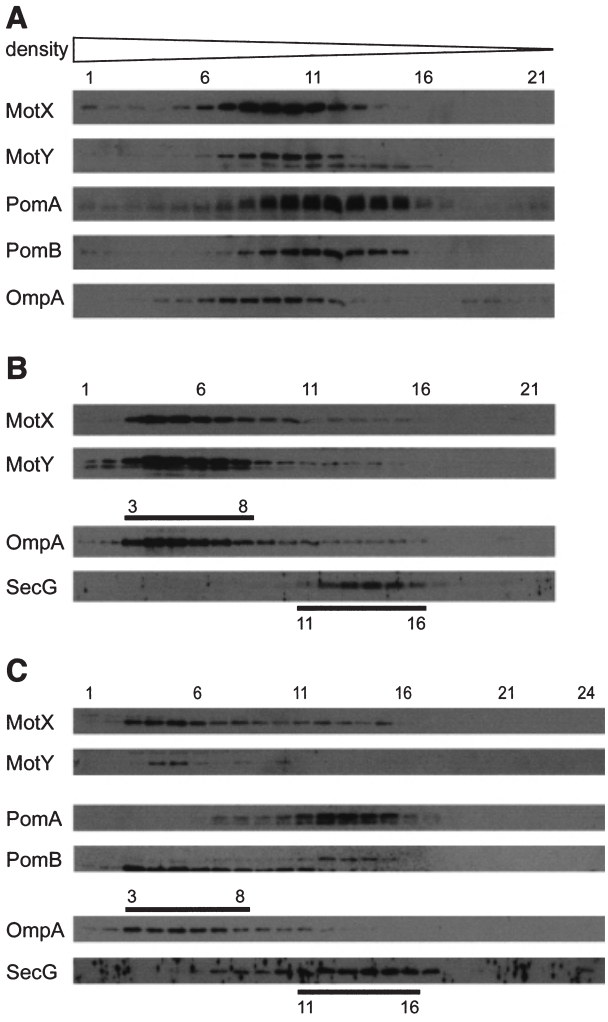

Fig. 2. Membrane localization of motor proteins. Membrane preparations were analysed by sucrose density gradient centrifugation. The gradient was divided into 21 or 24 fractions from the bottom. MotX, MotY, PomA, PomB, OmpA and SecG in each fraction were detected by immunoblotting using the appropriate antiserum. The membrane was prepared from *Vibrio alginolyticus* strain NMB191 (Δ*pomAB*) harbouring plasmid pKJ701 (*motX*$^+$ *motY*$^+$ *pomAB*$^+$) (A), *E. coli* JM109 cells harbouring plasmid pKJ601 (*motX*$^+$ *motY*$^+$) (B) and *E. coli* JM109 harbouring plasmid pKJ701 (*motX*$^+$ *motY*$^+$ *pomAB*$^+$) (C). In B and C, OmpA and SecG were detected in fractions 3–8 and 11–16 respectively.

MotX may slightly change its localization upon coexpression with PomA/PomB.

Signal peptide cleavage of MotX and MotY

If MotX and MotY localize to the outer membrane, they must have an N-terminal signal sequence (Pugsley, 1993). MotX and MotY were partially purified from *V. alginolyticus* as described in *Experimental procedures*. Their N-terminal amino acid sequences were analysed by Edman degradation. The N-terminal sequence of MotX was determined to be NVADV, indicating that the precursor was cleaved between Ala^{21} and Asn^{22}. The N-terminus of MotY was determined to be VMGKR, indicating that the processed form of MotY begins with Val^{22}. Additionally, N-terminus of MotX protein prepared from *E. coli* expression system had the identical sequence with that from *V. alginolyticus*. This result confirmed that the same processing reaction of MotX takes place within the two bacteria.

