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Isolation of *Vibrio alginolyticus* sodium-driven flagellar motor complex composed of PomA and PomB solubilized by sucrose monocaprate

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The polar flagella of *Vibrio alginolyticus* have sodium-driven motors, and four membrane proteins, PomA, PomB, MotX and MotY, are essential for torque generation of the motor. PomA and PomB are believed to form a sodium-conducting channel. This paper reports the purification of the motor complex by using sucrose monocaprate, a non-ionic detergent, to solubilize the complex. Plasmid pKJ301, which encodes intact PomA, and PomB tagged with a C-terminal hexahistidine that does not interfere with PomB function, was constructed. The membrane fraction of cells transformed with pKJ301 was solubilized with sucrose monocaprate, and the solubilized materials were applied to a Ni-NTA column. The imidazole eluate contained both PomA and PomB, which were further purified by anion-exchange chromatography. Gel-filtration chromatography was used to investigate the apparent molecular size of the complex; the PomA/PomB complex was eluted as approx. 900 kDa and PomB alone was eluted as approx. 260 kDa. These findings suggest that the motor complex may have a larger structure than previously assumed.

INTRODUCTION

Many bacteria swim by rotating helical flagella in liquid environments. The torque of flagella is generated by a reversible rotary motor embedded in the cytoplasmic membrane at the base of each flagellar filament (Blair, 1995; Macnab, 1999). The motor is composed of the stator and rotor parts. The energy for torque generation comes from the transmembrane electrochemical potential of specific ions, sodium ions or protons. The flagellar motors can be classified into two types: proton-driven and sodium-driven, which are dependent on different coupling ions (McCarter, 2001; Yorimitsu & Homma, 2001). Although the specific mechanism is unknown, ions are thought to pass through the stator complex while the ion flux generates the rotational force between the stator and rotor.

Escherichia coli and *Salmonella typhimurium* have proton-driven motors that have been extensively studied (Berry & Armitage, 1999; Blair, 1995; Derosier, 1998). The stator complex consists of the cytoplasmic membrane proteins MotA and MotB, which are essential for torque generation. MotA has four transmembrane segments and a large cytoplasmic loop (Zhou *et al.*, 1995). MotB has a single transmembrane domain and a peptidoglycan-binding motif (Chun & Parkinson, 1988); there is an Asp residue in the putative transmembrane region, which is speculated to be protonated during torque generation. The protonation of the Asp residue induces a conformational change of the motor protein (Kojima & Blair, 2001). MotA and MotB together have been suggested to form a proton channel, based on extensive genetic and physiological evidence (Blair & Berg, 1990; Garza *et al.*, 1995, 1996; Sharp *et al.*, 1995a; Stolz & Berg, 1991).

Vibrio alginolyticus has two types of flagella in one cell: a lateral flagellum with a proton-driven motor and a polar flagellum with a sodium-driven motor (Atsumi *et al.*, 1992; Kawagishi *et al.*, 1995). In the polar flagellar motor, the following four proteins have been identified and found to be essential for torque generation: PomA, PomB, MotX and MotY (Asai *et al.*, 1997; Furuno *et al.*, 1999; Okabe *et al.*, 2001; Okunishi *et al.*, 1996). PomA and PomB are homologous to MotA and MotB, respectively, of the proton-driven motor. Thus it is thought that PomA and PomB form a complex and have similar function to MotA and MotB. The direct interaction between PomA and PomB has been demonstrated (Yorimitsu *et al.*, 1999). PomA was required for the stability of PomB. PomB also has an essential charged residue, Asp, which is highly conserved in the transmembrane region of the motor protein. MotX and MotY were first identified in *Vibrio parahaemolyticus* (McCarter, 1994a, b), and homologous genes have been identified only in *Vibrio* species. We suggest that MotX and MotY mutually stabilize each other and that MotX is more directly involved in motor function than MotY (Okabe *et al.*, 2001). MotY has a peptidoglycan-binding motif at the C-terminal region. The motif is also observed

Abbreviation: β-octylglucoside, n-octyl β-D-glucopyranoside.

in MotB and PomB. The roles of MotX and MotY have not always been clear. However, these proteins are located in the outer membrane (Okabe *et al.*, 2002) and may be essential for sodium recognition in the motor and/or for the fast rotation speed of flagella in *Vibrio* cells (Asai *et al.*, 2003). The maximal rotational speed of the sodium-driven motor is 1700 revolutions per second, whereas the maximal rotational speed of the proton-driven *E. coli* motor is 300 revolutions per second (Yorimitsu & Homma, 2001). The sodium-driven motor has advantages for the study of motor function because sodium-motive force can be easily manipulated. Moreover, the specific sodium channel inhibitors, amiloride and phenamil, can be used to study the mechanism of torque generation in this system (Atsumi *et al.*, 1990; Sugiyama *et al.*, 1988). Phenamil-resistant mutations were mapped near the cytoplasmic ends of the putative transmembrane segment of PomA and PomB (Jaques *et al.*, 1999; Kojima *et al.*, 1999). It is inferred that the high-affinity phenamil-binding site is located around the interface of the cytoplasmic ends of PomA and PomB.

We previously purified the components of the torque-generating unit, PomA and PomB, which were solubilized by the non-ionic detergent β-octylglucoside. We showed that the PomA/PomB complex exhibits significant sodium uptake activity (Sato & Homma, 2000a). From further experiments, however, we found that the complex dissociates easily with β-octylglucoside. In the present study, the membrane-associated motor proteins were solubilized and isolated by using sucrose monocaprate, another non-ionic detergent. We partly characterized the motor complex composed of PomA and PomB, which appears to be much larger than previously believed.

