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The *Vibrio* motor proteins, MotX and MotY, are associated with the basal body of Na^+-driven flagella and required for stator formation

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Summary

The four motor proteins PomA, PomB, MotX and MotY, which are believed to be stator proteins, are essential for motility by the Na^+-driven flagella of *Vibrio alginolyticus*. When we purified the flagellar basal bodies, MotX and MotY were detected in the basal body, which is the supramolecular complex comprised of the rotor and the bushing, but PomA and PomB were not. By antibody labelling, MotX and MotY were detected around the LP ring. These results indicate that MotX and MotY associate with the basal body. The basal body had a new ring structure beneath the LP ring, which was named the T ring. This structure was changed or lost in the basal body from a Δ*motX* or Δ*motY* strain. The T ring probably comprises MotX and MotY. In the absence of MotX or MotY, we demonstrated that PomA and PomB were not localized to a cell pole. From the above results, we suggest that MotX and MotY of the T ring are involved in the incorporation and/or stabilization of the PomA/PomB complex in the motor.

Introduction

Many bacteria can swim using flagella, which function as a rotary machine like a corkscrew. The flagellar motor uses the electrochemical potential of specific ions (H^+ or Na^+) across the cytoplasmic membrane as the energy source (Yorimitsu and Homma, 2001; Blair, 2003). The flagellum consists of the filament, the hook and the basal body. In Gram-negative bacteria, the basal body, which is the supramolecular complex consisting of the rotor and the bushing, consists of the rod and the four ring structures, L, P, MS and C rings. The L, P and MS rings are thought to associate with the outer membrane, the peptidoglycan layer and the inner (cytoplasmic) membrane respectively. The C ring is located on the cytoplasmic face of the MS ring. The MS and C rings and the rod are thought to act as the rotor, and the L and P rings are thought to act as the molecular bushing for the rod (Macnab, 1996; Derosier, 1998).

mot mutants, which have paralysed phenotypes for motility (Mot^-), can produce flagella but cannot rotate them. Five genes, *motA*, *motB*, *fliG*, *fliM* and *fliN*, are responsible for the Mot^- phenotype in *Escherichia coli* and *Salmonella*. *motA* and *motB* are involved only in motor function. On the other hand, *fliG*, *fliM* and *fliN* are also involved in flagellar formation and switching of rotational direction because null mutations give a flagella-defective phenotype (Fla^-), and some mutations cause the motor to rotate in only one direction (Che^-). Genetic and physiological evidence has suggested that MotA and MotB, which are cytoplasmic membrane proteins, together comprise the stator complex of the motor and function as a proton channel complex to generate torque (Blair, 2003). MotA has four transmembrane segments and a large cytoplasmic loop and MotB has a single transmembrane domain and a peptidoglycan-binding motif through which the motor is anchored to the cell wall (De Mot and Vanderleyden, 1994). MotB has an Asp residue in the putative transmembrane region, which is speculated to be protonated during torque generation (Zhou *et al*., 1998a). PomA and PomB are orthologues of MotA and MotB, respectively, in the Na^+-driven polar flagellum of *Vibrio alginolyticus* (Asai *et al*., 1997). The purified PomA/PomB complex was shown to have sodium-conducting activity *in vitro* (Sato and Homma, 2000a) and is predicted to be in a 4A:2B stoichiometry complex (Sato and Homma, 2000b; Yorimitsu *et al*., 2004). The PomA/PomB complex is thought to function as the Na^+ channel and is referred to as a stator similar to the MotA/MotB complex (Asai *et al*., 1997). The cytoplasmic domains of MotA and PomA are thought to interact with FliG, one of the rotor components, to generate torque for the flagellar motor in the H^+- and Na^+-driven

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motors (Zhou *et al*., 1998b; Yakushi *et al*., 2006). However, such an interaction has not yet been biochemically observed (Driks and DeRosier, 1990). The stator proteins must be installed into the flagellar structure as a stator complex that surrounds the rotor. Recently, it has been shown that the flagellar structure and C-terminal domain of PomB are required for the localization of the PomA/PomB complex at the cell pole where the flagellum is generated (Fukuoka *et al*., 2005; Yakushi *et al*., 2005).

Two additional genes, *motX* and *motY*, are also required for motility in the Na^+-driven *Vibrio* flagella, and are thought to be stator proteins because null mutants have a Mot^- phenotype (Gosink and Häse, 2000; Yagasaki *et al*., 2006). MotY has a putative peptidoglycan-binding motif in its C-terminal region as do MotB and PomB. The functions of MotX and MotY have not yet been clarified.

Recently, MotX and MotY were shown to localize to the outer membrane and to interact with each other (Okabe *et al*., 2002; 2005). MotX affects the membrane localization of PomB when MotY is absent, suggesting that MotX interacts with PomB (Okabe *et al*., 2005). In this study, we show that MotX and MotY are associated with the flagellar basal body of *V. alginolyticus* and form a T ring. We demonstrated the interaction of the stator proteins with the basal body in the flagellar motor. We propose that the T ring formed by the MotX and MotY proteins is required for incorporation and stabilization of the PomA/PomB complex in the motor complex.

Results

Purification of the hook-basal body of V. alginolyticus

Vibrio alginolyticus cells have a single polar flagellum and multiple lateral flagella. To obtain a large amount of the hook-basal bodies of the sodium-driven polar flagella, we used the *flhG* mutant (KK148) which forms multiple polar flagella and was isolated from the lateral flagella defective mutant, VIO5 (Kusumoto *et al*., 2006). The fractions of purified hook-basal bodies were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue to visualize proteins (Fig. 1A). We reproducibly detected 11 bands with molecular masses of 62, 50, 47, 46, 40, 33, 32, 31, 28, 26 and 25 kDa. The banding pattern on the SDS-PAGE gel was similar to that of the purified hook-basal bodies from *Salmonella typhimurium* (Aizawa *et al*., 1985). We chose the major 50 kDa band and determined its N-terminal amino acid sequence by Edman degradation. The obtained sequence was SYVSL, which corresponds to the hook protein, FlgE, of *Vibrio parahaemolyticus*, which is a closely related species.