Altering the membrane localization of MotX and MotY

As described above, when expressed alone, most of MotY seemed to be in the periplasm as a soluble protein. On the other hand, when MotX and MotY were coexpressed, the both localized to the outer membrane (Fig. 2). We speculate that properties of MotY were altered by MotX. Figure 1 shows that, without MotX, most of MotY is recovered in the periplasmic fraction, however, some could be in the membrane fraction (data not shown). Membrane fraction of strain JM109 harbouring plasmid pKJ401 (*motX*$^+$) or pKJ502 (*motY*$^+$) was fractionated by sucrose density gradient centrifugation (Fig. 3). Comparing Figs. 2B and C, and 3A, MotX seems to be detected in a strange fraction, in which it does not correspond with either OmpA or SecG, in the absence of MotY. This result leads to three possibilities that MotX is present in (i) neither membrane (ii) the both membranes (iii) an intermediate membrane other than the inner and the outer membranes. In contrast, MotY was detected exclusively in the outer membrane fraction even in the absence of MotX (Fig. 3B).

MotX and MotY colocalize to the outer membrane

Previous studies and Fig. 1 showed that MotY is targeted to the membrane in the presence of MotX (McCarter, 1994b; Okabe *et al*., 2001). As shown in Figs 1 and 3A, MotX expressed by itself is in the membrane fraction, but its distribution corresponded with neither SecG nor OmpA. However, MotX coexpressed with MotY mostly localizes to the outer membrane (Figs 2B and C). These results indicate that both MotX and MotY are required for localization of either one to the outer membrane.

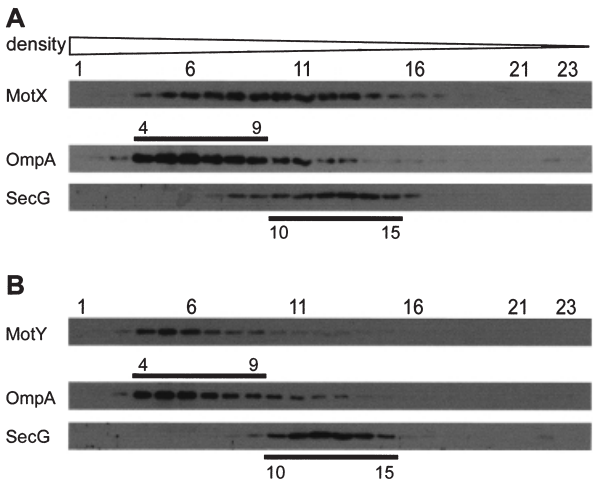

Fig. 3. Membrane localization of MotX and MotY solely expressed. Membranes were prepared from *E. coli* JM109 cells harbouring either plasmid pKJ401 (*motX*$^+$, A) or pKJ502 (*motY*$^+$, B) and analysed by sucrose density gradient centrifugation. The gradient was divided into 23 fractions from the bottom. MotX, MotY, OmpA and SecG in each fraction, which was separated by SDS-PAGE, were detected by immunoblotting. OmpA and SecG were detected in fractions 4–9 and 10–15 respectively.

To elucidate the biochemical properties of MotX and MotY, we attempted to solubilize them with several detergents. Membrane fractions of strain NMB94 (*motX*) expressing MotX (Fig. 4A) or of strain VIO542 (*motY*) expressing MotY (Fig. 4B) were used. Like *E. coli*, the latter strain mostly produces soluble MotY, but a little fraction of MotY is recovered in the membrane fraction, and the membrane-bound MotY can be not released by washing with buffer not containing any detergents (data not shown). MotX could not be solubilized any of the following four detergents: Triton X-100, β-octylglucoside, sucrose monocaprate and CHAPS. On the other hand, MotY was solubilized by all four detergents (Fig. 4). Membrane fractions from VIO542 cells expressing both MotX and MotY (Fig. 4C) and from NMB191 (Δ*pomAB*) expressing MotX, MotY, PomA and PomB (Fig. 4D) from a plasmid were also treated with detergents. Under these conditions, MotY was not solubilized. MotY did not become solubilized upon coexpression with MotX. This property was not changed by the simultaneous expression of PomA/PomB. These results may indicate that the membrane-binding manner of MotY would be different in strains having and lacking MotX. Also, a physical interaction between MotX and MotY exists.