METHODS

Bacterial strains, plasmids, growth conditions and media. The strains and plasmids used are shown in Table 1. *V. alginolyticus* cells were 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 VPG medium (1 %, w/v, polypeptone, 0·4 %, w/v, K_2HPO_4, 3 %, w/v, NaCl, 0·5 %, w/v, glycerol). For the swarm assay, VPG-0·3 % agar plates were used. When necessary, kanamycin was added to the final concentration of 100 μg ml^{-1} for *V. alginolyticus*.

Plasmid construction. Plasmid pKJ201, a pSU41-based plasmid, was constructed to carry *pomB-his*$_6$ under the control of the *lac* promoter. A 0·9 kb DNA fragment including the *pomB* open reading frame with a 3′ attachment of 5′-GAAGAAGTGCCGGTAATTCA-ACATCACCATCACCATCACTAAGAGCTCAAG-3′ (stop as His$_6$-end; the underlining indicates the created *Sac*I restriction sites) was prepared by PCR. The fragment was cloned into pSK603 (pSU41 carrying the wild-type *pomB* gene; Kojima *et al.*, 1999) digested by *Hpa*I and *Sac*I. The total inserts were confirmed by DNA sequencing. The fragment of pKJ201, digested by *Hpa*I and *Sac*I, was replaced with the fragment of pYA303 carrying *pomA* and *pomB* under *lac* promoter control. The resultant plasmid pKJ301, carrying *pomA* and *pomB-his*$_6$, was constructed. Other plasmids used are listed in Table 1.

Association of PomA and PomB. Cells were harvested and washed with V-buffer (25 mM Tris/HCl, pH 7·5, 10 mM $MgSO_4$, 300 mM NaCl). The washed cells were suspended in 400 ml 20TMPD (20 mM Tris/HCl, pH 8·0, 5 mM $MgSO_4$, 0·5 mM PMSF and 1 mM DTT) and mixed with a homogenizer (Polytron, type PT3000), with the rotation speed set at 10 000 r.p.m. for 2 min. After adding DNase I to 20 μg ml^{-1}, 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 20 min at 4 °C) and the membrane fraction was recovered from the supernatant by centrifugation at 200 000 ***g*** for 2 h at

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description*	Reference
V. alginolyticus		
NMB190	Rif^r Pof$^+$ Laf$^-$, Δ*pomA*	Asai *et al.* (1999)
NMB191	Rif^r Pof$^+$ Laf$^-$, Δ*pomAB*	Yorimitsu & Homma (2001)
NMB192	Rif^r Pof$^+$ Laf$^-$, Δ*pomB*	Asai *et al.* (2003)
Plasmids		
pSU41	*kan*(Km^r) P_{lac} *lacZα*	Bartolome *et al.* (1991)
pKS101	pSU41 0·8 kb *Bam*HI–*Bam*HI (*his*$_6$-*pomA*)	Sato & Homma (2000a)
pKS102	pSU41 1·9 kb *Bam*HI–*Sac*I (*his*$_6$-*pomA*/*pomB*)	Sato & Homma (2000a)
pSK603	pSU41 1·1 kb *Bam*HI–*Sac*I (*pomB*)	Kojima *et al.* (1999)
pKJ201	pSU41 1·1 kb *Bam*HI–*Sac*I (*pomB-his*$_6$)	This study
pYA303	pSU41 1·9 kb *Bam*HI–*Sac*I (*pomAB*)	Kojima *et al.* (1999)
pKJ301	pSU41 1·9 kb *Bam*HI–*Sac*I (*pomA*/*pomB-his*$_6$)	This study
pMMB206	*cat*(Cm^r) IncQ *lacI*^{q} Δ*bla* $P_{tac\text{-}lac}$ *lacZα*	Morales *et al.* (1991)

*Cm^r, chloramphenicol-resistant; Km^r, kanamycin-resistant; P_{lac}, *lac* promoter; $P_{tac\text{-}lac}$, tandemly located *tac* and *lac* promoters; Rif^r, rifampicin-resistant; Pof$^+$, polar flagella formation; Laf$^-$, lateral flagellar formation defect.

4 °C. The membrane pellet was suspended in 20TNPDG (20 mM Tris/HCl, pH 8·0, 0·15 M NaCl, 0·5 mM PMSF, 1 mM DTT and 10 %, w/v, glycerol). Sucrose monocaprate or β-octylglucoside was added to the membrane fraction suspended in 20TNPDG to give final concentrations of 2·5 % and 10 mg membrane protein ml^{-1}. Two millilitres of the suspension was mixed for 30 min at 4 °C and centrifuged for 30 min at 100 000 ***g***. Imidazole was added to each supernatant at 5 mM final concentration, then the solubilized membrane preparation was mixed with 25 µl Ni-NTA agarose (QIAGEN) for 1 h at 4 °C. The loaded resin was extensively washed with 20TNPDG containing 5 mM imidazole and 0·5 % sucrose monocaprate or 1·25 % β-octylglucoside. Bound proteins were eluted with 100 µl 20TNPDG containing 400 mM imidazole and 0·5 % sucrose monocaprate or 1·25 % β-octylglucoside.

