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Cell-free Synthesis of the Torque-Generating Membrane Proteins, PomA and PomB, of the Na^+-driven Flagellar Motor in *Vibrio alginolyticus*

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Flagellar motor proteins, PomA and PomB, are essential for converting the sodium motive force into rotational energy in the Na^+-driven flagella motor of *Vibrio alginolyticus*. PomA and PomB, which are cytoplasmic membrane proteins, together comprise the stator complex of the motor and form a Na^+ channel. We tried to synthesize PomA and PomB by using the cell-free protein synthesis system, PURESYSTEM. We succeeded in doing so in the presence of liposomes, and showed an interaction between them using the pull-down assay. It seems likely that the proteins are inserted into liposomes and assembled spontaneously. The N-terminal region of *in vitro* synthesized PomB appeared to be lost, but this problem was suppressed by fusing GFP to the N-terminus of PomB or by mutagenesis at Pro-11 or Pro-12. A structural change of the N-terminal region of PomB by these modifications may prevent cleavage during protein synthesis in PURESYSTEM. The mutations did not affect the functioning of the motor. Using this system, biochemical analysis of PomA and PomB can be performed easily and efficiently.

Key words: bacterial flagellum, *in vitro* protein synthesis, ion-driven motor, membrane protein, stator.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimetylammonio] propanesulfonate; DTT, Dithiothreitol; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid.

The bacterial flagellar motor is a rotational molecular machine, which converts the electrochemical potential difference of a coupled ion, which is H^+ for *Escherichia coli* or *Salmonella* (*1*, *2*) and Na^+ for alkalophilic *Bacillus* or *Vibrio alginolyticus* (*3*), into rotational energy. The flagellar motor is composed of a stator part and a rotor part. It is believed that 10–12 functional units of the stator complexes surround the rotor (*4*, *5*). In the polar flagellum of *V. alginolyticus*, the cytoplasmic membrane proteins, PomA and PomB, together comprise the stator complex of the motor and function as a Na^+ channel to generate torque (*6*). The purified PomA/B complex was shown *in vitro* to have sodium-conducting activity, and is predicted to be in a 4A:2B stoichiometry complex (*7*). PomA has four transmembrane (TM) segments and a large cytoplasmic loop, while PomB has a single TM domain and a peptidoglycan-binding motif, which anchors the motor to the cell wall (*6*). An Asp residue of PomB in the TM region is speculated to be the Na^+ binding site during torque generation (*8*). *Escherichia coli* has MotA and MotB which are orthologs of PomA and PomB, respectively and the MotA/B complex functions as the H^+ channel (*9*, *10*). The cytoplasmic domains of MotA and PomA are thought to interact with FliG, one of the rotor components and to generate the rotary force (*11–14*). It is still unknown what happens at the interaction sites involved in torque generation in the H^+- and Na^+-driven motor. A periplasmic region just above the MotB TM region is proposed to form a plug in the H^+ channel (*10*, *15*). When the MotA/B complex is produced, no reduction in growth is observed with wild-type MotB, but this occurs if there are mutations in the plug region, suggesting that the opening/closing of the H^+ channel cannot be regulated in the plug mutants.

Protein purification from cells is essential for biochemical or structural analysis. However, some proteins, especially membrane proteins, are difficult to isolate from cells. A cell-free protein synthesis system, which contains all components required for transcription, translation and energy regeneration to synthesize the target proteins *in vitro*, has been developed (*16*, *17*). This system allows us to produce proteins that are either toxic to the cell, or impair its growth when overexpressed. Moreover, using cell-free protein synthesis allows us to skip several steps required for protein production or purification, thereby save time. By using this method, it is expected that comprehensive biochemical analysis of many mutant proteins can be carried out more easily. There are several kinds of cell-free protein synthesis system, which are based on, for example, cell extracts derived from *E. coli*, wheat germ or rabbit reticulocyte (*16*, *18*, *19*). Alternatively, there is a system, named PURESYSTEM, which contains all purified and reconstituted components required for protein synthesis (*20*, *21*). These cell-free protein synthesis systems have usually been used for synthesizing soluble proteins.

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Recently, however, membrane proteins, such as GPCRs (G-protein coupled receptors), EmrE (multidrug efflux pomp), cytochrome b_5 and MtlA (mannitol permease) and so on, have been synthesized using this system and successfully used in experiments (*22–26*).

