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Multimeric Structure of PomA, a Component of the Na^+-driven Polar Flagellar Motor of *Vibrio alginolyticus**

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Four integral membrane proteins, PomA, PomB, MotX, and MotY, are thought to be directly involved in torque generation of the Na^+-driven polar flagellar motor of *Vibrio alginolyticus*. Our previous study showed that PomA and PomB form a complex, which catalyzes sodium influx in response to a potassium diffusion potential. PomA forms a stable dimer when expressed in a PomB null mutant. To explore the possible functional dependence of PomA domains in adjacent subunits, we prepared a series of PomA dimer fusions containing different combinations of wild-type or mutant subunits. Introduction of the mutation P199L, which completely inactivates flagellar rotation, into either the first or the second half of the dimer abolished motility. The P199L mutation in monomeric PomA also altered the PomA-PomB interaction. PomA dimer with the P199L mutation even in one subunit also had no ability to interact with PomB, indicating that the both subunits in the dimer are required for the functional interaction between PomA and PomB. Flagellar rotation by wild-type PomA dimer was completely inactivated by phenamil, a sodium channel blocker. However, activity was retained in the presence of phenamil when either half of the dimer was replaced with a phenamil-resistant subunit, indicating that both subunits must bind phenamil for motility to be fully inhibited. These observations demonstrate that both halves of the PomA dimer function together to generate the torque for flagellar rotation.

Bacterial flagella are the organelles responsible for motility. Flagellar rotation is driven by a reversible rotary motor embedded in the cytoplasmic membrane at the base of each flagellar filament (1–3). Energy for rotation derives from the transmembrane electrochemical potential of specific ions. Two types of motors, proton-driven (2) and sodium-driven (4), have been investigated. The proton-driven motors of *Escherichia coli* and *Salmonella typhimurium* have been studied intensively. The stator part of the torque generator consists of two cytoplasmic membrane proteins, MotA and MotB, which contain four and one transmembrane-spanning segments, respectively (5–8). There exists much genetic and physiological evidence that suggests that MotA and MotB together form a proton channel (9–12), surrounding the rotor (13), and this complex is believed to be anchored to the cell wall via a peptidoglycan-binding domain of MotB (8, 14, 15). It is thought that ions passing through those proteins somehow generate torque (16), which is transmitted to the rotor part of the motor, the FliG protein (17, 18). Together with FliM and FliN (19, 20), FliG forms the switch complex, which is essential for torque generation, flagellar assembly, and controlling the direction of motor rotation (21–25).

Bacteria such as alkalophilic *Bacillus* and *Vibrio* species use the electrochemical gradient of sodium to drive flagellar rotation (4). The sodium-driven motor has advantages for studying the motor function, because sodium-motive force can be easily manipulated, and amiloride and phenamil work as specific inhibitors of their function (26, 27). Four proteins essential for torque generation, PomA, PomB, MotX, and MotY, were recently identified in the polar flagellar motor of *Vibrio alginolyticus* (28, 29). PomA and PomB are homologous to MotA and MotB and contain four and one transmembrane segments, respectively. Both MotX and MotY, which were first identified in *Vibrio parahaemolyticus* (30, 31), have a putative single transmembrane segment; they are unique to the sodium-type motor, and their function is unknown so far. We recently showed that PomA and PomB functionally interact with each other in a molar stoichiometric ratio of 2 PomA:1 PomB and together form a sodium-conducting channel (32). Furthermore, PomA forms a stable homodimer when expressed in the absence of PomB, implying that this may be the absolute number of PomA subunits in the functional complex.