Preparation of the motor complex. A culture of bacteria was grown to stationary phase in 18 litres of VC medium. The membrane fraction was prepared as above and suspended in 20TNPDG. This suspension was homogenized with a Polytron homogenizer and sucrose monocaprate was added to 2 % final concentration. The suspension was mixed on ice and centrifuged for 20 min at 10 000 ***g***. After addition of imidazole to 10 mM final concentration, the clarified extract was mixed with Ni-NTA agarose (QIAGEN), incubated at 4 °C for 1 h with gentle mixing, and then packed into the column. The loaded resin was washed with 20TNPDG containing 0·5 % sucrose monocaprate and 10 mM imidazole. Elution was conducted with 20TNPDG containing 0·5 % sucrose monocaprate and 400 mM imidazole. The eluate from the Ni-NTA column was diluted twofold with 20TPDG (20 mM Tris/HCl, pH 8·0, 0·5 mM PMSF, 1 mM DTT and 10 %, w/v, glycerol) and applied to a Q-Sepharose HiTrap Q HP column (Pharmacia) equilibrated with 20TPDG containing 75 mM NaCl. The column was washed with 20TPDG containing 75 mM NaCl and bound material was eluted with a 75–700 mM linear gradient of NaCl in 20TPDG.

Gel-filtration chromatography. A Superose 6 PC 3.2/30 gel-filtration column (Pharmacia) was equilibrated with 20TNPDG containing 0·5 % sucrose monocaprate. A 50 µl sample was applied to the Superose 6 column and eluted with the same buffer as used for the equilibration, at 30 µl min^{-1} flow. The sample was recovered as 60 µl fractions 25 min after the sample injection.

Sucrose density-gradient centrifugation. The suspension was layered on 5–30 % linear sucrose gradient in 20TNPDG containing 0·5 % sucrose monocaprate. Centrifugation was carried out in a Beckman SW41Ti rotor for 22 h at 24 000 r.p.m. and 6 °C.

SDS-PAGE and immunoblotting. These were performed as previously described (Okabe *et al.*, 2001). The antipeptide antibodies against PomA and PomB, which are referred to as PomA91 and PomB93, respectively, were prepared as previously described (Yorimitsu *et al.*, 1999).

Amino acid sequence of the N terminus. Proteins separated by SDS-PAGE were transferred to a PVDF membrane and stained by Coomassie blue R250. The protein band corresponding to PomB was excised and analysed with a peptide sequencer (analysis done by APRO Science Co., Tokushima, Japan).

$^{22}Na^{+}$ uptake by proteoliposomes. Proteoliposomes were reconstituted by the detergent dilution method as follows. A sample was mixed with 5·0 mg *E. coli* phospholipids (Avanti Polar Lipids) in 20 mM Tris/HCl, pH 8·0, 200 mM KCl, 0·5 %, w/v, sucrose monocaprate, 10 %, w/v, glycerol. The mixture (100 µl) was sonicated briefly and incubated on ice for 20 min and rapidly diluted (40-fold) into the dilution buffer (20 mM Tris/HCl, pH 8·0, 200 mM KCl). After 15 min at 4 °C with gentle shaking, proteoliposomes formed were recovered by centrifugation at 200 000 ***g*** for 1 h, resuspended in 100 µl dilution buffer, frozen in dry ice/ethanol and stored at −80 °C. For assays, the suspension was thawed at room temperature.

The standard incubation mixtures contained the following, in 500 µl, at 30 °C: (i) 20 mM Tris/HCl, pH 8·0, 200 mM choline chloride, 1 mM $^{22}NaCl$ (0·4 µCi ml^{-1}; 14·8 kBq ml^{-1}), and (ii) proteoliposomes loaded with 200 mM KCl (50 µl). Components (i) and (ii) were separately incubated at 30 °C for 3 min; they were then mixed and valinomycin was added at the final concentration of 2 µM to form a diffusion membrane potential. At intervals, 90 µl of the reaction mixture was passed through Dowex 50WX8-100 (Sigma) prewashed with 20 mM Tris/HCl buffer, pH 8·0, containing 200 mM choline chloride, to trap the unincorporated $^{22}Na^{+}$. The radioactivity of the flow-through fraction was determined by a γ-counter.

RESULTS

Association of PomA with PomB in different detergents

The membrane fractions of NMB190 transformed with pKS101 and NMB191 transformed with pKS102 were solubilized with β-octylglucoside or sucrose monocaprate. It seemed that the membrane was more effectively solubilized by sucrose monocaprate because the amount of PomA and PomB protein detected was greater with this detergent. The solubilized membrane proteins were passed through a Ni-NTA agarose column and the column was washed. Bound proteins were eluted with buffer containing imidazole (Fig. 1, lanes 5). The PomA protein was detected by immunoblotting after solubilization by detergent. However, the PomB protein was only detected after sucrose monocaprate solubilization, even when both proteins were overproduced from pKS102. This indicates that the complex of PomA and PomB was dissociated by β-octylglucoside. The amount of PomA recovered was greater with sucrose monocaprate. This may reflect the efficiency of the solubilization from the membrane fraction.

Construction of PomB-His_6 and its function

The binding efficiency of His_6-PomA to Ni-NTA agarose was not good enough to allow suitable recovery of PomA/PomB complexes, so we tried to make a His-tagged PomB protein. The hexahistidine sequence was inserted at the C terminus of PomB, where it was predicted to localize in the periplasmic space or bind to the peptidoglycan. We constructed plasmid pKJ201, which encodes PomB with an attached hexahistidine sequence (PomB-His_6) at the C terminus. pKJ201 complemented the *pomB* null mutant; the complementation profile was similar to that observed with pSK603, which encodes wild-type PomB (Fig. 2). In addition, the swimming speed of cells harbouring pKJ201 was similar to that of wild-type cells. Thus, the attachment of the hexahistidine tag does not interfere with PomB function. This hexahistidine tag was transferred to the plasmid pYA303 carrying *pomA* and *pomB*; the resultant plasmid was named pKJ301. The motility of the *pomB* mutant (NMB192) and the *pomA pomB* mutant (NMB191) was restored by pKJ301.