In order to understand the flagellar motor function, it has been a long-term aim to know how sodium ions flow through the PomA/PomB complex and how the opening and closing the PomA/B channel is regulated. As the purification and functional reconstitution of membrane proteins of PomA and PomB is still not easy, we took advantage of the cell-free protein synthesis system as described above. In this study, we tried to produce the flagellar motor proteins, PomA and PomB, using a cell-free protein synthesis system.

MATERIALS AND METHODS

Bacteria and Media—The strains used are shown in Table 1. *Vibrio 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 VPG medium [1% (w/v) polypeptone, 0.4% (w/v) K_2HPO_4, 3% (w/v) NaCl, 0.5% (w/v) glycerol]. *Escherichia coli* was cultured in LB broth [1% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl]. Ampicillin was added to a final concentration of 100 μg/ml for *E. coli*. Chloramphenicol was added to a final concentration of 2.5 μg/ml for *V. alginolyticus* and 25 μg/ml for *E. coli*.

Table 1. **Bacterial strains and plasmids.**

Strain or plasmid	Genotype or description	Reference
Vibrio alginolyticus strains		
NMB191	Rif^r, Pof$^+$, Laf$^-$ Δ*pomAB*	(*27*)
Escherichia coli strains		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44* *relA1* λ$^-$Δ(*lac-proAB*) (F' *traD36* *proAB lacI*^{q} *lacZ*Δ*M15*)	(*32*)
BL21(DE3)	T7 expression host	(*33*)
Plasmids		
pET22b(+)	T7 expression vector, Amp^r	Novagen
pBAD33	Cm^r, P*BAD*	(*34*)
pHFAB	*pomA* and *pomB* in pBAD33	(*30*)
pET-*pomB*	*pomB* in pET22b(+)	In this study
pET-GB	*Gfp-pomB* in pET22b(+)	In this study
pHFGBA2	*his$_6$-gfp-pomB* and *pomA* in pBAD33	(*30*)
pYA303	*pomA* and *pomB* in pSU41	(*35*)

Rif^r, rifampicin resistant; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; P*BAD*, *araBAD* promoter; Pof$^+$, normal polar flagellar formation; Laf$^-$, defective in lateral flagellar formation.

Plasmid Construction—Plasmids and primers are listed in Tables 1 and 2, respectively. Fragments containing *pomB* with an NdeI site at the 5′ and BamHI at the 3′ were amplified using PCR, and these fragments were inserted into the NdeI and BamHI sites of pET22b(+) (Novagen, Germany); this plasmid was named pET-*pomB*. Site-directed mutagenesis in *pomB* was performed by using the QuickChange procedure (Promega). Fragments containing *gfp-pomB* were amplified by 1st PCR using *gfp*(+) PURE forward and *pomB*-BamHI(−), and then by 2nd PCR using universal primer(+) containing XbaI site and *pomB*-BamHI(−) and the 1st PCR products as the template. These fragments were inserted into the XbaI and BamHI sites of pET22b(+) and named pET-GB.

Preparation of Liposome—L-α-Phosphatidylcholine type II-S from soybean (Sigma, USA) was dissolved in PA3-buffer [10 mM HEPES–KOH pH 7.5, 5 mM $MgCl_2$, 10% (w/v) glycerol] to a final concentration of 44 mg/ml, stirred at RT for 30 min and then sonicated. Liposomes for the Na^+-uptake assay were prepared using PA3-buffer containing 200 mM KCl. The prepared samples were frozen using liquid N_2 and stored at −80°C.

Protein Synthesis and Purification—PURESYSTEM classic II mini (Post Genome Institute, Japan) was used for the protein synthesis. PCR products of *pomA* were amplified from pHFAB by using primers pomA(+) pure forward and pomA(−) pure reverse and added 750 ng as template DNAs in the reaction solution. PCR products of *pomB* or their mutant genes were also amplified from pHFAB, pET-*pomB* or its mutant genes by primers pomB(+) pure forward pomB(−) pure reverse, his6-pomB(+) pure forward and pomB-his6(−) pure reverse and added 1500 ng as template DNAs. The detergents were added at the critical micelle concentration (CMC) or the liposome to a final concentration of 4.4 mg/ml. The protein synthesis reaction was incubated at 30°C for 1.5 h. Then, the reaction solution containing the detergent was diluted twice by 40 mM HEPES–KOH pH 7.5 containing the detergent at CMC, 1/10 volume of Ni-NTA agarose was added