In this study, we describe a fusion protein that contains different combinations of wild-type PomA or mutant (P199L or D148Y) subunits that we have used to evaluate whether adjacent PomA domains may be required for torque generation. Our results demonstrate that PomA functions as an even number of subunits in a single functional complex and that both subunits contribute to PomA-PomB assembly.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Growth Conditions, and Media—V. alginolyticus strains NMB190 (Rif^r, Pof^+, Laf^-, Δ*pomA*) (33) and VIO5 (Rif^r, Pof^+, Laf^-) were used. *E. coli* strain JM109 (*recA1, endA1, gyrA96, thi*$^-$*, hsdR17, relA1, supE44,* λ$^-$*,* Δ*(lac-proAB);* (*F′, traD36, proAB, lacI*^{q}*, lacZ*Δ*M15*)) was used for DNA manipulations. *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 swarm assay, a VPG-0.3% agar plate was used. *E. coli* cells were cultured at 37 °C in LB medium. When necessary, kanamycin was added to a final concentration of 100 μg/ml for *Vibrio* cells or 25 μg/ml for *E. coli* cells. Plasmid pKS101, a pSU41-based plasmid, was constructed to carry *his*$_6$*-pomA* under the *lac* promoter control. A 0.8-kb[1] DNA fragment including the *pomA* open reading frame with a 5′ attachment of 5′-ATTGGATCCATGCATCACCATCACCATCACATGGATTTAGCAACCCTATTA-3′ (initiates as Met-

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[1] The abbreviations used are: kb, kilobase pair(s); NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis.

His_6; the underline indicates the created (*Bam*HI and *Eco*T22I) restriction sites) was originally prepared by polymerase chain reaction and cloned into *Bam*HI-digested pSU41. Plasmid pKS105, encoding the tandem PomA with a six-histidine linker, was generated by engineering an *Eco*T22I site at the 3′-end of the *pomA* clone and then linked to the 5′-end of *his*$_6$-*pomA* via *Eco*T22I. pKS106 (PomA-PomA), a pSU41-based plasmid with a kanamycin resistance marker, encoded the tandem PomA under *lac* promoter control. An oligonucleotide with the sequence 5′-ATGCATTATCCGTACGATGTTCCAGATTATGCAATAG-AAGGTCGCATGCAT-3′ was inserted into the *Eco*T22I site of pKS105, allowing an in frame insertion of the sequence MHYPYDVPDYAIEGR into the 3′-end of the *pomA* clone. The final linking sequence was MHYPYDVPDYAIEGRM-His_6, *i.e.* a hemagglutinin tag and six histidines. Plasmid pKS107 (PomA-P199L) was prepared by the insertion of a 0.8-kb *Dra*I fragment of pKS106 into the corresponding site of pMK101-P199LKm. pMK101-P199LKm was constructed by the insertion of a 0.8-kb *Bam*HI fragment of pMK101-P199L (34) into the corresponding site of pSU41. A 1.0-kb *Hin*dIII fragment of pKS106 was ligated with pMK101-P199LKm that had been partially digested with *Hin*dIII, creating pKS108 (P199L-PomA). Plasmid pKS109 (P199L-P199L) was constructed by the insertion of a 0.8-kb *Sph*I fragment of pKS108 into the corresponding site of pKS107. pKS111 carrying the His_6-PomA P199L gene was created by insertion of a 0.6-kb *Dra*I–*Eco*RI fragment of pMK101-P199LKm into the corresponding site of pKS101. Plasmid pKS113 (PomA-D148Y) was constructed by the insertion of a 0.8-kb *Dra*I fragment of pKS106 into the corresponding site of pYA301-D148Y (35). A 1.0-kb *Hin*dIII fragment of pYA301-D148Y was ligated with pKS106 that had been partially digested with *Hin*dIII, creating pKS114 (D148Y-PomA). pKS115 (D148Y-D148Y) was constructed by the insertion of a 0.8-kb *Sph*I fragment of pKS114 into the corresponding site of pKS113. All inserts were confirmed by DNA sequencing.

Measurement of Swimming Speed—Cells were harvested at late logarithmic phase and resuspended in an equal volume of TMN medium containing 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose, 50 mM NaCl, 250 mM KCl. The cell culture was diluted 100-fold into TMN medium containing various concentrations of NaCl, and then motility of the cells was observed at room temperature under a dark field microscope and recorded on video recorder. Swimming speed was determined as described (36). The average swimming speed was obtained by measuring at least 20 swimming tracks.

