Random mutagenesis of the pomA gene encoding a putative channel component of the Na⁺-driven polar flagellar motor of Vibrio alginolyticus

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Random mutagenesis of the pomA gene encoding a putative channel component of the Na⁺-driven polar flagellar motor of Vibrio alginolyticus. On the basis of their similarity to MotA and MotB, which are the proton-conducting components of the H⁺-driven motor, they are thought to form the Na⁺-channel complex and to be essential for mecanochemical coupling in the motor. To investigate PomA function, random mutagenesis of the pomA gene by using hydroxylamine was carried out. We isolated 37 non-motile mutants (26 independent mutations) and most of the mutations were dominant; these mutant alleles are able to inhibit the motility of wild-type cells when greatly overexpressed. The mutant PomA proteins could be detected by immunoblotting, except for those with deletions or truncations. Many of the dominant mutations were mapped to the putative third or fourth transmembrane segments, which are the most conserved regions. Some mutations that showed strong dominance were in highly conserved residues. T1861 is the mutation of a polar residue located in a transmembrane segment that might be involved in ion translocation. P199L occurred in a residue that is thought to mediate conformational changes essential for torque generation in MotA. These results suggest that PomA and MotA have very similar structures and roles, and the basic mechanism for torque generation will be similar in the proton and sodium motors.

Keywords: flagella, sodium ion, Vibrio, motor, pomA

INTRODUCTION

Bacterial flagella are the organelles responsible for motility. At the base of each filament, a rotary motor is embedded in the cytoplasmic membrane, and bacteria can swim by rotating their helical flagellar filaments. The motor is powered by the electrochemical gradient of a specific ion, H⁺ or Na⁺, across the cytoplasmic membrane (Blair, 1995; Imae & Atsumi, 1989). The study of the flagellar motor has been intensively done in the H⁺ motors of Escherichia coli and Salmonella typhimurium. These studies have focused mainly on five core proteins involved in the motor function. In the rotor part of the motor, three soluble proteins, FliG, FliM and FliN, are the components for force generation, flagellar assembly and controlling the direction of motor rotation (Yamaguchi et al., 1986; Sackett et al., 1992; Irikura et al., 1993). Together they form a complex, called the ‘switch complex’ or ‘C-ring’ (Francis et al., 1994). Among them, FliG has a direct role in force generation (Lloyd et al., 1996). The stator part of the motor, two integral membrane proteins, MotA and MotB, are the components essential for force generation (Dean et al., 1984; Stader et al., 1986; Block & Berg, 1984; Blair & Berg, 1988). They have four and one transmembrane segments, respectively (Zhou et al., 1995; Chun & Parkinson, 1988), and form a H⁺-conducting channel complex responsible for coupling ion translocation to force generation (Blair & Berg, 1990; Stolz & Berg, 1991; Sharp et al., 1995a; Garza et al., 1995, 1996). The MotA/MotB channel complex is believed to be anchored to the cell wall by the peptidoglycan-binding domain of MotB (Chun & Park-
Intensive mutational analysis of MotA, MotB and FliG of *E. coli* revealed some critical residues involved in torque generation. It was shown that most of the dominant mutations occurred in the transmembrane domains of MotA and MotB (Blair & Berg, 1991; Blair et al., 1991). In particular, Asp32 of MotB, a conserved acidic residue in the membrane segment, was suggested to be the residue conveying protons (Zhou et al., 1998a). Other recent mutational analyses indicated that three charged residues of FliG predicted to be on one surface, Arg281, Asp288, Asp289, and two cytoplasmic charged residues in the membrane segment, is suggested to be the residue conveying protons (Zhou et al., 1998a). It was shown that most of the acidic residue in the membrane segment, is suggested to be the residue conveying protons (Zhou et al., 1998a).

The Na+-driven motor has some advantages for study compared with the H+-driven type. Sodium-motive force can be manipulated more easily than proton-motive force, and amiloride and its analogues work as specific inhibitors of the motor (Sugiyama et al., 1997). It was speculated that these charged residues of MotB and PomB were homologous to the proton-motive force, and amiloride and its analogues work as specific inhibitors of the motor (Sugiyama et al., 1997). Therefore, PomA and PomB are thought to form a Na+-channel complex in the motor. Recently, mutations which cause the resistance to the specific inhibitor, phenamil (an amiloride analogue), were identified in PomA and PomB, supporting the suggestion that these proteins are Na+-motor-specific channel components (Kojima et al., 1999). On the other hand, both MotX and MotY, which have been identified in *Vibrio parahaemolyticus* (McCarter, 1994a, b) and have putative single transmembrane segments, are not homologous to the proton-type motor proteins, except that MotY has a peptidoglycan-binding motif in the C-terminal region, as do MotB and PomB. MotX was also inferred to be the Na+-channel component of the motor because overproduction of MotX is lethal to *E. coli* in proportion to the external Na+ concentration and because this lethality is reversed by the addition of amiloride (McCarter, 1994a).