Discussion

The sodium-driven polar flagellum of *Vibrio* is completely paralysed by *motX* or *motY* mutation, suggesting that MotX and MotY proteins are essential for the sodium-driven motor. Mutations of *pomA* or *pomB* confer the same

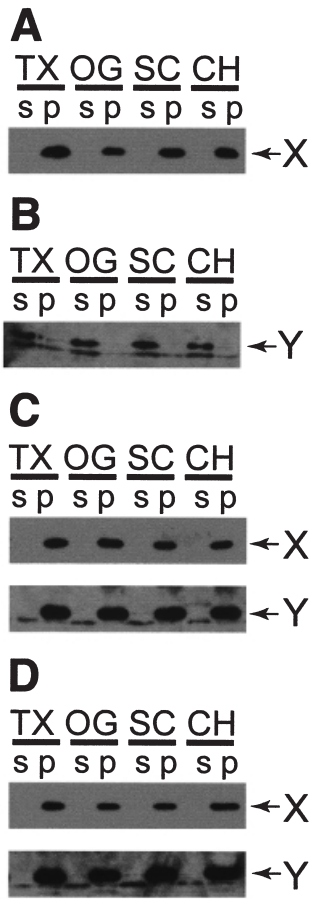

Fig. 4. Solubilization of MotX and MotY with several detergents. Membranes from *V. alginolyticus* NMB94 (*motX94*) cells harbouring plasmid pKJ401 (*motX*$^+$, A), VIO542 (*motY542*) cells harbouring plasmid pIO6 (*motY*$^+$, B), plasmid pKJ601 (*motX*$^+$ *motY*$^+$, C), and NMB191 (Δ*pomAB*) cells harbouring plasmid pKJ701 (*motX*$^+$ *motY*$^+$ *pomAB*$^+$, D) were solubilized with 2.5% each of Triton X-100 (TX), β-octylglucoside (OG), sucrose monocaprate (SC) or CHAPS (CH). The supernatant (s) and precipitate (p) were prepared by ultracentrifugation (200 000 *g*, 10 min) and subjected to SDS-PAGE and immunoblotting using anti-MotX antiserum (A, and upper panels of C and D) and anti-MotY antiserum (B, and lower panels of C and D).

polar-flagellar-motility phenotype, as PomA and PomB proteins form a complex to conduct sodium ions. On the other hand, the function of MotX and MotY is obscure, which have been believed to be located in the inner membrane. In this paper, we concluded that MotX and MotY colocalize to the outer membrane from the following observations: (i) MotX and MotY did not have their putative transmembrane segments, suggesting they are cleaved during translocation across the inner membrane; (ii) They localized to the outer membrane in *E. coli*, whereas PomA/PomB were in the inner membrane. In the *Vibrio* cell, they were detected in the fraction different from that of PomA/PomB, which may suggest their location in the outer membrane; (iii) The outer membrane localizations of MotX and MotY were dependent on each other. Furthermore, MotY could not be solubilized in the presence of MotX, supporting their interaction in the outer membrane. The L-ring component, FlgH, is a flagellar protein located in the outer membrane (Macnab, 1996). This protein is thought to participate in the structure of flagellum, bushing for rotating rod in the outer membrane, rather than in the motor rotation. Therefore, we suggest that MotX and MotY are the first example of outer membrane motor proteins.