Co-elution Assay—Cells of NMB190 expressing His_6-tagged PomA derivative were cultured at 30 °C under strong aeration in VPG medium, harvested, washed with buffer (20 mM Tris-Cl, pH 8.0, 5 mM $MgSO_4$, 10% (w/v) sucrose), and resuspended (0.2 g, wet weight/ml) in 20 mM Tris-Cl, pH 8.0, containing 1 mM dithiothreitol, 5 mM $MgSO_4$, 30 μg/ml DNase I, and 0.5 mM phenylmethylsulfonyl fluoride. Membrane vesicles were prepared by subjecting the suspension to a single passage through a French press (5501-M Ohtake Works) at 4000 p.s.i. 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. The membrane pellet was suspended in buffer A (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% (w/v) glycerol) containing 5 mM imidazole, pH 8.0, and 2.5% (w/v) β-octyl glucoside. The suspension was stirred for 30 min at room temperature and centrifuged for 20 min at 10,000 × *g*. The clarified extract was mixed with Ni^{2+}-NTA-agarose (QIAGEN) prewashed with the same buffer, incubated at 4 °C for 1 h with gentle mixing, and then packed into the column. The loaded resin was washed with buffer A containing 40 mM imidazole, pH 8.0, and 1.25% (w/v) β-octyl glucoside. Elution was conducted with buffer A, containing 500 mM imidazole, pH 8.0, and 1.25% (w/v) β-octyl glucoside. The Ni^{2+}-NTA-purified material was analyzed by SDS-PAGE followed by immunoblotting.

RESULTS

Construction and Expression of PomA Dimers—Our previous studies suggested that the native PomA protein alone forms a stable dimer in detergent extract (32). The possibility that the dimerization of PomA subunits may be required for torque generation was approached by constructing a series of tandem dimer PomA proteins. The construction was performed by linking two *pomA* open reading frames in frame with 22 residues as a linker (Fig. 1*A*). The resulting plasmid encodes a 528-residue polypeptide (PomA-PomA) with an N-terminal subunit continuous with a C-terminal subunit. As shown in Fig. 1*B*, NMB190 *(*Δ*pomA)* cells expressing the tandem PomA gained the ability to swarm on a soft agar plate. Compared with wild-type monomeric PomA, tandem PomA slightly reduced the swarming ability. This is also shown in Fig. 6*B*, where NMB190 cells with tandem PomA had significantly reduced swimming speed compared with cells expressing wild-type monomeric PomA. Since the C terminus of *Salmonella* MotA, the PomA homolog, has been shown to be important for function (37), diminished swarm ability may be due to restricted flexibility around the C terminus of the N-terminal half of tandem PomA. To demonstrate that swarming was due to the activity of full-length tandem PomA and not to monomeric PomA resulting from partial translation or proteolytic cleavage of the PomA fusion, an immunoblotting was carried out with membranes from NMB190 expressing the tandem fusion protein. As shown in Fig. 2*B*, tandem PomA migrates around 50 kDa, as do SDS-resistant dimeric aggregates of monomer PomA (Fig. 2*B*, *lane 2*) that we have observed routinely (38). No other immunoreactive species is observed on the immunoblots compared with wild-type endogenous PomA (*lanes 7–9*), particularly around 25 kDa, where monomeric PomA migrates. The immunoreactive materials at slightly larger than the monomer size (Fig. 2*B*, *lanes 3* and *6*) are the nonspecific degradation products during membrane preparation.

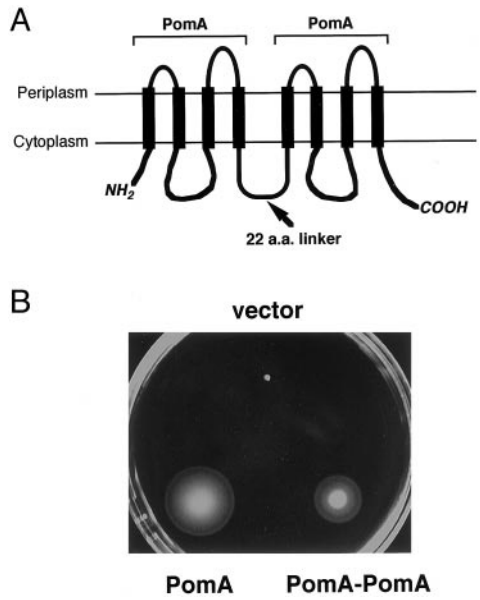

FIG. 1. **Membrane topology and function of dimeric PomA fusion protein.** *A*, schematic diagram of a PomA-PomA dimer. Two PomA monomers joined tail-to-head in tandem are expressed as a single polypeptide. The *arrow* indicates the position of the connecting site; the linking sequence is MHYPYDVPDYAIEGRM-His_6 (22 amino acids). *B*, swarming abilities of NMB190 (Δ*pomA*) cells expressing nothing (*vector*), His_6-PomA (*PomA*), or tandem PomA (*PomA-PomA*). Overnight cultures were spotted on VPG-0.3% agar plate containing kanamycin and incubated at 30 °C for 5 h. *a.a.*, amino acid.