Thus, the H+ motor and the Na+ motor have some similar and some distinct aspects. We hypothesize that the basic mechanism for torque generation will be common in the H+-driven and Na+-driven motors. If so, residues critical for motor function will also be common between the two types of motors. In this study, we carried out random mutagenesis of the *pomA* gene in order to identify residues responsible for the function of PomA.

**METHODS**

**Bacterial strains, growth conditions and media.** *V. alginolyticus* VIO5 (Laf- PomA+) and VIO586 (Laf- PomA+) were used as the host strains to express the wild-type and mutant proteins (the Laf phenotype is the presence of lateral flagella) (Okunishi et al., 1996, Asai et al., 1997). For DNA manipulation, we used the *E. coli* strain DH5α (Grant et al., 1990). *V. alginolyticus* cells were cultured in VC medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K2HPO4, 3% NaCl, 0.2% glucose) at 30 °C and *E. coli* cells were cultured in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C. For swarm assays, *Vibrio* cells were inoculated on 0.25% agar VPG plates (0.25% agar in VPG medium containing 1% polypeptone, 0.4% K2HPO4, 3% NaCl, 0.5%, w/v, glycerol). When necessary, chloramphenicol was added to a final concentration of 2.5 μg ml^-1^ for *Vibrio* cells and 25 μg ml^-1^ for *E. coli* cells.

**Plasmids.** For isolation of *pomA* mutants, the *pomA* gene in the BamHI fragment of plasmid pYA301 was subcloned into pSU21, a chloramphenicol-resistant vector (Bartolome et al., 1991), and named pMK101. The BamHI fragment in pMK101 was inserted in the opposite orientation to the lac promoter in pSU21. This BamHI fragment was also cloned in the reverse orientation and the resultant plasmid was named pMK201.

**Isolation of *pomA* mutants.** Plasmid pMK101 was treated with 2 M hydroxylamine in 0.1 M potassium phosphate (pH 6.0), 1 mM EDTA for 4 h at 50 °C. After the treatment, plasmids were diluted in 10 mM Tris/HCl (pH 7.5) containing 1 mM EDTA, and dialysed overnight to remove hydroxylamine. Then, plasmids were precipitated with ethanol and transformed into the *pomA* mutant VIO586. We selected Pom- (polar flagellar motility defective) transformants from the chloramphenicol-resistant and swarm-deficient ones as described previously (Okunishi et al., 1996). The Pom- phenotype was confirmed by isolating mutant plasmids and retransforming them into VIO586.

**Dominance.** To assay the dominance of the plasmid-borne *pomA* mutants, each plasmid was transformed into the PomA+ strain VIO5. Transformants were inoculated on a 0.25% agar VPG plate and incubated for 6 h at 30 °C. The diameters of the swarms of each mutant were measured and their mean diameter was normalized to the diameter of the swarm formed on the same plate by VIO5/pMK201 (PomA+) cells. The relative swarm size of each mutant was obtained from at least three independent experiments, and the dominance was determined from the mean value of them. The swarm size of wild-type cells was 0.9 ± 0.06 cm. The mutants were categorized into four types; severely dominant (+ + + +; 0–20% of wild-type swarm size), strongly dominant (+ + +; 21–45% of wild-type), slightly dominant (+; 46–70% of wild-type) and recessive (+; 71–100% of wild-type).

**DNA manipulations and sequencing.** Routine DNA manipulations were carried out according to standard procedures (Sambrook et al., 1989). Restriction endonucleases and other enzymes for DNA manipulations were purchased from Takara Shuzo and New England Biolabs. The nucleotide sequence was determined by the dideoxy chain termination method using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 377 DNA sequencer (Perkin Elmer).

**Electroporation.** Transformation of *Vibrio* cells by electroporation was carried out as described previously (Kawagishi et al., 1994).