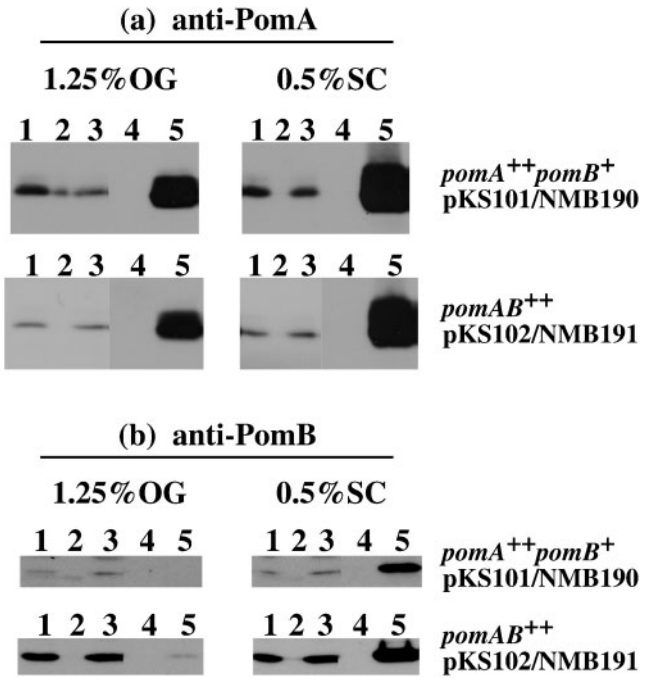

Fig. 1. Stability of the complex solubilized by detergents. Membrane fractions (lanes 1) of NMB190 transformed with pKS101 (*his*$_6$-*pomA*) and NMB191 transformed with pKS102 (*his*$_6$-*pomA pomB*) were prepared. Sucrose monocaprate (SC) and β-octylglucoside (OG) were added to the membrane fraction and centrifuged for 30 min at 100 000 ***g*** and the unsolublized pellet (lanes 2) and solubilized supernatant (lanes 3) were recovered. The solubilized membrane was mixed with Ni-NTA agarose and the loaded resin was washed with 20TNPDG containing 5 mM imidazole and 0·5 % sucrose monocaprate or 1·25 % β-octylglucoside (lanes 4). Bound proteins were eluted with 20TNPDG containing 400 mM imidazole and 0·5 % sucrose monocaprate or 1·25 % β-octylglucoside (lanes 5). Proteins in each elute were separated by SDS-PAGE and immunoblotting was performed with anti-PomA antibody (a) and anti-PomB antibody (b).

Separation of the motor complex composed of PomA and PomB

The membrane fractions of NMB191 transformed with pKJ301 were prepared, solubilized with sucrose monocaprate and applied to a Ni-NTA agarose column. The agarose column was washed and bound proteins were eluted with buffer containing imidazole. The PomB protein was detected by staining with Coomassie brilliant blue but the PomA protein was not. The N-terminal five amino acids (MDDED) of the stained protein matched the predicted sequence of PomB. Both PomA and PomB were detected by immunoblotting (data not shown).

The eluate from the Ni-NTA agarose resin was applied to an anion-exchange column and eluted with a NaCl gradient. Each fraction was analysed by SDS-PAGE followed by staining with Coomassie brilliant blue (Fig. 3a) and by

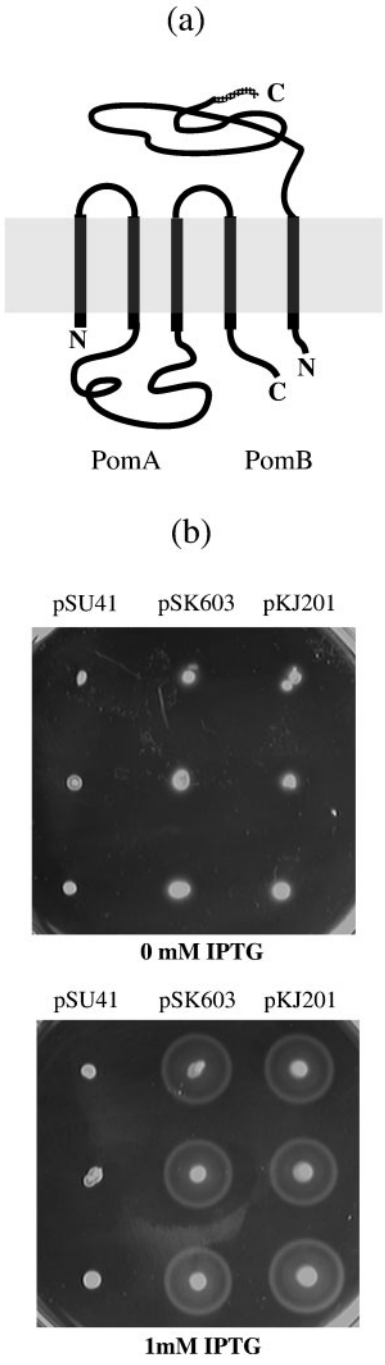

Fig. 2. (a) Predicted membrane topology of PomA and PomB. Thick black lines indicate the predicted transmembrane regions. The dotted line at the C-terminal region of PomB indicates the added hexahistidine. (b) Swarming ability by cells encoding PomB-His$_6$. Swarming abilities of NMB192 (Δ*pomB*) cells, which were co-transformed with pMMB206 (*lacI*^{q}) plus pSU41 (vector), pSK603 (*pomB*$^+$), or pKJ201 (*pomB-his*$_6$$^+$) were examined. Overnight cultures were spotted on VPG-0·3 % agar plates containing kanamycin, chloramphenicol with (lower photograph) or without (upper photograph) IPTG and incubated at 30 °C for 4 h.