Function of PomA Dimers Containing Different Combinations of Wild-type or Mutant Subunits—In addition to wild-type PomA dimer, the following mutant dimers were created: PomA-P199L, P199L-PomA, and P199L-P199L (Fig. 2*A*). As a measure of function, the ability of PomA dimer and mutant dimers to swarm in a soft agar plate was assayed in *V. alginolyticus* NMB190 (Δ*pomA*). Fig. 3*A* shows the swarm abilities conferred by PomA dimer (PomA-PomA) and dimers containing mutation P199L. Previous mutational studies have shown that a conserved proline residue in PomA (Pro^{199} in the protein from *V. alginolyticus* and Pro^{222} in *E. coli* MotA), which is important for function, since mutations of this residue completely prevent flagellar rotation and showed strong negative dominance (34). When this inactivating mutation was introduced into the N-

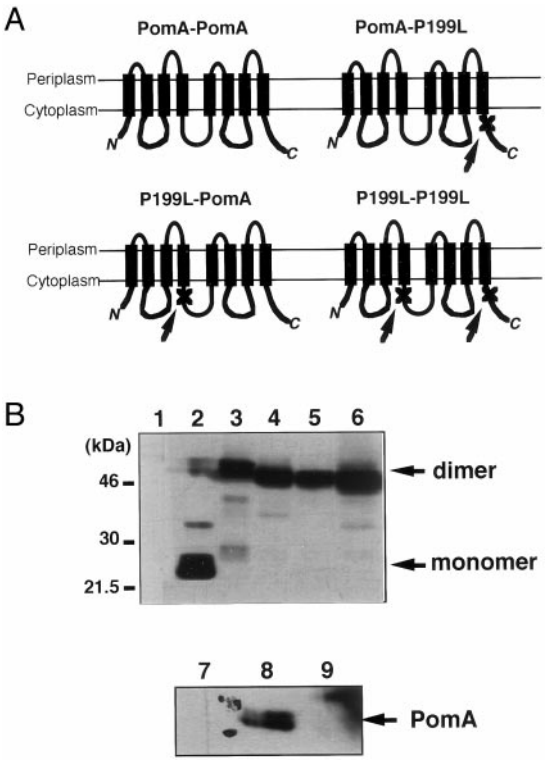

FIG. 2. **Expression of tandem PomA.** *A*, schematic diagrams of various PomA-PomA dimers. Two PomA monomers joined tail-to-head in tandem are expressed as a single polypeptide. The *arrows* indicate the positions where site-specific substitutions were introduced. *B* (*upper panel*), membrane vesicles (each 10 μg of protein) of NMB190 (Δ*pomA*) harboring a vector plasmid (*lane 1*), a plasmid encoding His_6-PomA (*lane 2*), tandem PomA dimer (*lane 3*), or tandem PomA dimer mutants (*lanes 4–6*) were subjected to SDS-PAGE and immunoblotting with anti-PomA antibody. *B* (*lower panel*), membrane vesicles (each 20 μg of protein) of NMB190 (Δ*pomA*) harboring a vector plasmid (*lane 7*), VIO5 ($PomA^+$) harboring a vector plasmid (*lane 8*), or NMB190 (Δ*pomA*) harboring a plasmid encoding tandem PomA dimer (*lane 9*) were subjected to SDS-PAGE and analyzed with immunoblotting as above.