Table 2. **Primers for PCR.**

Primer	Sequence
pomA(+) PURE forward	5′-AAGGAGATATACCAATGGATTTAGCAACCCTATTAGG-3′
pomA(−) PURE reverse	5′-TATTCATTACTCGTCAATCTCAAGGG-3′
pomB(+) PURE forward	5′-AAGGAGATATACCAATGGATGATGAAGATAACAAATGCG-3′
pomB(−) PURE reverse	5′-TATTCATTATTGAATTACCGGCACTTCTTCGCTG-3′
his6-pomB(+) PURE forward	5′-AAGGAGATATACCAATGCATCACCATCACCATCACATGGATGATGAAGATAACAAATGCG-3′
pomB-his6(−) PURE reverse	5′-TATTCATTAGTGATGGTGATGGTGATGTTGAATTACCGGCACTT-3′
gfp(+) PURE forward	5′-AAGGAGATATACCAATGGTGAGCAAGGGCGAGG-3′
universal primer(+)	5′-GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTT TGTTTAACTTTAAGAAGGAGATATACCA-3′
NdeI-pomB(+)	5′-GGAATTCCATATGGATGATGAAGATAACAAATGCG-3′
pomB-BamHI(−) revevrse	5′-CGCGGATCCTTATTGAATTACCGGCAC

(Qiagen, Germany), and was shaken at 4°C for 1 h. The resin-bound proteins and ribosomes were removed by an ultrafiltration device (Microcon YM-100, Millipore, USA). The reaction solution containing liposomes was diluted twice by 40 mM HEPES–KOH pH 7.5 and sonicated for 30 s in a bath. The samples were then diluted twice by 100 mM $MgCl_2$ and stood on ice for 10 min. Liposome was precipitated by centrifugation at 10,000*g* for 10 min. The pellet was re-suspended in SDS loading buffer.

Detection of Proteins—The samples were mixed with SDS loading buffer and boiled at 95°C for 5 min. SDS–PAGE and immunoblotting, using the antibodies against PomA, PomB, His-probe (Santa Cruz, USA) and GFP (BD living colors, USA), were performed as described previously (*27*).

Solubilization of Proteins—The reaction solution for the protein synthesis was mixed at the final concentration of 1% (w/v) CHAPS and incubated on ice for 10 min. The solubilized samples were centrifuged at 10,000*g* for 10 min at 4°C and then the supernatants were centrifuged at 200,000*g* (TLA-100.3, Beckman, USA) for 1 h at 4°C to remove insoluble material.

Pull-down Assay—The protein synthesis was carried out as above with slight modifications. PURESYSTEM advance (Post Genome Institute, Japan) was used instead of PURESYSTEM classic II mini (Post Genome Institute, Japan), the PCR products of *pomA* template and liposome were changed to 150 ng and the final concentration of 2.2 mg/ml, respectively. His_6-tag was added to the C terminus of PomB protein. The reaction solution for the protein synthesis was mixed at the final concentration of 1% (w/v) CHAPS and incubated for 10 min on ice. The solubilized samples were centrifuged at 10,000*g* for 10 min at 4°C. Ni-NTA agarose was added to the supernatant, and the mixture was incubated at 4°C for 30 min. The Ni-NTA agarose was washed by centrifugation three times with wash buffer [50 mM HEPES–KOH pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1% (w/v) CHAPS and 50 mM imidazole] at 4°C. The associated proteins were eluted by elution buffer [50 mM HEPES–KOH pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1% (w/v) CHAPS and 300 mM imidazole] at 4°C. The proteins were detected by immunoblotting as described above.

Size Exclusion Chromatograph Assay—A Superdex 200 10/300 GL size exclusion column (GE Healthcare, Sweden) was equilibrated with size exclusion buffer [50 mM HEPES–KOH pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1% (w/v) CHAPS and 1mM DTT]. Solubilized samples, as prepared above, were applied to the Superdex 200 10/300 GL column and eluted with the size exclusion buffer.

$^{22}Na^+$-uptake Assay—A total of 630 μl of the assay buffer containing 20 mM HEPES/KOH pH 7.5, 200 mM choline chloride, 5 mM $MgCl_2$ and 1 mM $^{22}NaCl$ (14.8 kBq/ml) and 70 μl of the cell-free reaction mixture were mixed and incubated at 30°C for 10 min. Valinomycin was added at the final concentration of 25 μM to form a diffusion membrane potential. At intervals, 90 μl of the reaction mixture was sampled out and passed through Dowex 50WX8-400 (Sigma, USA) to trap the unincorporated $^{22}Na^+$. The radioactivity of the flow-through fraction was measured using a γ-counter.