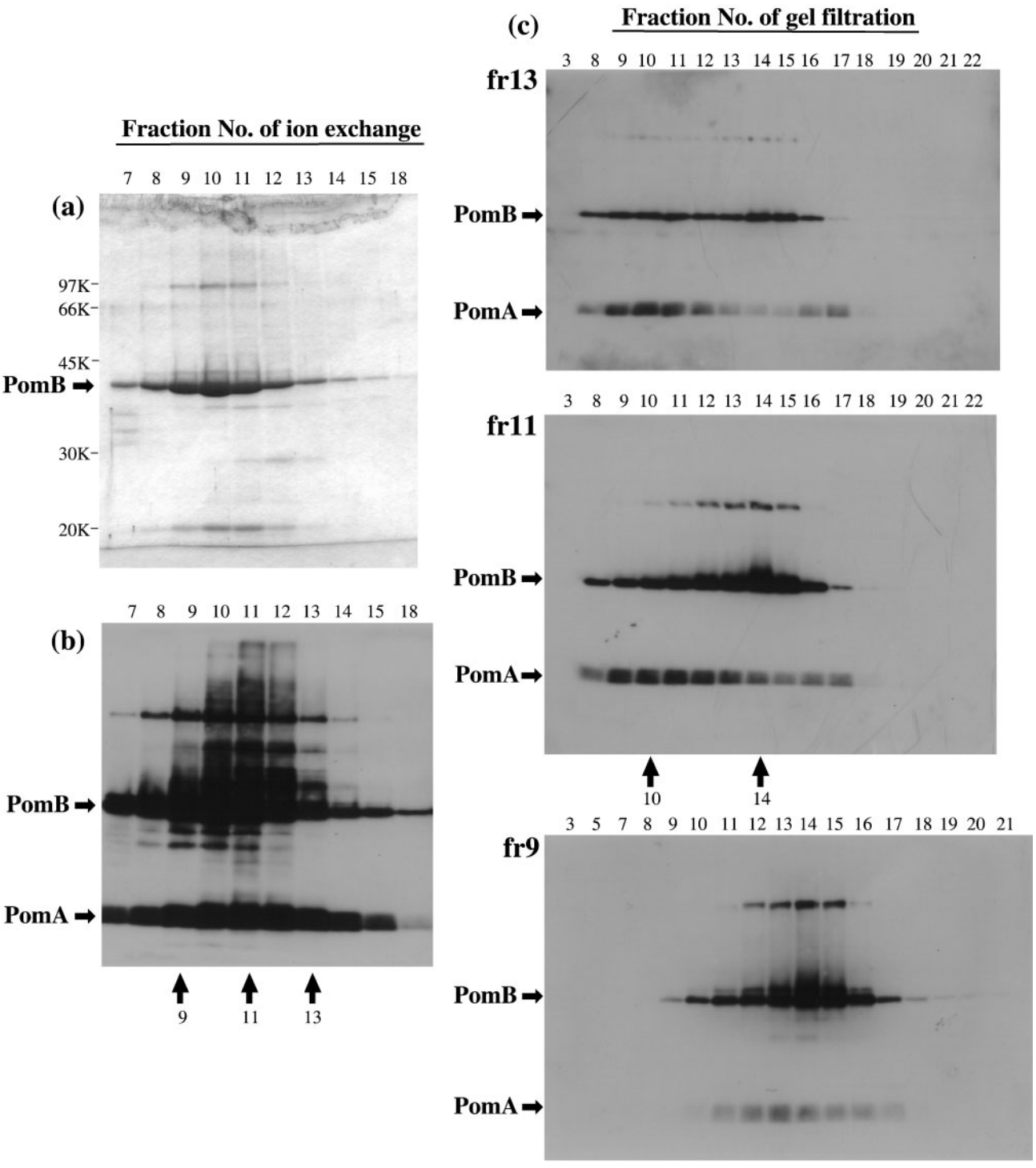

Fig. 3. Isolation of the PomA/PomB complex. The membrane fraction from NMB191 transformed with pKJ301 (*pomA pomB-his*$_6^+$) was solubilized with sucrose monocaprate and subjected to Ni-NTA chromatography. The eluate was applied to a Q Sepharose HP anion-exchange column and eluted with a linear gradient from 75 to 700 mM NaCl. The proteins of each fraction were separated by SDS-PAGE and were detected by Coomassie brilliant blue (a) or by immunoblotting with the anti-PomA and the anti-PomB antibodies (b). Fractions 9, 11 and 13 from the anion-exchange chromatography were analysed on a Superose 6 column, and PomA and PomB in each fraction were detected by immunoblotting with anti-PomA and the anti-PomB antibodies (c).

immunoblots using antibodies generated against PomA and PomB (Fig. 3b). PomB was efficiently recovered. PomA and PomB mostly co-eluted and the main peaks were at approximately 0·3 M NaCl. However, the elution profiles of PomA and PomB, at the peak fractions 11 and 10, respectively, did not completely correspond with each other.