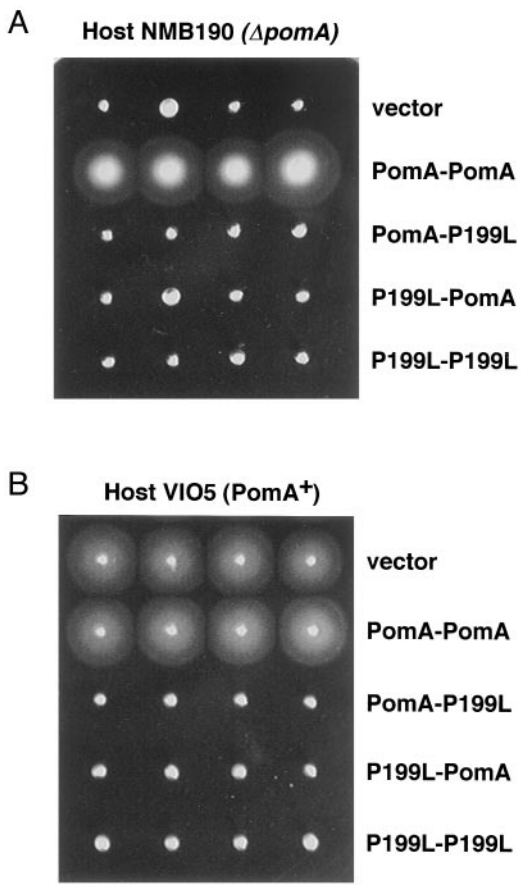

FIG. 3. **Functions of PomA dimer mutants.** *A*, swarming abilities of the PomA dimer mutants. Four independent clones of NMB190 (Δ*pomA*) each carrying the PomA dimers were cultured overnight, spotted on a VPG-0.3% agar plate containing kanamycin, and incubated at 30 °C for 5 h. *vector*, NMB190/pSU41 used as a negative control. *B*, dominant-negative effects of the mutant PomA dimers. Overnight cultures of four independent clones of VIO5 ($PomA^+$) each expressing PomA dimers from a multicopy plasmid were spotted on a VPG-0.3% agar plate containing kanamycin and incubated at 30 °C for 5 h.

terminal (P199L-PomA) or the C-terminal (PomA-P199L) half of the dimer, swarm activity was completely diminished. PomA dimer with the mutation in both halves (P199L-P199L) was also completely inactive (Fig. 3*A*). Under the dark field microscope (39), no flagellar rotation was observed with cells producing P199L-containing dimers (data not shown).

We also investigated for dominant-negative effects of the mutant PomA dimers. The plasmids encoding PomA dimer or mutant dimers were introduced into wild-type cells, VIO5 ($PomA^+$), and the transformants were inoculated on a soft agar plate. As shown in Fig. 3*B*, all PomA dimers containing the P199L mutation in either half of the dimer reduced the swarming ability of VIO5 ($PomA^+$) cells, whereas PomA homodimer resulted in no significant reduction in swarm size compared with wild-type cells. These results suggest that PomA dimers with the P199L mutation compete with the wild-type PomA for occupying the functional site of the torque-generating unit. Taking the results together, it is highly possible that PomA dimers are inserted in the membrane at the functional site. Furthermore, the inactivation of half of the dimer interferes with the function of the other half, implying that the two halves of the dimer function together.

Interaction of PomA Dimer and PomB—Our previous studies showed that PomA and PomB functionally interact with each other (38). To investigate whether PomA dimers have the ability to interact with PomB, a co-elution assay was performed (Fig. 4*A*). Membrane fractions of NMB190 (Δ*pomA*) expressing various PomA dimers were prepared, solubilized with β-octyl glucoside, and passed through a Ni^{2+}-NTA-agarose column. The column was washed, bound protein was eluted with buffer containing imidazole, and the eluate was analyzed by immunoblotting using antibodies against PomA and PomB (Fig. 4*A*). Although only His_6-PomA dimer is overexpressed in the cells, the protein complex contains only endogenous PomB, which should therefore reflect the native interaction between PomA and PomB. PomB co-eluted with PomA dimer (PomA-PomA; *lane 1*), whereas no PomB band was observed with PomA dimers containing the P199L mutation in either or both halves (P199L-PomA, PomA-P199L, or P199L-P199L; *lanes 2–4*).

Although Pro^{222} of *E. coli* MotA has been proposed to function as the regulator for the conformational changes of the MotA-MotB complex (40), its specific role still remains unclear. To examine the interaction between monomeric PomA P199L and PomB, a His_6 tag was attached to the N terminus of PomA and P199L mutant monomer, and the co-elution assay was performed as above (Fig. 4*B*). When the eluates were analyzed by immunoblotting, PomB was found to have co-isolated with wild-type His_6-PomA (*lane 6*), but no detectable PomB was co-sedimented with His_6-P199L (*lane 7*). The results described above suggest that as well as the Pro^{199} of PomA being essential for PomA-PomB interaction, probably affecting the C-terminal tail of PomA, both halves of the PomA dimer contribute to form the functional PomA-PomB complex.