The putative transmembrane region of each protein, MotX or MotY, exhibits relatively low similarity among the *Vibrio* species (Okabe *et al*., 2001). This implies that this region may not have important roles in structure and function. Such assumptions are reasonable because we demonstrated that the hydrophobic N-terminal sequence of MotX and MotY was not present in the mature protein. It can be speculated that the peptide was cut during translocation across the inner membrane, probably in a Sec machinery-dependent manner (Pugsley, 1993). We conclude that the predicted transmembrane regions are signal sequences for export. The protein translocation mechanism in *V. alginolyticus* seems to be equivalent to the *E. coli* Sec system, because several Sec factors of *V. alginolyticus* show high similarity to those of *E. coli* and also can function in *E. coli* (Tokuda *et al*., 1990; Kunioka *et al*., 1998; Nishiyama *et al*., 1998). In this study, MotX proteins from the two expression systems of *E. coli* and *V. alginolyticus* had the identical N-terminus, suggesting functionally homologous enzymes are relevant to the processing of secretory proteins in the organisms. From these lines of evidence, we speculate that primarily translated forms of MotX and MotY are translocated across the inner membrane and signal sequences are cleaved to convert the mature proteins and localize to the outer membrane of *V. alginolyticus*. MotX and MotY homologues have not been reported from any other bacteria than *Vibrio* species, including alkalophilic *Bacillus* species that have sodium-driven flagella. That is reasonable, because *Bacilli* do not have an outer membrane. *Rhizobium meliloti* requires a periplasmic motor protein MotC for the proton-driven flagellar rotation (Platzer *et al*., 1997). This protein, however, has significant sequence homology with neither MotX nor MotY.

The secondary-structures of MotX and MotY from *V. alginolyticus* were predicted by the method of Chou and Fasman (Chou and Fasman, 1974). This method showed that MotX is more α-helical than MotY, which exhibits an extensive β-strand structure (data not shown). Outer membrane proteins often form a β-barrel structure in their membrane-spanning domains (Pugsley, 1993; Koebnik *et al*., 2000). A three-dimensional structure prediction program, 3D-PSSM based on the SCOP (http://www.sbq.bio.ic.ac.uk/~3dpssm/) (Kelley *et al*., 2000), showed that MotY has similarities to OmpA trans-

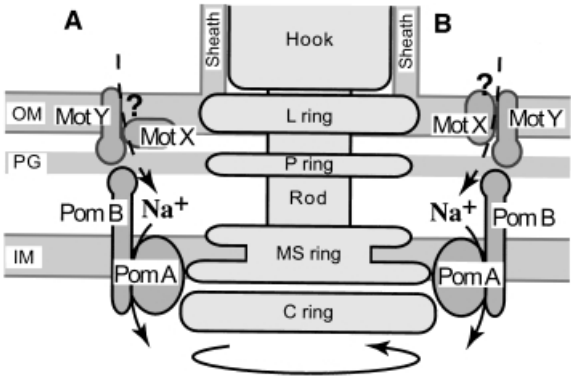

Fig. 5. A hypothetical model for the sodium-driven motor. The sodium-driven polar flagellum of *Vibrio* is sheathed. The energy source for flagellar rotation is provided by an electrochemical gradient across the inner membrane. MotX and MotY colocalize to the outer membrane and may participate in conduction of sodium ions in concert with PomA/PomB. From the predicted structure, MotX seems not to have enough β-sheet to span the outer membrane (A), but may alter its conformation upon interaction with MotY (B). IM, inner membrane; PG, peptidoglycan; OM, outer membrane.

membrane domain with a relatively high *E*-value (8.26E-05). As this domain of OmpA (1–171 residues), of which the structure has been determined, loses its C–terminal peptidoglycan association region (Pautsch and Schulz, 1998), the similarities are restricted in the region embedded in the outer membrane. On the other hand, there is no candidate similar to MotX with such a high *E*-value. Structure prediction may infer that MotY is an integral outer membrane protein and MotX has too short β-sheet structure to span the outer membrane (Fig. 5A).

MotY has a peptidoglycan-binding motif in its C-terminal portion that was proposed to anchor the protein to the peptidoglycan (McCarter, 1994b; Okunishi *et al*., 1996), thus forming a bridge between the outer membrane and the cell wall (Fig. 5). When MotY is expressed by itself, most of that remains in the periplasm but the rest moves exclusively to the outer membrane. This result corroborates the earlier observation that MotY is a membrane protein that is not stable in the absence of MotX (McCarter, 1994a; Okabe *et al*., 2001). Noteworthily, in contrast with MotX, a limited portion of MotY itself is able to precisely localize to the outer membrane in the absence of MotX (Fig. 3B). On the other hand, most of MotX seems to associate with neither membrane, but rather is detected in intermediate fractions between the inner and outer membranes, when expressed by itself (Fig. 3A). From this result, three possible behaviours of MotX without MotY are speculated as follows. First, MotX associates with neither membrane, but rather with some large molecule, such as protein complex and the peptidoglycan. Second, MotX associates with the both membranes, some are in the outer and others are in the inner membrane. Finally, the third possibility is an intermediate membrane. Further experiments may help to understand the ambiguous localization of MotX expressed by itself. Whatever may happen in the absence of MotY, MotX coexpressed with MotY localizes to the outer membrane and seems to interact therein. Also, both MotX and MotY seem to be mutually required for their precise outer membrane localization. In such situation, the both proteins are present, MotX may be embedded in the outer membrane (Fig. 5B).