A new ring structure in the basal body of V. alginolyticus

Purified hook-basal bodies from *V. alginolyticus* were observed by electron microscopy, and their overall structure was similar to those of other Gram-negative bacteria (Fig. 1B). The structure of the hook-basal body of Gram-negative bacteria is composed of three rings and an axial rod connected to the hook. Interestingly, in the *V. alginolyticus* basal body, we found an additional new ring structure that appeared as an appendage on the periplasmic side of the P ring, and it was named the T ring. The diameter and axial dimension of the T ring were 31 ± 2 (SD) nm and 8 ± 1 (SD) nm ($n = 31$) (Fig. 1C). Such T rings have not been previously observed in the basal bodies of other Gram-negative bacteria. When the basal body structure was compared with that from *Salmonella*, the LP ring from *V. alginolyticus* looked like two thin rings. The diameter of the LP ring was 33 ± 3 (SD) nm ($n = 31$). The LP ring of the *V. alginolyticus* polar flagellum is larger than that of *Salmonella* flagella, which is approximately 27 nm (Sosinsky *et al*., 1992). When the hook-basal body preparation from *V. alginolyticus* and that from *E. coli* were compared directly, the diameter of the LP rings of the *V. alginolyticus* polar flagella was clearly larger than that of the *E. coli* flagella (Fig. 1D). When the structure of the purified hook-basal bodies of strain KK148, which is the *flhG* mutant with multiple flagella, were compared with those of the parent strain VIO5 by electron microscopy, we did not see any differences between the two basal body structures (data not shown).

Detection of MotX and MotY in the hook-basal body

We analysed components of the purified hook-basal bodies by immunoblotting using the anti-PomA, anti-PomB, anti-MotX and anti-MotY antibodies. MotX and MotY were detected in the purified hook-basal body, whereas PomA and PomB were not (Fig. 2A). These results suggest that MotX and MotY are associated with the basal body. Recently, it was shown that the overexpressed MotX and MotY tend to oligomerize by themselves (Okabe *et al*., 2005). We examined the hook-basal body fraction further by using sucrose density gradient centrifugation to see whether MotX and MotY co-fractionate with the basal body proteins. The P ring component, FlgI, was used as a marker protein for the basal body. The fractionation profiles of both MotX and MotY corresponded well to that of the FlgI protein (Fig. 2B). We propose that MotX and MotY are components of the flagellar basal body.

Localization of MotX and MotY on the basal body

In order to directly observe the localization of MotX and MotY in the basal body, we performed immunoelectron microscopy using anti-MotX or anti-MotY antibodies and gold-labelled anti-rabbit IgG as the secondary antibody