Size-separation of the motor complex

Some of the fractions from anion-exchange chromatography were also analysed by gel-filtration chromatography using a Superose 6 column, which has a separation range from 5 kDa to 5000 kDa (Fig. 3c). A PomA/PomB complex of high molecular size was recovered when ion-exchange fraction 11 or 13 was applied to the gel-filtration column. However, fraction 9, which was eluted from the anion-exchange column at a lower concentration of NaCl, contained very little high molecular size complexes. PomA eluted around fraction 10 of the gel-filtration column. PomB alone seemed to be eluted at peak fraction 14 in gel-filtration chromatography. Elution profiles of UV absorbance for the gel-filtration chromatography are shown in Fig. 4. The peaks of the molecular size of fractions 10 and 13 were estimated as about 900 kDa and 260 kDa, respectively, when soluble globular proteins were used as

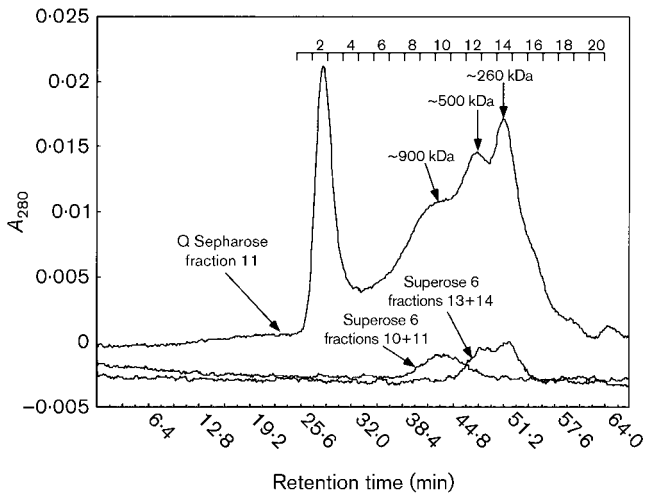

Fig. 4. Elution profiles on gel-filtration chromatography. Fraction 11 from the anion-exchange chromatography was mixed with blue dextran, and applied to the Superose 6 column; the A_{280} was measured in the eluate. Similarly, samples consisting of pooled fractions 10 and 11 or fractions 13 and 14 from the gel-filtration column used to separate fraction 11 of anion-exchange column were analysed again by the same Superose column without blue dextran. The numbers on the scale above the UV absorbance trace correspond to the gel-filtration chromatography fractions. A standard curve was made from the elution time of molecular size standards: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and ribonuclease A (14 kDa). The molecular size of the peaks was estimated as shown by arrows.

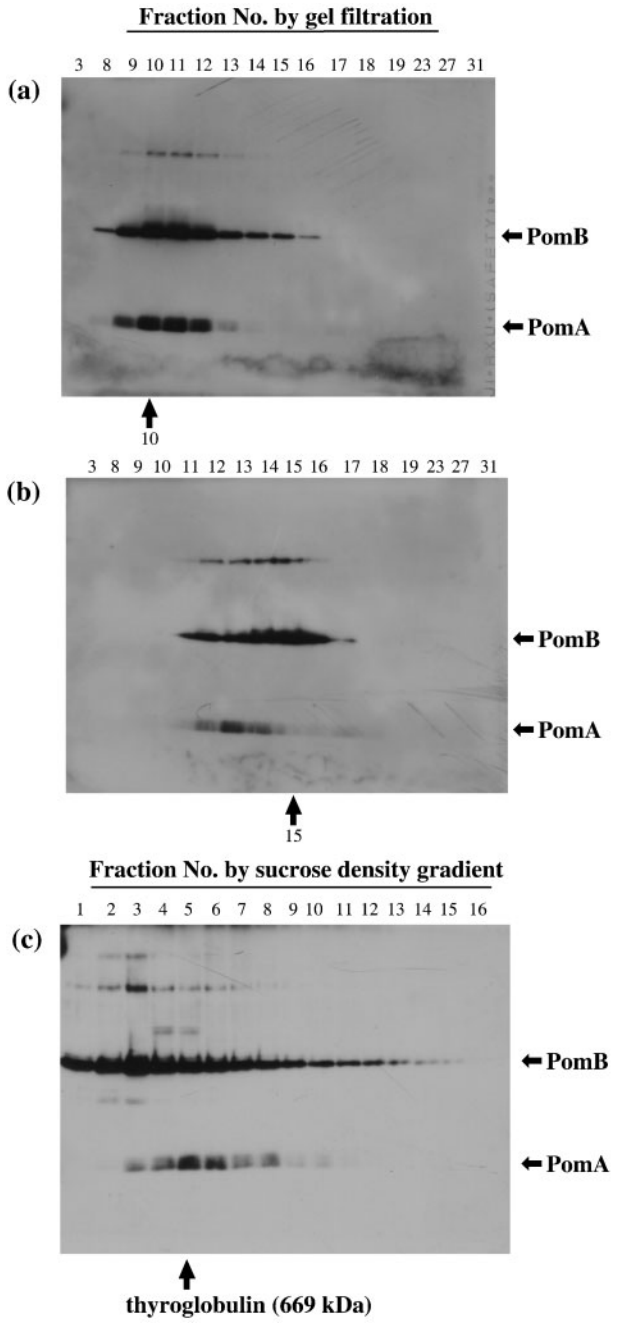

Fig. 5. Separation of the purified PomA/PomB complex by gel-filtration chromatography and sucrose density-gradient centrifugation. Samples consisting of pooled fractions 10 and 11 (a) or pooled fractions 13 and 14 (b) of the gel-filtration column used to separate fraction 11 of the anion-exchange column were analysed again on the same gel-filtration column. (c) Purified PomA/PomB complex from fraction 10 of (a) was analysed by sucrose density-gradient centrifugation (5–30 %). The gradient was divided into 23 fractions from the top; the arrow indicates the fraction in which the thyroglobulin (669 kDa) peak was detected. PomA and PomB of each fraction, which were separated by SDS-PAGE, were detected by immunoblotting with the anti-PomA and the anti-PomB antibodies.

standards. The ion-exchange fraction 11 was applied to the gel-filtration column and the resulting fractions 10 and 11 or fractions 13 and 14 of the gel-filtration column were pooled. Each pooled fraction was separated again by the same Superose column (Figs 4 and 5). PomA and PomB were separated at similar fraction positions as before, suggesting that the complex is fairly stable. These experiments show that the structures composed of PomA and/or PomB separated by gel-filtration chromatography have different molecular sizes. The size of the huge complex of PomA and PomB was confirmed by sucrose density-gradient centrifugation (Fig. 5c). The PomA/PomB complex isolated by the Ni-NTA and by gel-filtration chromatography was reconstituted into proteoliposomes. However, we could not detect sodium uptake activity in any samples used (data not shown).