Swarm Assay—VPG-0.25% agar plate [1% (w/v) bactotrypton, 0.4% (w/v) K_2HPO_4, 3% (w/v) NaCl, 0.5% (w/v) glycerol and 0.25% (w/v) bactoagar] was used for *V. alginolyticus* swarm assays. Chloramphenicol and arabinose were added at the final concentration of 2.5 μg/ml and 0.02% (w/v), respectively. An aliquot (1 μl) of overnight culture was spotted onto a plate, which was then incubated at 30°C.

RESULTS

Synthesis of PomA and PomB in the Presence of Detergent or Liposome—To synthesize PomA and PomB, we used the cell-free synthesis system of PURESYSTEM (*20*, *21*). We used the mixed *pomA* and *pomB* PCR products as the template DNAs in the cell-free system. It has been shown that PomA and PomB interact with each other and that their interaction is important for the stability of the proteins (*27*). We could detect the proteins in the reaction mixture even without detergent by immunoblotting using anti-PomA or PomB antibodies (Fig. 1). The PomA signal was detected as doublet bands, this may be a character of the very hydrophobic PomA protein from *V. alginolyticus* and not because of the degradation (*27*). After the reaction, the proteins for the cell-free synthesis were removed by treatment with Ni-NTA agarose and the ribosomes were removed using an ultrafiltration device. Using this procedure, however, the synthesized proteins were lost completely. This is probably because PomA and PomB aggregated. To handle the membrane proteins, we added some detergents to the cell-free system at the critical micelle concentration. PomA and PomB were synthesized in the presence of 0.46% (w/v) CHAPS, 0.125% (w/v) sucrose

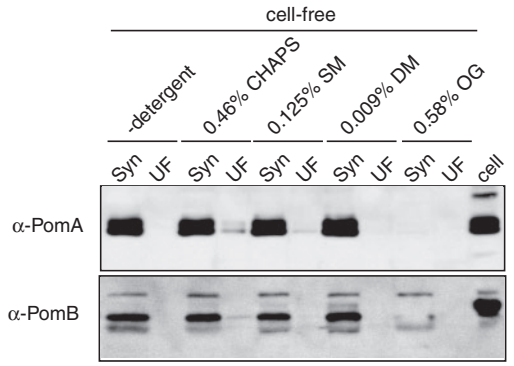

Fig. 1. **Cell-free synthesis of PomA and PomB in the absence or presence of detergents.** The cell-free synthesis was performed using PCR products from the *pomA* and *pomB* genes as template DNA. The detergent, 0.46% (w/v) CHAPS, 0.125% (w/v) sucrose monocaprate, 0.009% (w/v) *n*-dodecyl-β-D-maltoside or 0.58% (w/v) *n*-octyl-β-D-thioglucoside, which are the CMC, was added in PURESYSTEM. Cell, *Vibrio* whole-cell producing PomA and PomB from pYA303; cell-free, cell-free protein synthesis; Syn, a whole fraction of the protein synthesis; UF, a soluble fraction after chelating Ni-NTA agarose binding proteins and ribosomes by an ultrafiltation device filtrating the molecular mass of >100 kDa. *Vibrio* cells (cell) were cultured at 30°C for 3 h in VPG medium. The cells collected by centrifugation were suspended in SDS loading buffer at a concentration corresponding to an A_{660} of 50. The 5 μl preparations of the synthesized proteins were mixed with 5 μl of SDS loading buffer. Immunoblotting to detect PomA and PomB was performed using anti-PomA antibody (PomA1312) and anti-PomB antibody (PomB93), which were previouly prepared (*27*).

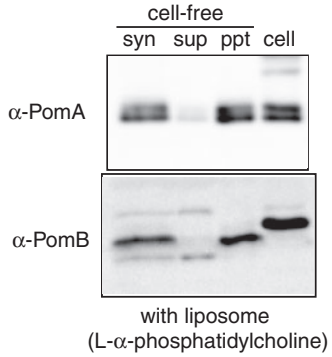

Fig. 2. **Cell-free synthesis of PomA and PomB in the presence of liposome made of L-α-phosphatidylcholine.** The cell-free synthesis was performed using PCR products from the *pomA* and *pomB* genes as the template DNA. Cell, *Vibrio* whole-cell producing PomA and PomB from pYA303; cell-free, cell-free protein synthesis; syn, a whole fraction of the protein synthesis; ppt, a precipitation fraction when liposome was precipitated by adding magnesium at a final concentration of 50 mM; sup, the supernatant fraction. PomA and PomB were detected as Fig. 1.