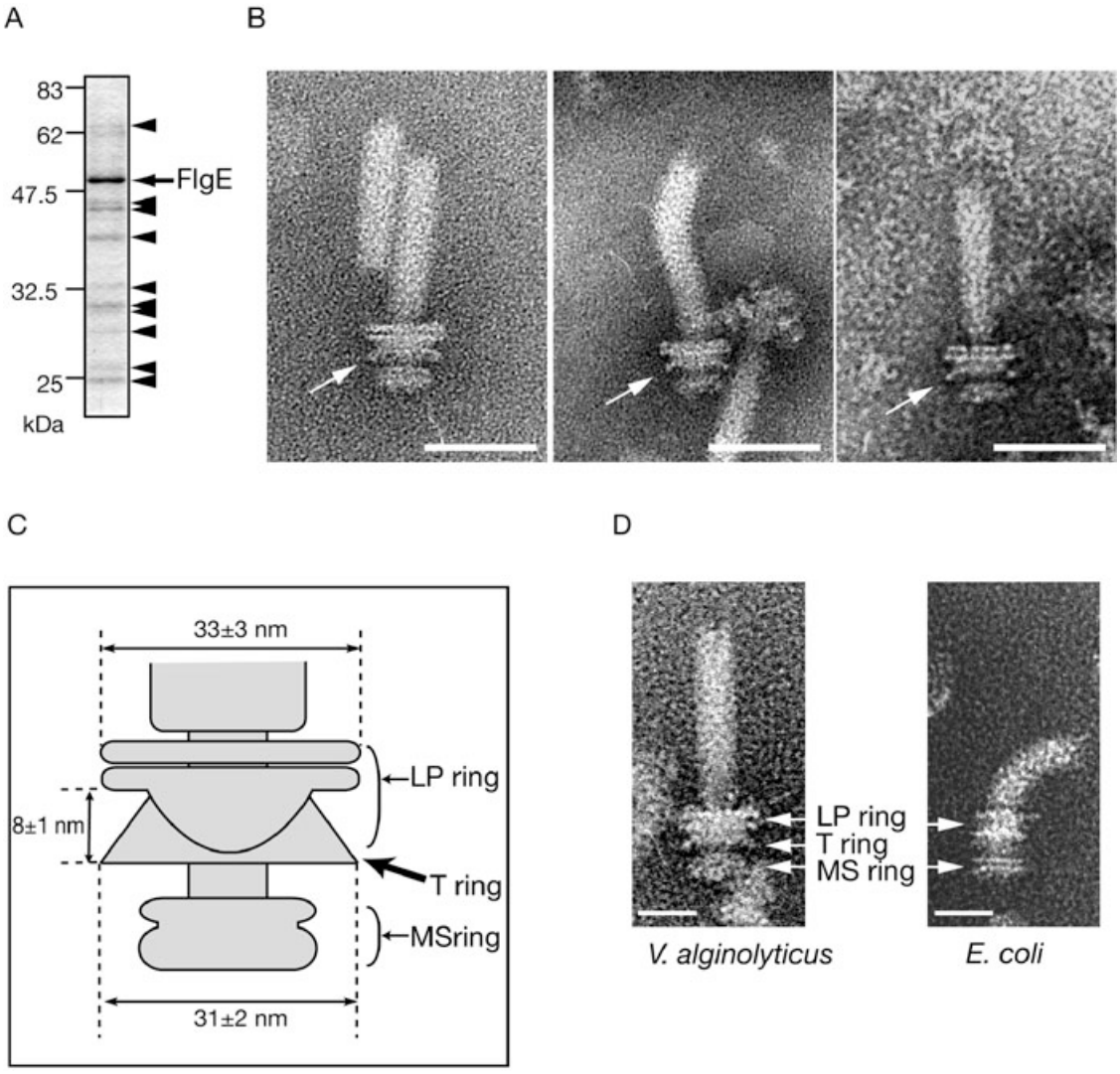

Fig. 1. A. Purification of the hook-basal bodies of *V. alginolyticus* (KK148). The proteins of the purified hook-basal body were separated by SDS-PAGE. The 50 kDa band and the other 10 bands with molecular masses of, 62, 47, 46, 40, 33, 32, 31, 28, 26 and 25 kDa are shown with an arrow and arrowheads respectively. The N-terminal amino acid sequence, SYVSL, of the 50 kDa protein was determined by Edman degradation, identifying the hook protein FlgE.
B. Electron microscopy image of the hook-basal bodies from *V. alginolyticus* strain KK148. The new structure named the T ring is indicated by the arrow. The hook-basal bodies were negatively stained with 2% phosphotungstic acid (pH 7.4). Bar, 50 nm.
C. Cartoon of the flagellar basal body of *V. alginolyticus*. The diameter of the LP ring is 33 ± 3 (SD) nm ($n = 31$). The diameter and axial dimension of the T ring of the flagellar basal body are 31 ± 2 (SD) nm and 8 ± 1 (SD) nm ($n = 31$).
D. Comparison of the basal bodies purified from *V. alginolyticus* (left: KK148) and *E. coli* (right: RP437). Bar, 25 nm.

(Fig. 3). When the basal bodies from the parental strain KK148 were treated with anti-MotX or anti-MotY antibodies, the fuzzy structure of the antibody was observed around the LP ring or the T ring and gold particles mainly attached around the basal body or tended to be present around the LP ring or the T ring. On the other hand, when the same treatment was carried out with the basal bodies isolated from the Δ*motX* Δ*motY* strain (TH3), which we constructed from strain KK148, gold particles were not localized around the basal body or the LP ring. These results strongly suggest that MotX and MotY are associated with the basal body. Thus, we hypothesized that the T ring detected as appendages around the P ring is composed of MotX and MotY.

The basal body structure from ΔmotX *or* ΔmotY *cells*

If the T ring is composed of MotX and MotY, the basal bodies from Δ*motX* and/or Δ*motY* cells might have an altered appearance. Therefore, we constructed Δ*motX* (TH1) and Δ*motY* (TH2) strains from KK148 and observed their basal bodies by electron microscopy. The

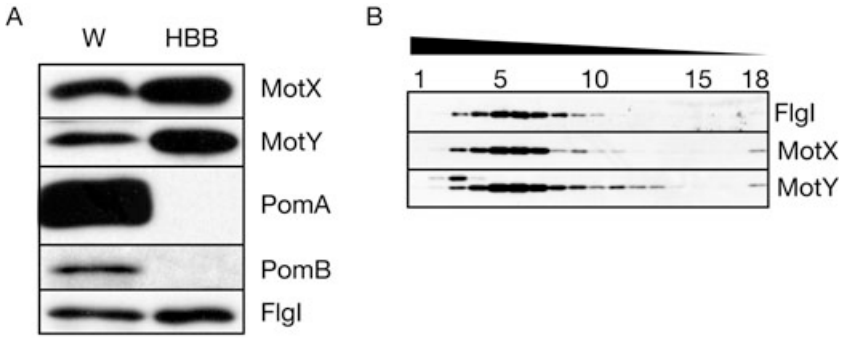

Fig. 2. Co-purification of MotX and MotY with the basal body.
A. Whole cell (W) and the purified hook-basal bodies (HBB) of *V. alginolyticus* strain KK148 were subjected to SDS-PAGE, followed by immunoblotting using anti-MotX, anti-MotY, anti-PomA, anti-PomB and anti-FlgI antibodies.
B. The purified hook-basal bodies were separated by sucrose density gradient centrifugation. The gradient was divided into 18 fractions from the bottom. MotX, MotY and FlgI in each fraction were subjected to SDS-PAGE and detected by immunoblotting using anti-MotX, anti-MotY and anti-FlgI antibodies.

basal body of strain TH1 still had an appendage, but the structure seemed to be smaller than the T ring observed in the parental strain (Fig. 4A). Therefore, the appendage may be derived from the MotY protein. We detected the MotY protein in the purified hook-basal body of strain TH1 by immunoblotting (Fig. 4B). The basal body of strain TH2 did not have a T ring (Fig. 4A), and MotX protein was also not seen in whole cell extracts or the purified hook-basal body preparation by immunoblotting (Fig. 4B). It has been suggested that MotX is rapidly degraded in the absence of MotY (Okabe *et al*., 2001; Yagasaki *et al*., 2006). The T ring was not observed in the basal bodies purified from strain TH3 or strain TH2, in which no MotX protein was detected (Fig. 4A). These results demonstrate that MotX and MotY are responsible for the T ring structure.