**Immunoblots of PomA.** Immunoblotting was carried out as described previously (Nishioka et al., 1998). Cells were
harvested and suspended in distilled water at an OD_{600} of 10. The suspension was electrophoresed through a 10% SDS-PAGE gel. For the detection of the mutant PomA proteins, we used an anti-PomA peptide antibody (PomA91), which was generated against peptides derived from three segments of PomA with an additional cysteine at the N terminus to keyhole limpet haemocyanin; 1, CKKDALTDERHTQGDVFRAFDVAP (in the cytoplasmic domain between TM2 and 3); 2, CTTLYGAILSNMVFPHPADKLSLRDDQET (in the TM4); 3, CQDGQNPVIDSYLKNYLNEGK (in the C-terminal cytoplasmic domain).

RESULTS
Isolation of PomA mutants

We first subcloned the pomA gene from the plasmid pYA301 (pomA', Kmr') into the vector pSU21 (Cm'), because transformation of Vibrio cells with the Cm' vector was more efficient than with the Kmr vector. Random mutagenesis of the pomA gene was carried out by treating the resultant plasmid, pMK101 (pomA', Cm'), with hydroxylamine. The mutagenized plasmids were introduced into the pomA mutant VIO586, which has non-motile polar flagella, and each transformant was inoculated on semisolid agar plates. Approximately 1% of the Cm' clones obtained were deficient for swarming in this screen, and we isolated 43 mutants by this procedure. These mutants were classified into two groups: (I) cells completely impaired in polar flagellar motility on semisolid agar (Pom'); 37 clones), and (II) cells that swarm much more slowly than wild-type (6 clones). Fig. 1 shows typical swarms of the type I mutants (alleles 1 and 3) and type II mutants (alleles 2 and 4). We expected that type II mutants might swim slowly. However, when each of them was grown in liquid broth and examined under the microscope, only a small fraction of the cells were swimming, at the same speed as wild-type cells, and most cells were immotile. All of the type II mutants showed this phenotype. On the other hand, all of the type I mutants were completely immotile when grown in broth and observed under the microscope. We characterized the type I mutants further.

Positions of pomA mutations

The nucleotide changes in all the type I pomA alleles were determined by sequencing. The results are summarized in Table 1. In six mutants (alleles 3, 7, 23, 24, 26 and 37) we could not find any nucleotide changes in the pomA coding region. These mutations might occur in regions that affect the expression of pomA. Nucleotide changes in the other mutants were of the type expected for hydroxylamine mutagenesis (GC to AT transitions). The mutants were grouped into four categories (Table 1). Deletions or terminations were found in ten mutant alleles. Single and double amino acid substitutions were found in 16 and 5 mutants, respectively. Double mutations were detected mainly in the cytoplasmic domain, and interestingly, two were in adjacent residues. On the other hand, it is noteworthy that most of the single mutations were mapped to the putative third or fourth transmembrane segments, which are highly conserved between PomA and MotA of R. sphaeroides or E. coli (Asai et al., 1997) (Fig. 2). Actually, all of the mutated residues in the single mutants, except for Ser193, are conserved in MotA. Five mutations were found in residues that also gave a dominant, swarm-deficient phenotype when mutated in motA in E. coli. These residues are Gly8, Gly154, Thr186 and Pro199 (mutations in MotA were G6S, G6D, G176S, T209W and P222L, respectively). Most of the single mutations were substitutions of a non-charged residue by a polar or charged one, and others were substitutions by residues with large side chains.

Dominance of pomA mutations

Next, we investigated the dominant-negative effects of the mutant PomA proteins. All the type I mutant pomA alleles were introduced into VIO5 (pomA') cells, and fresh transformants were inoculated on semisolid agar plates. None of the mutant plasmids caused any significant reduction in swarm size in this assay (data not shown). The pomA fragment in pMK101 is inserted in the opposite orientation to the lac promoter, and so although the protein is expressed at levels sufficient to complement a pomA defect, it is not expected to be overexpressed (for discussion of an analogous construct expressing motY, see Okunishi et al., 1996). To determine whether higher-level expression of mutant pomA alleles in trans from multicopy plasmids in PomA' cells might affect the dominance of the mutation, we reversed the orientation of the wild-type and all the
Table 1. Nucleotide and amino acid changes of PomA mutants

Nucleotide changes were identified as described in Methods. Dominance was determined by swarm size relative to that of wild-type (see Fig. 4). Loop1-4 is the region between TM1 and TM2 (periplasm), loop2-3 is the region between TM2 and TM3 (cytoplasm), and loop3-4 is the region between TM3 and TM4 (periplasm). NE; not examined.