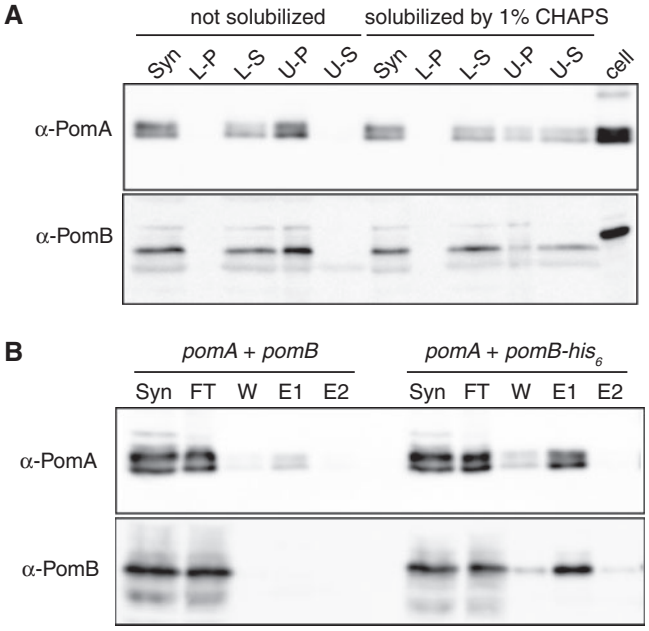

Fig. 3. **Biochemical profiles of PomA and PomB.** (A) Solubilization of proteins and liposomes by CHAPS. After synthesizing PomA and PomB in the presence of liposome, CHAPS was added at the final consentration of 1% (w/v). After removing the insoluble materials by low-speed centrifugation, the supernatant was ultracentrifuged. Syn, a whole fraction of the protein synthesis; L-P, ppt of low-speed centrifugation; L-S, sup of low-speed centrifugation; U-P, ppt of ultracentrifugation; U-S, sup of ultracentrifugation; cell, *Vibrio* whole-cell producing PomA and PomB from pYA303. (B) Interaction between PomA and PomB detected by pull-down assay. Ni-NTA agarose was added into the solubilized solution by CHAPS. PomB-His_6 bound to the resin was eluted by 300 mM imidazole. Syn, a whole fraction of the protein synthesis; FT, the flow-through fraction; W, the wash fraction; E1, the first elution fraction; E2, the second elution fraction. The cell-free synthesis was performed using PCR products from the *pomA* and *pomB* genes or the *pomA* and *pomB-his*$_6$ genes as template DNA. PomA and PomB were detected as Fig. 1.

monocaprate or 0.009% (w/v) *n*-dodecyl-β-D-maltoside, but not in the presence of *n*-octyl-β-D-thioglucoside (Fig. 1). Only in the presence of CHAPS, were PomA and PomB recovered as soluble forms after ultrafiltration. Under all conditions, synthesized PomB was detected as a protein with smaller molecular mass than that of the control PomB produced *in vivo*.

Next, we carried out the protein synthesis by adding liposomes made of L-α-phosphatidylcholine. PomA and PomB were produced and were detected in the fraction containing lipids precipitated by adding magnesium at a final concentration of 50 mM (Fig. 2, ppt) as reported previously (*28*). Therefore, PomA and PomB seem to be inserted into the liposomes as previously suggested (*25*, *26*). Synthesized PomB also had a smaller molecular mass in this condition.

Interaction Between PomA and PomB—Liposomes containing the synthesized PomA and PomB were solubilized by 1% CHAPS, and fractionated. They were detected in the supernatant, after ultracentrifugation, as the soluble fraction (Fig. 3A). Without CHAPS they were detected in the precipitate after ultracentrifugation (Fig. 3A). Next, we examined the interaction between PomA and PomB in the liposomes. The His_6-tag fused to PomB at the C-terminus was used to pull-down PomA. After PomA and PomB were synthesized by the cell-free system in the presence of liposomes, the proteins (solubilized by CHAPS) were applied to Ni-NTA agarose and the bound proteins were detected by immunoblotting using anti-PomA and PomB antibodies (Fig. 3B). As a result, PomA from the cell-free system was detected together with the His_6-tag fused-PomB. This suggests an interaction between PomA and PomB in the cell-free system.