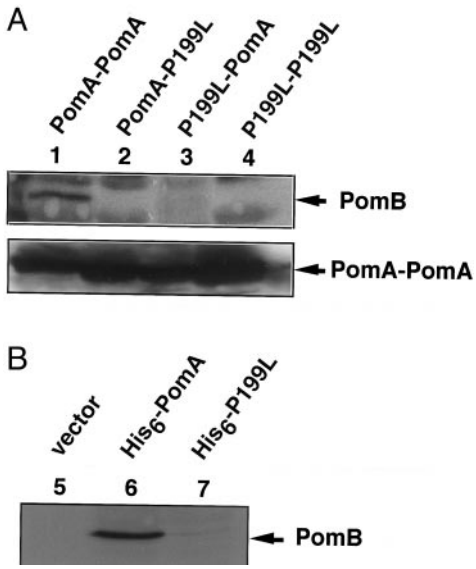

FIG. 4. **Co-elution analysis of PomA dimer.** *Upper panel*, detergent extract from membrane vesicles of NMB190 (Δ*pomA*) expressing either PomA-PomA (*lane 1*), PomA-P199L (*lane 2*), P199L-PomA (*lane 3*), or P199L-P199L (*lane 4*) were incubated with Ni^{2+}-NTA-agarose resin as described under "Materials and Methods," and eluates were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting with anti-PomB93 or anti-PomA1312 antibody (38). *Lower panel*, solubilized membrane vesicles of NMB190 (Δ*pomA*) expressing either nothing (*lane 5*), His_6-PomA (*lane 6*), or His_6-P199L (*lane 7*) were mixed with Ni^{2+}-NTA resin, and eluates were analyzed with SDS-PAGE and immunoblotting as above.

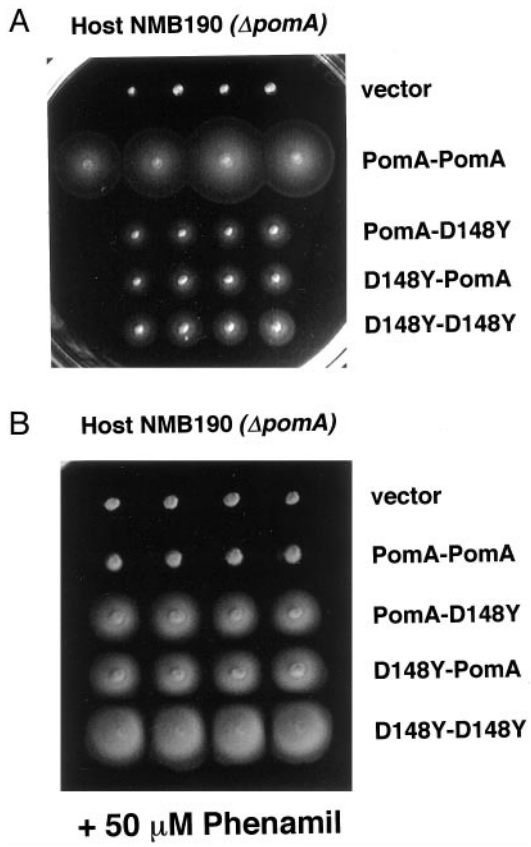

FIG. 5. **Effect of phenamil on torque generation driven by PomA dimer (PomA-PomA) or dimers with D148Y mutations (D148Y-PomA, PomA-D148Y, or D148Y-D148Y).** Four independent clones of NMB190 (Δ*pomA*) expressing the given PomA dimers were cultured overnight and spotted on a VPG-0.3% agar plate (100 mM NaCl) containing kanamycin (*A*) or both kanamycin and 50 μM phenamil (*B*) and incubated at 30 °C for 6 and 12 h, respectively. *vector*, NMB190/pSU41 used as a negative control.