Polar localization of PomA/PomB complex requires MotX and MotY

Recently, it has been suggested that MotX interacts with the PomA/PomB complex as well as MotY (Okabe *et al*., 2005). In the present study, we showed that MotX and MotY were associated with the basal body or the LP ring, therefore they might be rotor or bushing components. Although we know that MotX and MotY are essential for the function of the sodium-driven motor, their roles are not clear. Now, we speculate that MotX and MotY are involved

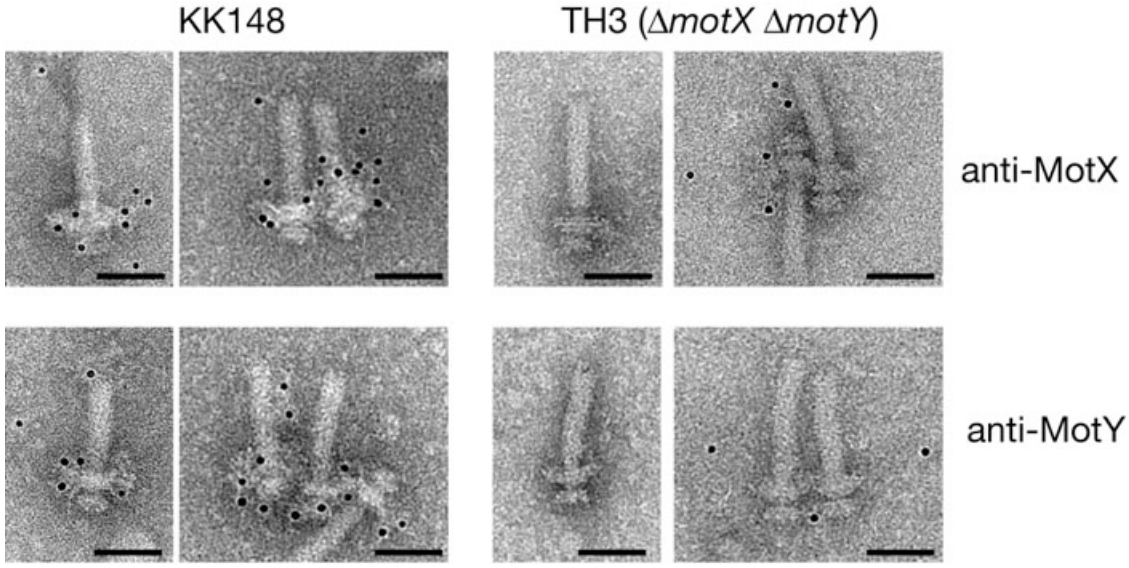

Fig. 3. Immunogold labelling of the basal bodies using anti-MotX and anti-MotY antibodies. The basal bodies from KK148 (left) or TH3 (Δ*motX* Δ*motY*) (right) were treated with anti-MotX (upper) or anti-MotY (lower) antibodies, which were prepared as described previously (Yagasaki *et al*., 2006), and 5 nm colloidal gold-conjugated anti-rabbit IgG antibody. The samples were negatively stained with 2% phosphotungstic acid (pH 7.4). Bar, 50 nm.