To examine molecular sizes of PomA and PomB from the cell-free system, we solubilized the synthesized PomA and PomB using CHAPS and fractionated them by size exclusion column. When only PomA was synthesized and applied to the size exclusion column, it was eluted in a wide range of the fractions with the peak around fraction 22 (Fig. 4A). When only PomB was synthesized, it was eluted in the fraction of molecular size of ~75 kDa, estimated using the molecular size standards (Fig. 4B). When PomA and PomB were synthesized together, profiles of each protein were essentially same as that of PomA or PomB only (Fig. 4C). On the other hand, when they were incubated for 20 h at 30°C following protein synthesis, PomB was detected in fractions of 18–20, whose molecular size are ~440–158 kDa, and PomA was shifted to be eluted in the same fractions and the higher molecular fractions (Fig. 4D). It is likely that PomA and PomB oligomerized in the liposomes. We carried out measurement of Na^+-uptake activity of PomA and PomB using the cell-free system and radioisotope, $^{22}Na^+$. However, we could not detect a significant Na^+-uptake into the liposomes (data not shown).

Molecular Mass of in vitro *Synthesized PomB*—PomB synthesized by the cell-free system had a smaller molecular mass than that produced in the cells. To investigate

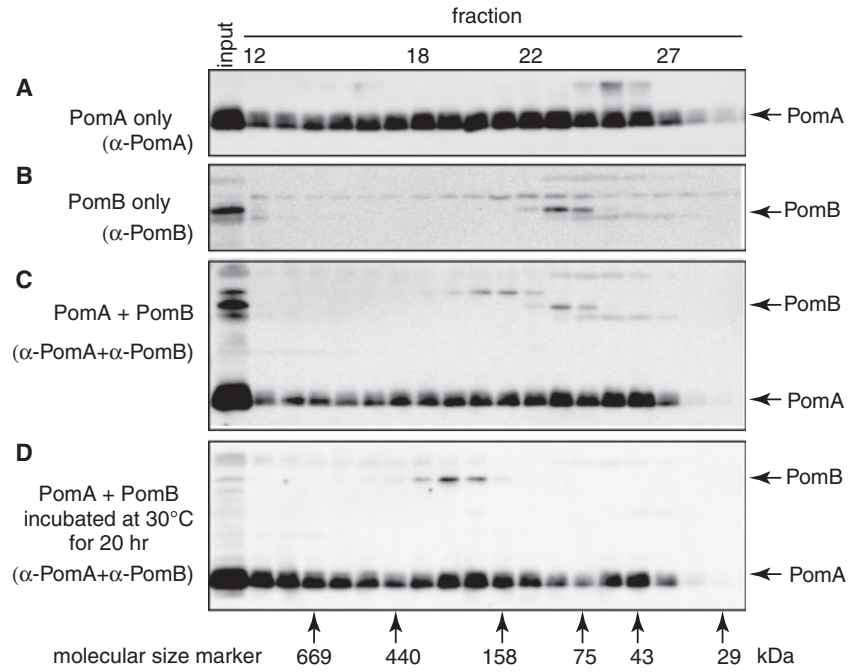

Fig. 4. **Size exclusion chromatography of PomA and/or PomB from cell-free synthesis.** (A) PomA only, (B) PomB only and (C) PomA and PomB together were synthesized, and then solubilized immediately after the protein synthesis by using CHAPS and applied to superdex 200 10/300 GL size exclusion column. (D) PomA and PomB together were synthesized and incubated for 20 h at 30°C, and then solubilized and applied as above. PomA and PomB were detected as Fig. 1.