Fractions from gel-filtration chromatography were analysed by SDS-PAGE without the reducing reagent 2-mercaptoethanol (Fig. 6). The peak fraction 14 from the gel-filtration chromatography mainly contained the PomB dimer connected by disulfide cross-linking.

Effects of SDS and DTT on the PomA/PomB complex

We performed gel-filtration chromatography after the addition of SDS or DTT (Fig. 7). The approximately 900 kDa complex seemed to be dissociated by the SDS treatment. PomA eluted at fraction 17, which corresponded to approximately 60 kDa. On the other hand, PomB of the high molecular size complex disappeared and the approximately 260 kDa complex remained as a result of this SDS treatment. The PomA complex appeared to be unaffected by the DTT treatment. PomB continued to be eluted at fraction 9 or 10, corresponding to approximately 900 kDa. However, the amount of PomB at this size was reduced. The main peak of PomB corresponded to approximately 260 kDa and shifted one fraction later, which corresponded to about half of the original size.

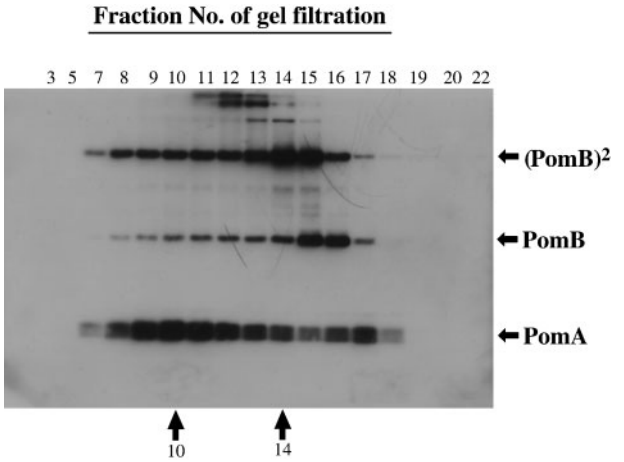

Fig. 6. SDS-PAGE profiles without reducing reagent. Fraction 11 from anion-exchange chromatography was analysed on a Superose 6 column. Proteins in each fraction were separated by SDS-PAGE without 2-mercaptoethanol and PomA and PomB were detected by immunoblotting.

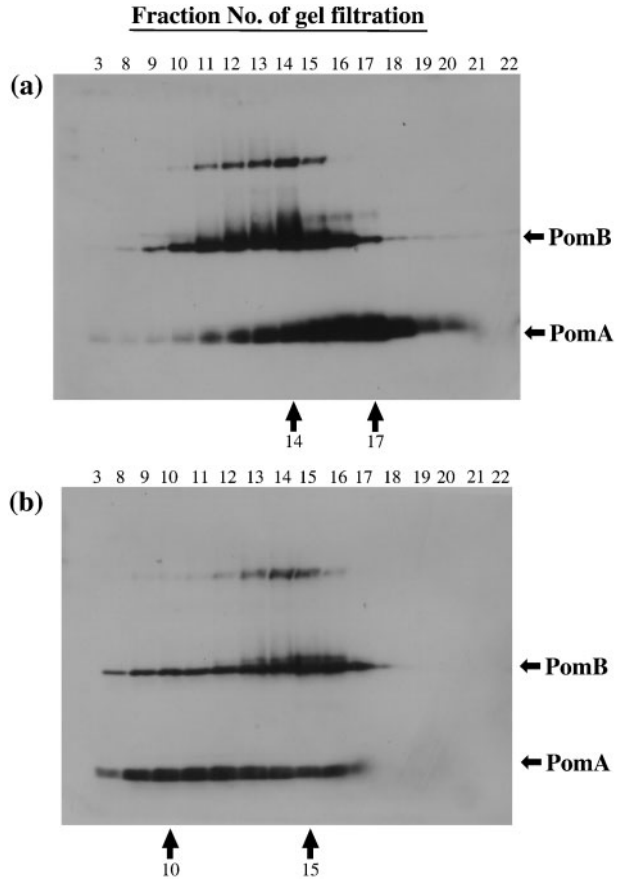

Fig. 7. Treatment with SDS or DTT for the PomA/PomB complex. (a) A 20% SDS solution was added at 1/20 to fraction 11 of anion-exchange chromatography and the mixture was incubated at 65 °C for 5 min. (b) A 0·5 M DTT solution was added at 1/10 to fraction 11 of anion-exchange chromatography. The SDS- (1%) or DTT- (50 mM) treated sample was analysed on a Superose 6 column. PomA and PomB in each fraction were separated by SDS-PAGE and detected by immunoblotting.