Although an interaction between MotX and MotY can be suggested by Fig. 4, a major question remains to be answered, how might MotX and MotY contribute to motor function in the outer membrane? One possibility is that they make a pore for sodium ions in the outer membrane. MotX has been reported as a sodium channel that can be inhibited by amiloride, a sodium channel blocker (McCarter, 1994a). Many outer membrane proteins with a β-barrel structure form pores that allow water, ions and various small molecules to pass across the bilayer. The predicted secondary and tertiary structure of MotY may be embedded in the outer membrane to form a structure like porin. Sodium ions may pass through the structure and may be delivered to other flagellar motor components, such as PomA and PomB (Fig. 5). Figure 2C suggests the possible interaction between MotX and PomA/PomB, supporting such speculations. There are reports pointing out that MotA and MotB show moderate sequence similarity to ExbB and ExbD, which are the inner membrane proteins functioning in cooperation with TonB and outer-membrane receptors to drive active transport of certain nutrients across the outer membrane (Cascales *et al*., 2001; Kojima and Blair, 2001). This similarity implies that the electrochemical potential in the inner membrane may be transferred to MotX and MotY in the outer membrane by function of PomA/PomB to drive the flagellar rotation therein.

We have observed that overexpression of MotX suppressed *motY* mutations (Okabe *et al*., 2001). MotY may function as a kind of chaperon for MotX, or vice versa. The polar flagellum of *Vibrio* is covered by a membranous sheath that extends from the outer membrane (Furuno *et al*., 2000). MotX and MotY could be present in the sheath and affect its formation coordinate with the rotation of flagellum. The both proteins have been found being in the outer membrane in this study. The precise roles of them are not clear at present. However, they should function in the outer membrane to rotate the sodium-driven flagellar motor.

Experimental procedures

Bacterial strains and growth conditions

The strains of *Vibrio alginolyticus* and *Escherichia coli* used

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype or description	Reference
Vibrio alginolyticus strains		
VIO542	Rif^r Laf$^-$ *motY542*	Okunishi *et al.* (1996)
NMB94	Laf$^-$ *motX94*	Okabe *et al.* (2001)
NMB191	Rif^r Laf$^-$ Δ*pomAB*	Yorimitsu *et al.* (1999)
Escherichia coli strains		
JM109	*recA1, endA1, gyrA96, thi$^-$, hsdR17, relA1, supE44,* λ$^-$, Δ(*lac-proAB*)*; F′, traD36, proAB, lacI^q,* Δ*M15*	Yanisch-Perron *et al.* (1985)
Plasmids		
pSU41	*kan* (Km^r) P_{lac} *lacZa*	Bartolome *et al.* (1991)
pMO200	pSU21, 1.0 kb *Eco*RI–*Eco*RI fragment (*motX*$^+$)	Okabe *et al.* (2001)
pMO401	pSU41, 700 bp *Cla*I–*Bam*HI fragment (*motX*$^+$)	Okabe *et al.* (2001)
pKJ401	pSU41, 700 bp *Xba*I–*Bam*HI fragment (*motX*$^+$)	This work
pIO6	pSU38, 1.0 kb *Hin*dIII–*Xba*I fragment (*motY*$^+$)	Okunishi *et al.* (1996)
pKJ502	pSU41, 1.0 kb *Sal*I–*Xba*I fragment (*motY*$^+$)	This work
pKJ503	pSU41, 1.0 kb *Sal*I–*Xba*I fragment (*motY-his*$_6^+$)	This work
pKJ601	pKJ502 (*motY*$^+$), 700 bp *Xba*I–*Bam*HI fragment (*motX*$^+$)	This work
pYA303	pSU41, 1.9 kb *Sph*I–*Sal*I flagment (*pomAB*$^+$)	Kojima *et al.* (1999)
pKJ701	pKJ601 (*motY*$^+$ *motX*$^+$), 1.9-kb *Sph*I–*Sal*I fragment (*pomAB*$^+$)	This work