Torque Generation of PomA Dimers Containing Phenamil-resistant Mutations—We further extended this approach to investigate the relationship between torque generation and sodium conductance. Our previous studies had shown that the substitution of Asp^{148} by Tyr (D148Y) in PomA results in resistance to phenamil, a specific inhibitor of the PomA-PomB sodium channel (35). Here, we have used this mutant to determine whether sodium conductance of both halves of the PomA dimer are required for torque generation. D148Y-containing mutant dimers were expressed as a 50-kDa protein, and no degradation products were observed (data not shown). Introduction of this mutation into either or both halves of the dimer reduced swarm activity (Fig. 5*A*). This is consistent with our previous observation that mutation at Asp^{148} results in impaired motility (35). 50 μM phenamil, which completely blocks sodium conductance of the PomA-PomB channel (35), completely inhibited motility driven by tandem PomA (PomA-PomA) (Fig. 5*B*). In contrast, NMB190 cells expressing tandem PomA with D148Y mutations in both halves (D148Y-D148Y) showed phenamil resistance. Interestingly, PomA heterodimers with either the N-terminal or the C-terminal half containing the D148Y mutation (PomA-D148Y or D148Y-PomA) retained activity in the presence of phenamil. To further examine the effect of sodium channel blockage on torque generation, the swimming speeds of cells harboring mutant PomA were measured in the presence or the absence of phenamil (Fig. 6*A*). In the absence of phenamil, NMB190 cells expressing tandem PomA with both halves containing the D148Y mutation had swimming speed reduced to about 80% compared with those expressing PomA-D148Y or D148Y-PomA. This is consistent with the control experiment shown in Fig. 6*B*, that the D148Y mutation significantly reduced the swimming speed compared with cells expressing wild-type monomeric PomA. When 50 μM phenamil was added to the cells expressing the PomA dimer with either or both halves of subunits containing D148Y mutations, all of the mutants showed similar motility. This suggests that both subunits of the dimer contribute to the phenamil binding. The results might also indicate that the individual halves of the dimer do not function independently but function together to conduct sodium ions.

DISCUSSION

We demonstrate here that an engineered fusion protein containing two PomA molecules can insert into the membrane and exhibit torque generation activity. Tandem PomA dimer provides a unique model system that permits coupled synthesis of two PomA molecules, with the advantage that both halves of the dimer are present at the identical level. A dimer containing wild-type PomA and mutant P199L was constructed, and the effect of the ability to rotate flagella was assessed. Inactivation of either half of the dimer caused complete loss of activity, indicating that both halves of the dimer are essential for torque generation. If the two halves of the dimer functioned independently, inactivation of either half would be expected to result in ~50% decrease in activity. In other words, the two halves of the PomA dimer appear to function together.

To examine the possibility that incorrect membrane insertion of either half of the dimer might occur, negative dominance was tested. Strong dominant negative effects were observed with both P199L-PomA and PomA-P199L. Although P199L-containing PomA dimers cannot bind to PomB (Fig. 4*A*), this result is quite reasonable, because it might be still able to interact with MotX and/or MotY, which are also essential for the rotation of the sodium-driven motor. If so, the mutant PomA dimers could interfere with MotX and/or MotY function