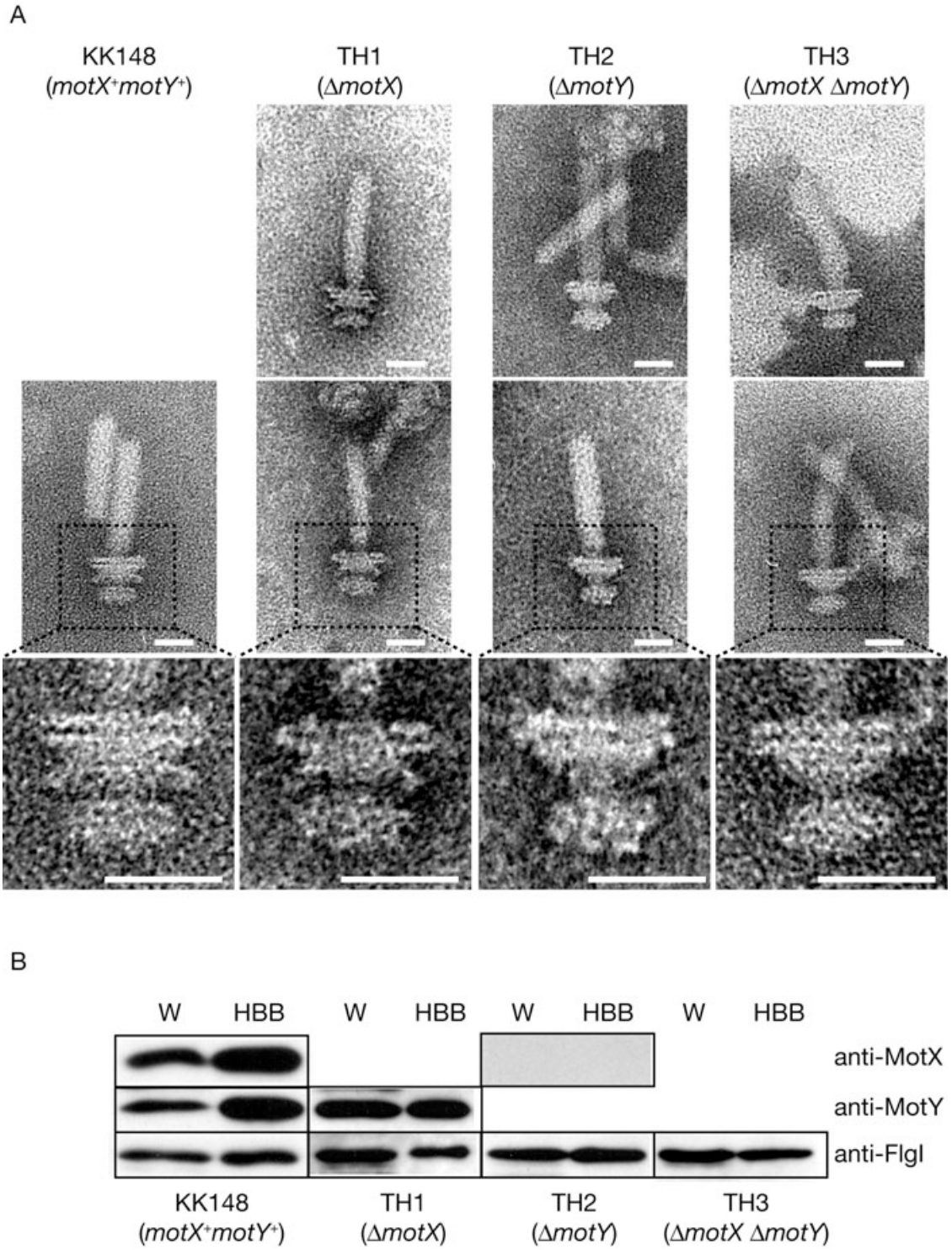

Fig. 4. The basal bodies from *V. alginolyticus motX* and/or *motY* deletion mutants.
A. Electron micrographs of the basal bodies from *V. alginolyticus* strains TH1 (Δ*motX*), TH2 (Δ*motY*) and TH3 (Δ*motX* Δ*motY*) (upper and middle) and enlarged images of the middle panel corresponding to the dotted frame (lower). The hook-basal bodies were negatively stained with 2% phosphotungstic acid (pH 7.4). Bar, 25 nm.
B. Whole cell (W) and the purified hook-basal bodies (HBB) of *V. alginolyticus* strains TH1 (Δ*motX*), TH2 (Δ*motY*) and TH3 (Δ*motX* Δ*motY*) were subjected to SDS-PAGE, followed by immunoblotting using anti-MotX, anti-MotY and anti-FlgI antibodies.

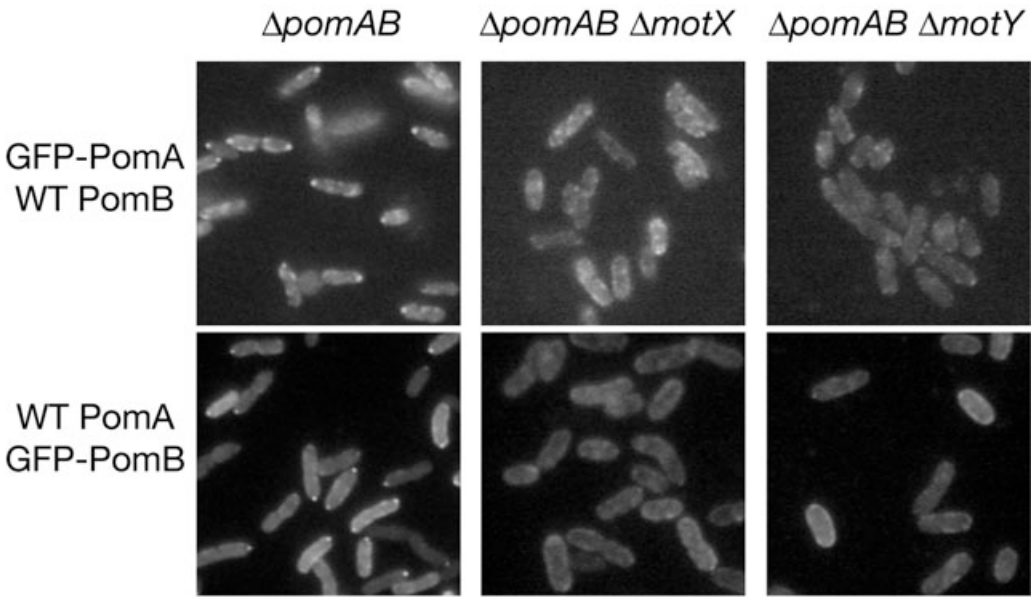

Fig. 5. Cellular localization of GFP-fused PomA or PomB in the *motX* and *motY* deletion strain. The cells harbouring plasmids encoding GFP-fused PomA and PomB (pHFGAB and pHFGBA2) were grown at 30°C for 4 h in VPG500 medium containing 0.02% (w/v) arabinose and were observed under a fluorescence microscope as described in *Experimental procedures*.

in the stabilization or incorporation of the PomA/PomB complex into the flagellar motor to connect to the rotor. To address this possibility, we examined the *in vivo* behaviour of GFP-fused PomA and PomB proteins, which have been shown to localize at a cell pole in a flagellum-dependent, partner protein-dependent and C-terminal region of PomB-dependent manner (Fukuoka *et al*., 2005). In the Δ*pomAB* strain, NMB191, the polar localization of GFP-PomA or GFP-PomB proteins was observed in the presence of the partner protein, PomB or PomA, as described previously (Fig. 5, left). On the other hand, the fluorescent GFP signals were diffused in most of the Δ*motX* (TH4) or Δ*motY* (TH5) cells, which were constructed from NMB191 (Fig. 5, middle and right). Although GFP-PomA produced in the TH4 or TH5 cells was diffuse, some fluorescent clusters were observed on the membrane. GFP-PomB produced in TH4 or TH5 cells was uniformly diffuse. Such intracellular localizations were also observed in cells deficient in the flagellum, the partner proteins or the C-terminal region of PomB as described in the previous report (Fukuoka *et al*., 2005). These results suggest that MotX and MotY are required for the polar localization of the PomA/PomB complex, at the base of the flagellum.