in this study are listed in Table 1. *V. alginolyticus* cells were cultured at 30°C on VC medium (0.5% tryptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.2% glucose) or 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 on Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). When necessary, kanamycin was added to a final concentration of 100 μg ml^{-1} (for *V. alginolyticus*) or 50 μg ml^{-1} (for *E. coli*).

Plasmid construction

DNA manipulations were carried out according to standard procedures (Sambrook *et al.*, 1989). Plasmids are listed in Table 1. Plasmid pKJ401 carrying *motX* was constructed by cloning a 700 basepair (bp) *Xba*I–*Bam*HI fragment of polymerase chain reaction (PCR) products using pMO200 as a template. The 5′-TTAGAT-3′ sequence, 18 bp upstream from the initiation codon of *motX*, was changed to 5′-TCTAGA-3′, to introduce the *Xba*I site. Also, the 5′-GGATCC-3′ sequence (*Bam*HI site) was introduced just adjacent to the termination codon of *motX*. Plasmid pKJ502 was constructed to carry a 1.0 kb *motY* by introducing *Sal*I (5′-GTCGAC-3′) and *Xba*I (5′-TCTAGA-3′) sites at 66 bp upstream of the initiation codon and just adjacent to the termination codon of *motY* respectively. A 700 bp *Xba*I–*Bam*HI fragment (containing *motX*) of pKJ401 was inserted into the corresponding sites of pKJ502 (carrying *motY*) to construct pKJ601, which carries an artificial *motY-motX* operon. A 1.9 kb *Bam*HI–*Sac*I fragment (*pomAB*$^+$) of pYA303 was inserted in the same restriction sites of pKJ601; the resultant plasmid pKJ701 carries a synthetic *motY-motX-pomAB* operon. Plasmid pKJ503 carried the gene encoding C-terminally hexahistidine-tagged MotY (MotY-His_6) in pSU41 by PCR using synthetic oligonucleotide primers. The nucleotide sequence of 5′-CATCACC ATCACCATCACTAATCTAGA-3′ (*Xba*I site is underlined) was inserted between the 3′-end of the *motY* open reading frame (ORF) and the *Xba*I site of the plasmid pKJ402. As this plasmid complemented the *motY* mutation of *Vibrio*, the C-terminal histidine tag does not perturb MotY function (data not shown). All motor genes of the plasmid constructed here do not have the native promoter and are under the control of the *lac* promoter–operator. Transformation of *Vibrio* cells by electroporation was carried out as described previously (Kawagishi *et al.*, 1994).

Membrane preparation and solubilization

Vibrio cells harbouring plasmids were cultured at 30°C on VPG medium. Cells were harvested and washed twice with 25 mM Tris-HCl, 10 mM $MgCl_2$, 300 mM NaCl, pH 7.5 and stored at –80°C until used. The frozen cells were thawed and resuspended (0.2 g ml^{-1}, wet weight) in 20 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg ml^{-1} of DNase I, pH 8.0. 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, 0.5 mM PMSF, 20% (w/v) glycerol, pH 8.0, homogenized, and stored at –80°C until used. The membrane preparations (10 mg ml^{-1}) were resuspended in 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM PMSF, 10% (w/v) glycerol, 5 mM imidazole, pH 8.0, and 2.5% (w/v) each of the following detergents: Triton X-100, β-octylglucoside (Dojin), sucrose monocaprate (Dojin), and 3-[3-cholamidopropyl-dimethylammonio] propanesulphonic acid (CHAPS) (Dojin). It was then incubated at 4°C for 10 min and centrifuged for 10 min at 200 000 *g* to separate the solubilized supernatant and the insoluble precipitate. Protein concentrations were determined by the BCA protein assay kit (Pierce).