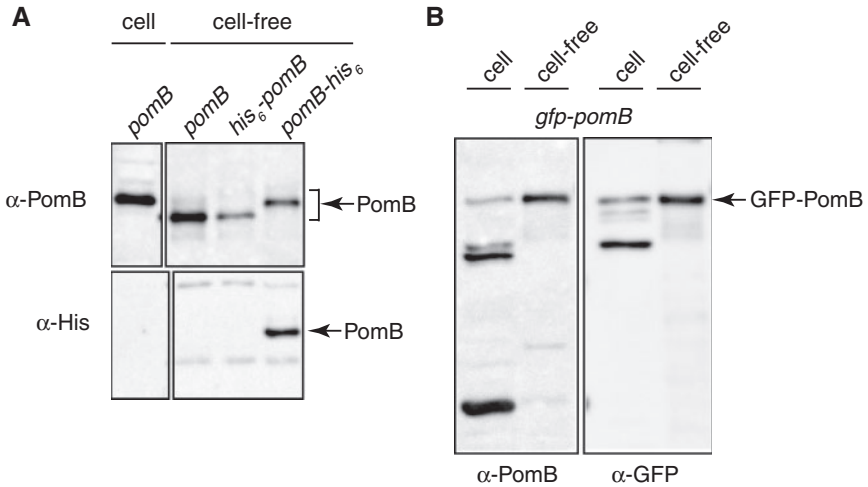

Fig. 5. **The cell-free synthesis of His_6-tag-fused PomB proteins at the N- or C-terminus (A) or GFP-fused PomB at the N-terminus (B).** Cell, *Vibrio* whole-cell producing PomA and PomB from pYA303 (A) or GFP-PomB from pET-GB (B); cell-free, cell-free protein synthesis. The cell-free synthesis was performed using PCR products from the *pomA* and *pomB* genes, the *pomA* and *pomB-his*$_6$ genes, the *pomA* and *his*$_6$-*pomB* genes or the *pomA* and *gfp-pomB* genes as template DNA. PomA and PomB were detected as Fig. 1. His_6-tag and GFP were detected by the antibodies against His-probe (α-His) and GFP (α-GFP), respectively.

the reason for this, we first synthesized PomB from the gene fused with *his*$_6$-tag at the N-terminus or C-terminus in the presence of liposome. PomB-His_6 but not His_6-PomB was detected using the anti-His-probe antibody (Fig. 5A). The molecular mass of the *in vitro* synthesized His_6-PomB was the same as that of PomB. These results suggest that the N-terminus of PomB was lost in the cell-free system. Next, we synthesized PomB from the gene fused with *gfp* at the N-terminus. *In vitro* synthesized GFP-PomB had the same molecular mass as *in vivo* synthesized GFP-PomB (Fig. 5B). The additional GFP fused at the N-terminus of PomB suppressed the reduction of PomB molecular mass.

Change of PomB Molecular Mass by the N-terminal Mutations—To investigate further the reduction of the

PomB molecular mass, we carried out site-directed mutagenesis to alter the stability of the N-terminal region of PomB or to disrupt a potential initiation site in the coding region of *pomB* mRNA. We made the Ala substituted mutant of PomB at Cys-10, Pro-11, Pro-12, Gly-14, Leu-15, Met-19 or Gly-20. PomB-P11A and P12A showed the native molecular mass. We made more mutants substituted to Gly and Asn at Pro-11 and Pro-12. These substitutions also suppressed the reduction of the molecular mass *in vitro* (Fig. 6A). The substitution at Pro-12 seems to have the strongest effect in preventing a change in molecular mass, as most of PomB had the same molecular mass as that produced *in vivo* and the remainder was smeared at the smaller molecular weight. We suspected that the Met-19 might become an initiation codon of *pomB* in the cell-free system, but the PomB-M19A mutant still had a smaller molecular mass (Fig. 6B). There seem to be no other potential initiation codons around the PomB N-terminal region. It is unlikely that the translation is initiated at another point.

We examined the *in vivo* function of the Pro mutants in PomB using swarming assay. When the Δ*pomAB Vibrio* cells producing PomB-P11/12A, PomB-P12A, PomB-P12G or PomB-P12N with PomA from plasmids were inoculated in the soft agar plates, their swarming abilities were the same as the wild-type strain (Fig. 7A). In the cells, PomB mutants were detected at the same level as wild-type PomB and their molecular masses were same as that of wild-type PomB (Fig. 7B). These results suggest that the mutations of PomB Pro-11 and Pro-12 are not likely to affect the stator function.

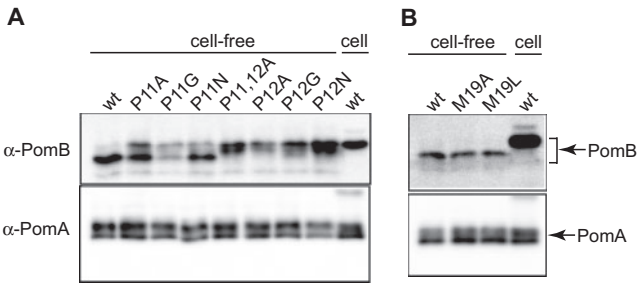

Fig. 6. **The cell-free synthesis of mutant PomB proteins.** The mutations in PomB are introduced into Pro-11 and/or Pro-12 (A) or Met-19 (B). Cell, *Vibrio* whole-cell producing PomA and PomB from pYA303; cell-free, cell-free protein synthesis. The cell-free synthesis was performed using PCR products from the *pomA* and *pomB* genes, the *pomA* and mutant *pomB* genes. PomA and PomB were detected as Fig. 1.