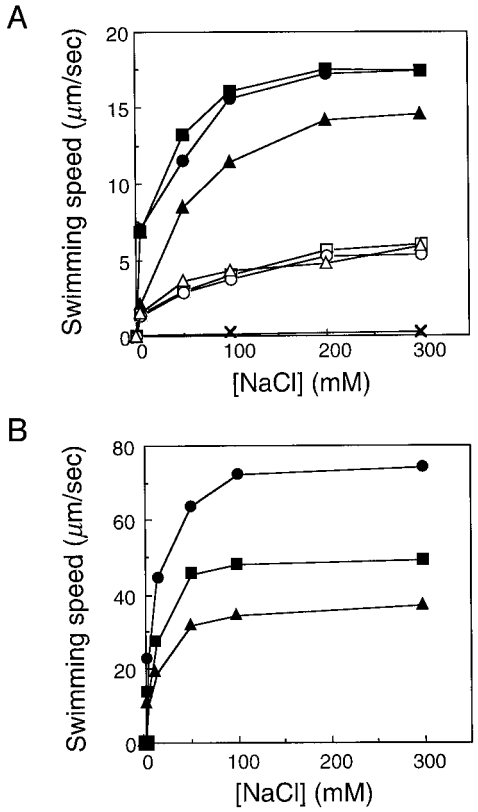

FIG. 6. **Sensitivity to phenamil of PomA dimers.** *A*, swimming speeds were measured in TMN medium in the presence or the absence of 50 μM phenamil as indicated under "Materials and Methods." Cells of NMB190 (Δ*pomA*) expressing PomA-D148Y (*squares*), D148Y-PomA (*circles*), or D148Y-D148Y (*triangles*) were collected at late logarithmic phase and suspended in TMN medium (pH 7.5, 50 mM NaCl). The cell suspension was diluted to 100-fold into TMN medium containing various concentrations of NaCl. *Filled* and *open* symbols indicate speeds in the absence or the presence of 50 μM phenamil, respectively. Cells of NMB190 (Δ*pomA*) expressing PomA-PomA (*crosses*) in the presence of 50 μM phenamil are also indicated. *B*, cells of NMB190 (Δ*pomA*) expressing PomA (*circles*), PomA-PomA (*squares*), or D148Y (*triangles*) were collected at late logarithmic phase, and swimming speeds were measured as above.

or sequester them away from the functional site. It is not clear that the wild-type PomA in the hetero-fused dimer folds like it would by itself or in the homo-fused dimer. Limited protease sensitivity of the dimer-expressed inverted membrane vesicles revealed that there were no differences of digestion pattern between wild-type fused dimer and mutant-containing dimer (data not shown), although further investigations are required. Can we exclude the possibility that a PomA dimer containing one wild-type PomA (P199L-PomA or PomA-P199L) might support rotation when this wild-type half of the mixed dimer is active, while other dimers impede rotation because the inactive (P199L) subunit is incorporated? This possibility is excluded for the following reason. If some of the active (wild-type) subunits of the P199L-bearing PomA dimers are incorporated into their correct sites in the motor to form a functional unit, those active subunits should form a PomA-PomB complex. However, we have shown here that all of the P199L-containing dimers cannot interact with endogenous PomB in Fig. 4*A*, which indicates that there are no such species as functional PomA-PomB complexes. Taken together, the results provide support for the contention that PomA is functional as an even number of subunits, either a dimer or a multimer of dimers.

The Pro^{199} residue of PomA is highly conserved in MotA homologs and is thought not to have a direct role in ion translocation but rather a structural role (34). Pro^{222} in *E. coli* MotA was investigated by intensive mutagenesis, leading to the conclusion that it might function to mediate conformational changes that couple the events occurring on the membrane and cytoplasmic domains during energy conversion (40, 41). We showed here that Pro^{199} of PomA also plays a critical role for PomA-PomB interaction. Monomeric PomA with the mutation P199L showed reduced ability to interact with PomB. It is noteworthy that this was also observed with PomA dimer containing the mutation in either half of the dimer. Suppose that PomA is functional as a monomer, and each molecule interacts with PomB. It is difficult then to imagine how the introduction of single P199L mutation into one half of the dimer could result in complete loss of PomA-PomB interaction. Function as a dimer is consistent with our previous estimate that the molar ratio of isolated functional PomA-PomB complex is 2 PomA:1 PomB (32). We therefore conclude that both subunits in PomA dimer contribute to the PomA-PomB interaction.

One more approach was used to investigate the functional impact of PomA dimer. Tandem PomA was constructed containing wild-type PomA and D148Y mutated PomA, and the effect of phenamil on torque generation was measured. The results indicate that the introduction of D148Y mutation to one half of the dimer results in no significant decrease in both swarm activity and swimming speed compared with dimer where both halves carry the phenamil-resistant mutation, suggesting the possibility that the individual halves of the dimer do not conduct sodium ions by themselves. Although it is not clear how many phenamils bind to this dimer molecule, it is highly probable that both halves of the PomA dimer function together to conduct sodium ions, and so both subunits cooperatively form a phenamil binding site.

Some integral membrane permeases such as the ribose transporter RbsC (42) form homodimers for their function. The FhuB, the ferric hydroxamate transporter component, functions as a single polypeptide containing two homologous repeats and is still active when those repeats are separated (43). Oligomerization of PomA may be an analogous process.

In the absence of high resolution images of the torque-generating unit, subunit stoichiometry can be tentatively assessed using biochemical methods. The results described above provide a framework for the subunit stoichiometry of the torque-generating unit, but a definitive answer will require further experimental documentation.

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