DISCUSSION

The flagellar motor consists of the stator and rotor. The stator is composed of MotA and MotB for the proton-driven motors or of PomA, PomB, MotX and MotY for the sodium-driven motors. The essential components are thought to be similar in the two systems, based on the following evidence. The structure of the basal body is similar (Bakkeva *et al.*, 1986). The proton-type MotA functions like the sodium-type PomA (Asai *et al.*, 1999). MotA and MotB can function as a proton motor with the

rotor of the *Vibrio* polar motor, which is originally of the sodium type (Gosink & Häse, 2000; Asai *et al.*, 2003). The structure and composition of the rotor part of the proton motor have been studied extensively by electron microscopic and biochemical analyses. At the early stage of the motor study, the rotor part (MS-ring) was thought to function as the source of force generation (Homma *et al.*, 1987). However, we now know that it is just a passive-rotating ring of the motor (DeRosier, 1998). A C-ring structure has been found under the MS-ring when isolated by a mild condition (Driks & DeRosier, 1990). Even in mild conditions, the stator parts or the Mot proteins were not associated with the rotor structure. We can assume that the interaction between the rotor and stator is not so strong because they have to move relative to one another to produce torque. It has been shown by a co-isolation experiment that MotA binds to FliG and to FliM (Tang *et al.*, 1996). This suggests that the rotor and stator interact under certain conditions so that a force is generated between them. We purified the stator complex or the PomA/PomB complex of *V. alginolyticus*. The complex did not contain MotX and MotY, or the rotor proteins FliG, FliM and FliN (this study; Sato & Homma, 2000a). We demonstrated sodium uptake activity in proteoliposomes reconstituted with the PomA/PomB complex, which was purified by using β-octylglucoside. The amount of protein of the complex recovered by this purification procedure was too low to proceed to further experiments for structural or biochemical analyses.

The purification procedure of the PomA/PomB motor complex was improved in this study. The detergent sucrose monocaprate allowed better solubilization of membrane fractions associated with the PomA/PomB complex; however, MotX or MotY was still not associated with this complex. We changed the position of the attached hexahistidine tag to motor components. Previously, the tag was fused to the N terminus of PomA. The binding efficiency of His_6-PomA to Ni-NTA agarose was not adequate. Therefore, we made a His-tagged PomB protein. The hexahistidine sequence was inserted at the C terminus of PomB, where it was predicted to localize in the periplasmic space or bind to peptidoglycans. PomB-His_6 functions like the wild-type PomB. Interaction with the rotor has been shown to be necessary to adjust the structure to a functional form of the stator, which is composed of A and B subunits (Garza *et al.*, 1996). We speculate that a small fraction of the motor complex in the overproduced condition was activated by interaction with the rotor because the functional rotors were not overproduced. Therefore, cells can survive in the overproduced condition. Similarly, the overproduction of MotA and MotB did not severely affect cell growth (Wilson & Macnab, 1990)

A complex composed of MotA and MotB is thought to be the force-generating unit. There is no direct evidence of this; however, the molar ratio of MotA and MotB is thought to be 1 : 1 and an ion-conducting pore forms with the transmembrane regions of each protein (Sharp *et al.*, 1995a, b). In *E. coli*, a systematic Cys-substitution against a single transmembrane segment of MotB was recently performed. Periodic formation of cross-links on one face of its α-helix with or without the co-expression of MotA was found (Braun & Blair, 2001). It has been proposed that the two copies of MotB form a dimer in the MotA/MotB complex and the two Asp residues are displayed on separate faces. A new topological model was presented from the assumption that the stoichiometry of the force-generating unit is a 4 : 2 complex of MotA and MotB (Blair, 2003). Furthermore, this stoichiometry of PomA and PomB was reported in one of our recent papers (Yorimitsu *et al.*, 2004). This model is consistent with the biochemical evidence that the ratio of PomA and PomB is 2 : 1. This has been determined from the intensity of the Coomassie blue staining. Also, the size of the complex estimated by gel filtration assay is 175 kDa (Sato & Homma, 2000a). This size corresponds to the complex of four PomA and two PomB proteins. It has been shown that PomA forms a stable homodimer and both halves of the dimer seem to function together to conduct sodium ions (Sato & Homma, 2000a, b).

The molecular size of the PomA/PomB-His_6 complex has been estimated to be greater than 900 kDa when solubilized and separated by the detergent sucrose monocaprate. When gel-filtration chromatography was used, PomB was eluted as an approximately 260 kDa complex, which is based on the analysis of fraction 9 by anion-exchange chromatography. The size of 260 kDa is much larger than the monomer size of PomB (37 kDa). In the presence of 50 mM DTT, the position of the main PomB eluted fraction was shifted to one corresponding to about half the size. Since PomB has three cysteine residues, the most probable explanation is that PomB forms a disulfide bridge between PomB molecules (Yorimitsu *et al.*, 2004). MotB is predicted to form a dimer with an estimated size of 74 kDa based on cross-linking experiments (Braun & Blair, 2001). However, the size of approximately 260 kDa is still much larger than the dimer size of PomB. The reliability of the estimated size of the membrane protein in the presence of the detergent is unknown. However, the size of the huge complex of PomA and PomB was confirmed by sucrose density-gradient centrifugation. On the other hand, the structure of MotB or PomB is expected to be very extended. Therefore, estimation of the molecular mass may be very difficult when globular proteins are used as standard proteins. Even with treatments of SDS and DTT, PomB was eluted at a size greater than 100 kDa as a globular protein. Currently, we cannot confirm that PomB still forms a complex.

Apparently, most of PomB does not associate with PomA. PomA and PomB proteins were co-purified in chromatography fractions with the highest molecular size (approx. 900 kDa), which suggests that they form the 900 kDa complex. This might not be an intact force-generating unit because the amount of PomA is smaller than that of PomB

in the high molecular size complex. Specifically, the PomB band was clearly detected by Coomassie blue R250 while the PomA band was not. This may suggest that PomA interacts with multiple PomB proteins or PomB complexes. In this condition, the association of PomA and PomB was probably destroyed. Even though the amount recovered improved, the sodium uptake activity was lost in this procedure. We suspect that the native PomA/PomB complex does not have ion-conducting activity, rather the disordered complex treated with certain detergents such as β-octyoglucoside does. We need to further clarify the biochemical character of the membrane components, PomA and PomB, or the complex, and to investigate improved conditions or detergents that maintain both association and activity.

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