DISCUSSION

We succeeded in the cell-free synthesis of the proteins PomA and PomB in the presence of several detergents or lipids (liposomes). Membrane proteins are generally difficult to produce using cell-free synthesis systems. Even without detergents PomA or PomB were synthesized by PURESYSTEM, but we were unable to handle them without detergents. Among the detergents used in this study, *n*-octyl-β-D-thioglucoside inhibits synthesis. CHAPS do not inhibit synthesis and can solubilize PomA and PomB.

The N-terminal region of *in vitro* synthesized PomB seems to be missing. Fused GFP at the N-terminus of PomB was not lost in the cell-free system. We speculate that the structure of the N-terminal region of PomB was affected by the GFP fusion and that the cleavage of the N-terminal region of PomB was difficult. This assumption was supported by the evidence that Pro-12 and Pro-11 mutations suppressed reduction of the molecular mass of PomB. Assuming that Pro residues greatly affect the protein structure, the structural change in the N-terminal region may prevent the cleavage. There should be little contamination by protease in PURESYSTEM because the proteins in PURESYSTEM are all purified. Even if the protease inhibitor was added to PURESYSTEM, the molecular mass was still small (data not shown). When the cell extracts from *E. coli* were added in PURESYSTEM, a certain fraction of synthesized PomB was detected in the full-length form (Terashima *et al*., unpublished results). This might suggest that some factors of *E. coli* affect the transcription of *pomB* mRNA, the translation of PomB or

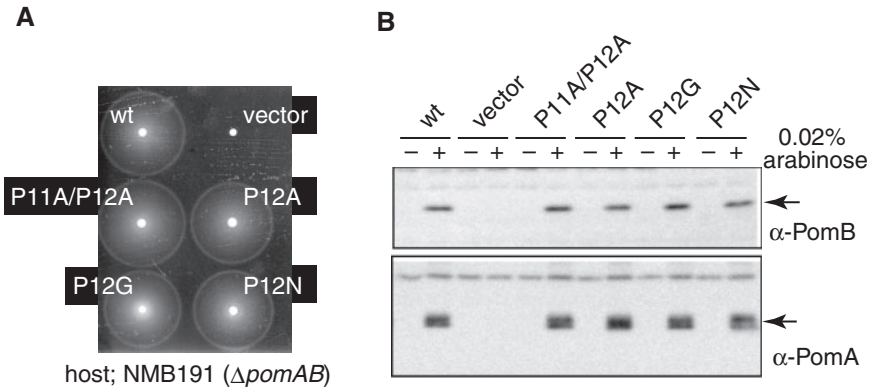

Fig. 7. **Function and expression of the *pomB* mutants *in vivo*.** (A) The swimming ability of Δ*pomAB* cells producing PomB Pro-12 mutants with wild-type PomA from plasmid was examined on the 0.25% soft agar plate incubated at 30°C for 6 h. (B) The Δ*pomAB* cells producing PomB Pro-12 mutants with wild-type PomA from plasmid were cultured in the absence or presence of 0.02% (w/v) arabinose at 30°C for 3 h. PomA and PomB were detected as Fig. 1. The arrows indicate PomA and PomB.

structural stability of the N-terminal region of PomB, preventing reduction of its molecular mass.

Although it is unclear how, *in vitro* synthesized PomA and PomB seem to be inserted spontaneously into the liposome and moreover, they interact with each other. Such an insertion of membrane protein has been observed and is independent on the Sec export system (*25*, *26*). We have solubilized the PomA/B complex from the membrane of *V. alginolyticus* cells using the detergents, *n*-octyl-β-D-thioglucoside (*7*), sucrose monocaprate (*29*) and CHAPS (*30*, *31*). The molecular sizes of the PomA/B complexes solubilized by their detergents were estimated as 175 kDa, 900 kDa and 550 kDa, respectively. The PomA/B oligomer, which was formed when the synthesized PomA and PomB were incubated for 20 h at 30°C, had similar high molecular sizes to the *in vivo* PomA/B complex solubilized by the detergents. However, we could not detect significant Na^+-uptake using the proteoliposome reconstituted with *in vitro* synthesized PomA and PomB. We had measured the Na^+ uptake activity of PomA/B complex using the proteoliposome reconstituted with PomA and PomB which were purified from *Vibrio* cells (*7*). In the present experiment, the amount and complex formation of the reconstituted PomA and PomB, buffer organization, etc. were, however, different from the previous condition. Therefore, protein synthesis, complex formation and measurement conditions must be further optimized. The synthesized PomA/B in this study might not be functional for the Na^+-conductive activity even though the complex formation seems to be normal. We are going to measure it using the mutant PomBs, which are not missing the N-terminal region.

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CONFLICT OF INTEREST

None declared.

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