Discussion

PomA, PomB, MotX and MotY are essential for motor function to rotate the Na^+-driven flagellar motor in *Vibrio*. In this study, we clarified that MotX and MotY were associated with the basal body at a position beneath the LP ring and formed a T ring structure based on the following results: (i) the basal body from *V. alginolyticus* had a T ring; (ii) the MotX and MotY proteins were detected in the basal body by immunoblotting; (iii) the periphery of the LP ring or the T ring was decorated with anti-MotX or anti-MotY antibody; and (iv) the basal bodies from Δ*motY* and Δ*motX* Δ*motY* strains lost the T ring. We think that the T ring is associated with the LP ring of the bushing rather than the rod of the rotor. It seems most likely that MotX and MotY associate with the LP ring and do not rotate because they function as stator proteins. The MotX and MotY proteins are translocated across the inner membrane in a signal sequence-dependent manner using the Sec machinery as are the L and P ring proteins, FlgH and FlgI, and they probably assemble onto the LP ring (Okabe *et al*., 2002). The unused MotX and MotY proteins for the T ring assembly can be isolated as outer membrane proteins.

We demonstrated that the GFP-PomA/PomB and PomA/GFP-PomB complexes were rarely localized at the cell pole in the absence of MotX or MotY. These results show that MotX and MotY are required for the polar localization of PomA and PomB and suggest that MotX and MotY have a role in incorporating the PomA/PomB complex into the flagellar motor. What roles do MotX and MotY have for incorporating the PomA/PomB complex? The basal body from the Δ*motX* strain has a partial T ring structure, which is likely to be composed of only the MotY proteins. In the Δ*motX* strain, the PomA/PomB complex appeared not to be incorporated into the flagellar motor because GFP-PomA and GFP-PomB did not localize to

the cell pole. MotX is likely to interact with PomB and recruit the PomA/PomB complex to the motor (Okabe *et al*., 2005). MotY may be required to anchor MotX to the basal body. We propose the incorporation process of PomA and PomB into the flagellar motor as the following scheme. A peptidoglycan-binding motif initially anchors PomB to the cell wall rather than to the motor. Because MotX seems to interact with PomB and MotY, the T ring composed of MotX and MotY appears to assist in this binding process. The T ring may link the PomA/PomB complex with the LP ring. MotX and MotY form a ring structure and may fix the PomA/PomB complex in the *Vibrio* polar flagellar motor. Subsequently, stator formation may be completed and may activate the PomA/PomB complex. The T ring composed of MotX and MotY might be important for generating high-speed rotation, which in the *Vibrio* motor reaches a maximum of 1700 Hz and an average of 1100 Hz (Magariyama *et al*., 1994). The rotation speeds of *E. coli* and *Salmonella* flagella are 300 Hz and 200 Hz respectively (Lowe *et al*., 1987; Kudo *et al*., 1990), and they do not have MotX and MotY paralogues.

A chimeric stator of PomA and PotB, whose periplasmic region was replaced by the corresponding region of MotB from *E. coli*, functioned in *Vibrio* cells without MotX or MotY, although the swarming ability was very poor (Asai *et al*., 2003). The periplasmic region, which contains the peptidoglycan-binding motif, may determine the requirement for MotX and MotY. It has been shown that MotY of *Pseudomonas aeruginosa* is important for flagellar rotation in that organism (Doyle *et al*., 2004). Moreover it has been shown that the MotY$_L$ of the H$^+$-driven lateral flagellar motor of *V. parahaemolyticus* is also important for lateral flagellar motility (Stewart and McCarter, 2003). As the whole genomes of many species of bacteria have been decoded, both putative *motX* and *motY* genes have been found in several species of bacteria, such as *Idiomarina loihiensis* (Hou *et al*., 2004) and *Photobacterium profundum* (Vezzi *et al*., 2005). In contrast, only putative *motY* genes have been found in *Pseudomonas syringae* (Buell *et al*., 2003) and *Legionella pneumophila* (Cazalet *et al*., 2004). It will be interesting to determine whether the flagellar basal body has an additional structure composed of the MotY protein in *Pseudomonas* or *Legionella* sp. As the *motX* gene has not been found in these species, the MotY protein of these species may have a different function from the *Vibrio* protein.

It has been suggested that the periplasmic protein MotC of *Sinorhizobium meliloti* interacts with MotB and regulates the rotation of the flagellar motor (Platzer *et al*., 1997). MotE, a periplasmic chaperone specific for MotC, affects the amount of MotC and also regulates motor rotation (Eggenhofer *et al*., 2004). These proteins do not have significant primary sequence homology with MotX and MotY, although their functions may be similar. Because MotX interacts with PomB, MotX might regulate some activity of the PomA/PomB complex: for example, opening and closing of the Na$^+$-channel of the PomA/PomB complex or the interaction between PomA and FliG through PomB, which is thought to be important for torque generation.

It will be necessary to analyse the stoichiometry of MotX and MotY in the basal body, and generate fine images of the basal body. The stoichiometry of MotX and MotY proteins with the basal body and the detailed shape of the T ring may help determine the number and/or arrangement of the PomA/PomB complexes with the motor. The PomA/PomB complex should be arranged around the basal body to carry out the interaction between PomA and FliG, which is thought to be essential for the rotation of the motor. However, as the PomA/PomB complex does not co-purify with the basal body, it has not been directly demonstrated that the PomA/PomB complex is arranged around the basal body. Although PomB has been suggested to interact with MotX, the PomA/PomB complex did not co-purify with the basal body of the *V. alginolyticus* polar flagellum. The purification method used in this study seemed likely to result in disruption of the interaction between MotX and PomB. If MotX could be bound to PomB in some way, for example, in a fusion or by chemical cross-linking, we would be able to co-purify the rotor with the stator and reconstitute the flagellar motor *in vitro*.

Experimental procedures

Bacterial strains, growth conditions and media

The strains and plasmids used are shown in Table 1. *V. alginolyticus* was cultured at 30°C in VC medium [0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.4% (w/v) K_2HPO_4, 3% (w/v) NaCl, 0.2% (w/v) glucose] or in VPG500 medium [1% (w/v) polypeptone, 0.4% (w/v) K_2HPO_4, 500 mM NaCl, 0.5% (w/v) glycerol]. *E. coli* was cultured in LB broth [1% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] or TG medium [1% (w/v) bactotryptone, 0.5% (w/v) NaCl, 0.5% (w/v) glycerol]. In the second selection to select mutants, *V. alginolyticus* was cultured at 30°C in sucrose-containing plates [1% (w/v) polypeptone, 30 mM NaCl, 55 mM KCl, 10% (w/v) sucrose, 1.25% (w/v) agar]. Chloramphenicol was added to a final concentration of 2.5 μg ml^{-1} for *V. alginolyticus* and 25 μg ml^{-1} for *E. coli*. Kanamycin was added to a final concentration of 100 μg ml^{-1} for *V. alginolyticus*.