Spheroplast formation

Conversion of *E. coli* cells into spheroplasts was performed as described previously (Osborn *et al*., 1972). Briefly, *E. coli* strain JM109 cells harbouring each plasmid were harvested and resuspended in 20 mM Tris-HCl, 0.75 M sucrose, 100 mM NaCl (pH 7.5). A twofold volume of 1.5 mM EDTA, 100 mM NaCl (pH 8.0) was gradually added, following the addition of lysozyme and DNase I, to convert to spheroplasts. The spheroplast suspension was centrifuged (10 000 *g* for 5 min) to separate periplasmic fraction from the spheroplasts. The precipitated spheroplasts were resuspended in an appropriate buffer and disrupted by sonication. Cytoplasmic and membrane fractions were prepared by ultracentrifugation (200 000 *g*, 10 min). Spheroplasting of *V. alginolyticus* was performed according to the method described previously (Nakamaru *et al*., 1999). The efficiency of spheroplast formation was determined from the fraction of round cells observed by phase-contrast microscopy.

Membrane localization of MotX and MotY

Escherichia coli strain JM109 harbouring plasmid pKJ401, pKJ502, pKJ601 or pKJ701 was grown on Luria–Bertani medium at 37°C to the mid-exponential phase. The expression of motor proteins was induced for 1 h by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). *V. alginolyticus* strain NMB191 harbouring plasmid pKJ701 was grown on VC medium and harvested in the mid-logarithmic phase. 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-HCl (pH 7.5) containing 1 mM EDTA. It was 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 21–24 fractions from the bottom to the top. OmpA (Yamada *et al*., 1989), SecG (Nishiyama *et al*., 1993), maltose binding protein (MBP) (New England Biolabs), LacI (Abo *et al*., 2000), MotX (Okabe *et al*., 2001), MotY (Okabe *et al*., 2001), PomA (Yorimitsu *et al*., 1999) and PomB (Yorimitsu *et al*., 1999) proteins in each fraction were detected by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with the respective antisera.

Preparation of MotX and MotY and determination of their N-terminal amino acid sequences

MotX was prepared from both *E. coli* strain JM109 harbouring plasmid pMO401 and *V. alginolyticus* strain NMB94 harbouring the plasmid. Membranes prepared as described above were suspended in buffer A (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 mM imidazole, pH 8.0) at 10 mg membrane protein per ml. The membranes were pretreated with 2.5% Triton X-100 on ice for 10 min, and the Triton-insoluble materials were recovered by ultracentrifugation (200 000 *g*, 10 min). The precipitates were resuspended in buffer A containing 3% (w/v) SDS in a tenth volume of the initial solution and treated at 80°C for 10 min. The SDS-solubilized material contained MotX. MotY was purified from the membrane fraction of the strain NMB94 harbouring plasmid pKJ503, which encodes C-terminally histidine-tagged MotY. The membranes (10 mg ml^{-1} protein) were treated with 2.5% Triton X-100 in buffer B (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 mM imidazole, pH 7.5). Triton-insoluble material was removed by ultracentrifugation (100 000 *g*, 30 min), and the supernatant was passed over Ni-NTA agarose. Bound His-tagged MotY was eluted with buffer B containing 0.05% Triton X-100 and 200 mM imidazole. Partially purified MotX and MotY were separated from impurities by SDS-PAGE and electrophoretic transfer to polyvinylidene difluoride membrane (Millipore) and stained with Coomassie blue R250. The bands corresponding to MotX and MotY were excised, and their N-terminal amino acid sequences were determined by Aproscience (Tokushima), based on Edman degradation. Five N-terminal amino acid sequences of MotX from both *E. coli* and *V. alginolyticus* expression systems were identical as described in *Results*.

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