Isolation of the flagellar basal bodies

The isolation of the flagellar basal bodies was carried out as described previously with several modifications (Aizawa *et al*., 1985; Kobayashi *et al*., 2003). An overnight culture (grown on VC medium) was inoculated at a 100-fold dilution

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype or description	Reference
V. alginolyticus strains		
VIO5	VIK4 *laf* (Rif^r Pof$^+$ Laf$^-$)	Okunishi *et al*. (1996)
KK148	VIO5 *flhG* (Rif^r multi-Pof$^+$)	Kusumoto *et al*. (2006)
TH1	KK148 Δ*motX* (Rif^r multi-Pof$^+$ Pom$^-$)	This study
TH2	KK148 Δ*motY* (Rif^r multi-Pof$^+$ Pom$^-$)	This study
TH3	KK148 Δ*motX* Δ*motY* (Rif^r multi-Pof$^+$ Pom$^-$)	This study
NMB191	VIO5 Δ*pomAB* (Rif^r Pof$^+$ Pom$^-$)	Yorimitsu *et al*. (1999)
TH4	NMB191 Δ*motX* (Rif^r Pof$^+$ Pom$^-$)	This study
TH5	NMB191 Δ*motY* (Rif^r Pof$^+$ Pom$^-$)	This study
E. coli strains		
SM10λpir	*thi thr leu tonA lacy supE recA::RP4-2-Tc::Mu Km λpirRK6*	Miller *et al*. (1988)
RP437	F$^-$*thi thr leu his met eda rpsL*	Parkinson and Houts (1982)
Plasmids		
pSU41	*kan* (Km^r) P_{lac} *lacZa*	Bartolome *et al*. (1991)
pMO401	pSU41, 0.7 kb ClaI-BamHI fragment (*motX*$^+$)	Okabe *et al*. (2001)
pIO6	pSU38, 1.0 kb HindIII-XbaI fragment (*motY*$^+$)	Okunishi *et al*. (1996)
pKJ601	pKJ502 (*motY*$^+$), 0.7 kb XbaI-BamHI fragment (*motX*$^+$)	Okabe *et al*. (2002)
pKY704	Suicide vector (Cm^r)	Xu *et al*. (1994)
pHFS401	pSU41, 2.0 kb EcoRV-XbaI fragment (*sacB*$^+$)	This study
pMO200	pSU21, 1.0 kb EcoRI-EcoRI fragment (*motX*$^+$)	Okabe *et al*. (2001)
pJN559	pMO200, 385 bp deletion in *motX*	This study
pTH101	pKY704, 0.6 kb EcoRI-EcoRI fragment (*motX*) from pJN559, 2.0 kb XbaI-XbaI fragment (*sacB*$^+$) from pHFS401	This study
pJN507	pUC18, 1.6 kb XbaI-HaeIII fragment (*motY*$^+$)	This study
pJN506	pJN507, 871 bp deletion in *motY*	This study
pTH102	pKY704, 0.8 kb EcoRI-EcoRI fragment (*motY*) from pJN506 2.0 kb XbaI-XbaI fragment (*sacB*$^+$) from pHFS401	This study
pHFGAB	*his6-gfp-pomA* and *pomB* in pBAD33	Fukuoka *et al*. (2005)
pHFGBA2	*his6-gfp-pomB* and *pomA* in pBAD33	Fukuoka *et al*. (2005)

Rif^r, rifampicin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; P_{lac}, *lac* promoter; Pof$^+$, normal polar flagellar formation; Laf$^-$, defective in lateral flagellar formation; multi-Pof$^+$, multiple polar flagellar formation.

into VPG500 medium (500 ml) and cultured at 30°C. After a subsequent 3.5 h cultivation, the cells were harvested in a sucrose solution (0.5 M sucrose, 50 mM Tris-HCl at pH 8.0) and converted into spheroplasts by adding lysozyme and EDTA to final concentrations of 0.1 mg ml^{-1} and 2 mM respectively. The spheroplasts were lysed by adding Triton X-100 to a final concentration of 1% (w/v). To reduce the viscosity of the lysed samples, $MgSO_4$ and DNase I were added to final concentrations of 5 mM and 0.1 mg ml^{-1} respectively. After the viscosity of the solution decreased, EDTA was added to a final concentration of 5 mM. Unlysed cells and cellular debris were removed by centrifugation at 17 000 *g* for 20 min. Polyethylene glycol 6000 and NaCl were added to the lysate to final concentrations of 2% and 100 mM respectively. The samples were then incubated at 4°C for 60 min. The suspension was centrifuged at 27 000 *g* for 30 min at 4°C and the pellet was resuspended in 6 ml of TET buffer [10 mM Tris-HCl at pH 8.0, 5 mM EDTA, 0.1% (w/v) Triton X-100]. To remove cellular debris, the suspension was centrifuged at 1000 *g* for 15 min at 4°C. The supernatant was centrifuged at 100 000 *g* for 30 min and the pellet was resuspended in 200 μl of TET buffer. To dissociate the flagellar filaments into monomeric flagellin, the suspension was diluted 30-fold in 50 mM glycine-HCl (pH 3.5) containing 0.1% (w/v) Triton X-100 and shaken for 60 min at room temperature. After the solution was centrifuged at 1000 *g* for 15 min at 4°C, the supernatant was centrifuged at 150 000 *g* for 40 min at 4°C and the pellet was resuspended in 100 μl of TET buffer. To isolate the basal bodies from *E. coli* RP437, an overnight culture was grown on LB medium and then a second culture was grown on TG medium for 6 h at 30°C. The basal bodies were isolated using the same procedure as described above, except for using 50 mM glycine-HCl (pH 2. 75) containing 0.1% (w/v) Triton X-100 at the filament dissociation step.

Electron microscopy

The isolated flagellar structures were negatively stained with 2% phosphotungstic acid (pH 7.4) and observed with a JEM-1200EX electron microscope (JEOL, Japan).

Determination of N-terminal amino acid sequence

The hook-basal bodies were prepared as described above. The proteins were separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore) and stained with Coomassie blue R250. The band corresponding to approximately 50 kDa was excised. The N-terminal amino acid sequence was determined by Aproscience (Tokushima, Japan), based on the Edman degradation method.

Detection of proteins in cells

Vibrio cells were cultured at 30°C for 3 h in VPG500 medium. The cells collected by centrifugation were suspended in SDS

loading buffer at a concentration corresponding to an A_{660} of 100. The 10 μl preparations of the purified basal body were mixed with 10 μl of SDS loading buffer. The samples were boiled at 90°C for 10 min. SDS-PAGE and immunoblotting were performed as described previously (Yorimitsu *et al*., 1999). The antibodies against MotX (MotXB0080), MotY (MotYB0079), PomA (PomA1312), PomB (PomB93) and FlgI were used as previously described (Nambu *et al*., 1999; Yorimitsu *et al*., 1999; Yagasaki *et al*., 2006).

Sucrose density gradient centrifugation

The preparation of basal bodies was applied to a 20–60% (w/w) stepwise sucrose gradient in TET. After centrifugation at 80 000 *g* for 13 h at 4°C, the gradient was divided into 18 fractions from the bottom to the top.

Gene disruption

The *motX* and *motY* deletion mutants were generated by homologous recombination using a suicide vector as described (Asai *et al*., 1999; Yagasaki *et al*., 2006). To make the Δ*motX* mutant, the sequences upstream (–227 to +72) and downstream (+457 to +756) of the *motX* coding region of pMO200 were amplified by PCR with an artificial StuI sequence (AGGCCT) and ligated with the StuI site, and the resultant plasmid was named pJN559. The 0.6 kb EcoRI fragment of the deleted *motX* gene was excised from pJN559 and introduced into the suicide vector pKY704 (Cm^r). To make the Δ*motY* mutant, the sequences upstream (–440 to –2) and downstream (+871 to +1237) of the *motY* coding region of pKJ507 were amplified by PCR with an artificial StuI sequence (AGGCCT) and ligated with the StuI site, and the resultant plasmid was named pJN506. The 0.8 kb XbaI-SacI fragment of the deleted *motY* gene was excised from pJN506 and introduced into the suicide vector pKY704 (Cm^r). The *sacB* gene ($sucrose^s$), which was obtained from pHFS401 using XbaI, was also introduced into pKY704 as a selection marker for the second recombination. The suicide plasmids, pTH101 (containing the Δ*motX* fragment) and pTH102 (containing the Δ*motY* fragment), were used to transform *E. coli* SM10λpir. The deletion alleles were introduced into KK148 by a conjugation-based method. Chloramphenicol-resistant mutants were selected first on VC plates, and then chloramphenicol-sensitive sucrose-resistant mutants were identified on sucrose-containing plates. We confirmed the deletion mutations in the chromosomal *motX* and *motY* genes based on the sizes of the amplified PCR fragments and the complementation tests with pMO401, pIO6 and pKJ601. These mutants were named TH1 (Δ*motX*), TH2 (Δ*motY*) and TH3 (Δ*motX* Δ*motY*). We also generated gene disruptions in NMB191 by the same methods; these mutants were named TH4 (Δ*pomAB* Δ*motX*) and TH5 (Δ*pomAB* Δ*motY*).

Immunogold labelling

The 3 μl basal body sample was mixed with 1 μl of buffer-A (10 mM Tris, 5 mM EDTA, 600 mM NaCl at pH 8.0) and with 1 μl of the antibody solution diluted 20-fold with buffer-B (10 mM Tris, 5 mM EDTA, 150 mM NaCl at pH 8.0). The mixture was placed on a carbon-coated copper-mesh grid and was left at room temperature for 1–2 h. The grid was washed with buffer-B, and 5 nm colloidal gold-conjugated anti-rabbit IgG antibody diluted twofold with 2 μl of buffer-C (20 mM Tris, 10 mM EDTA, 300 mM NaCl at pH 8.0) was put on the grid, and this sample was left at room temperature for 1–2 h. Then, the grid was washed with buffer-B, negatively stained with 2% phosphotungstic acid (pH 7.4) and observed with an electron microscope.

Observation of GFP-PomA and GFP-PomB

The cells harbouring plasmids encoding GFP-fused PomA and PomB (pHFGAB and pHFGBA2) were grown at 30°C for 4 h in VPG500 medium containing 0.02% arabinose. Cultured cells (1 ml) were harvested by centrifugation and suspended in 200 μl of V buffer (50 mM Tris, 5 mM $MgCl_2$, 300 mM NaCl, at pH 7.5). A small aliquot of the cell suspension was spotted onto a glass slide, and cells were fixed on the slide with an equal volume of 0.5% agarose dissolved in V buffer. Cells were observed by fluorescence microscopy (Olympus BX50). The fluorescent images of GFP-PomA and GFP-PomB were taken with a 1 s exposure. The images were recorded and processed using a digital camera (Hamamatsu Photonics, C4742-80-12AG) and imaging softwares (BD Biosciences, IPLab, Ver. 3.9.5 r2 and Adobe, Photoshop, Ver. 7).

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