<table>
<thead>
<tr>
<th>Allele number/type</th>
<th>Base change</th>
<th>Amino acid change</th>
<th>Dominance</th>
<th>Location</th>
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<tr>
<td>Deletion</td>
<td>A262-472</td>
<td>Frameshift</td>
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<td>Loop3-4-TM3</td>
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<td>Q54Termination</td>
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<td>Q226Termination</td>
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<tr>
<td>34</td>
<td>C694T</td>
<td>R232Termination</td>
<td></td>
<td>C-terminus</td>
</tr>
<tr>
<td>31</td>
<td>C578T/C694T</td>
<td>S193F/R232Termination</td>
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<td>TM4/C-terminus</td>
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<td>G85</td>
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<td>TM1</td>
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<td>G23A</td>
<td>G8D</td>
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<td>G154R</td>
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<td>G154E</td>
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<td>G157D</td>
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<td>(G115G)/T158I</td>
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<td>T186I</td>
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<td>TM4</td>
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<tr>
<td>30</td>
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<td>P199L</td>
<td>++</td>
<td>TM4</td>
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<tr>
<td>5</td>
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<td>(F198F)/P199L</td>
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<td>TM4</td>
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<td>14</td>
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<td>G221D</td>
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<td>C-terminus</td>
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<td>R247C</td>
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<tr>
<td>12</td>
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<td>M219I/D220N</td>
<td>++</td>
<td>C-terminus/C-terminus</td>
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</table>

As shown in Fig. 3(a), pomA in pMK201 complemented the PomA- phenotype of VIO586, indicating that function is normal when the PomA protein is expressed from this plasmid. Most of the mutant PomA remained nonfunctional in this assay. Exceptions were G176R and S193F, which were slightly motile, suggesting that these two mutations might be suppressed by overexpression of the proteins. As shown in Fig. 3(b), most of the mutant proteins reduced the swarming ability of VIO5 (PomA+) cells when they were overexpressed from the plasmids. To quantify the dominance of each mutant, the swarm assays were repeated, and relative swarm sizes for at least three measurements were averaged. The data are summarized in Fig. 4. Deleted or truncated PomA mutants exhibited recessive or slightly dominant phenotypes. On the other hand, most of the single or double mutants showed dominant effects. The strength of dominance was ranked as described in Methods (Table 1). The mutations in the transmembrane segments showed the strongest dominance (+++ or ++), consistent with the results of MotA mutagenesis.
Random mutagenesis of the *pomA* gene

**Fig. 2.** Amino acid alignments of PomA and sites of mutations isolated in this study. Mutated residues are marked by symbols. ● show sites of nonsense mutations, and arrows show the deletion in the allele 2 mutant. Circles show the sites of single mutations and paired symbols (△, ▽, □ or ○) show sites of double mutations. Residues giving dominant mutations in both PomA and MotA of *E. coli* are marked by a black circle. Abbreviations: VaPomA, *V. alginolyticus* PomA; RsMotA, *R. sphaeroides* MotA; EcMotA, *E. coli* MotA. White letters in black boxes show residues identical to PomA.

Expression of mutant PomA

To detect the mutant proteins expressed from the plasmid pMK201, we performed immunobots to the cell suspensions of the mutants by using a polyclonal anti-PomA peptide antibody (Fig. 5). We could detect PomA bands of the expected size (25 kDa) in all the strongly (+ +) or severely (+ + +) dominant mutants. We observed some small differences in the amounts of the proteins between the mutants. In addition to the 25 kDa band (arrow in Fig. 5), we detected a band at 45 kDa, in both the wild-type and the mutants. The significance of this band is discussed later. We could not detect the PomA protein in the wild-type strain itself (vector in Fig. 5a and b), or in recessive (−) or slightly dominant (+) mutants (for example, G154R in Fig. 5b;...
other data not shown), except for R247C and L131F/T132M. All of the deleted or truncated mutant proteins could not be detected. These mutant proteins might be degraded or synthesized in very small amounts. L131F/T132M, which was detected but not dominant, might have some defect in assembling into the appropriate position in the motor.

In some mutants, the PomA bands were found at positions different from the wild-type PomA. The T158I protein was observed at a higher position than wild-type, and in the G176E and R135Q/M169I mutants, both the 25 kDa and 45 kDa bands were at significantly higher positions. Some structural change caused by these mutations might affect the mobility of the proteins in electrophoresis.

**DISCUSSION**

### Isolation of pomA mutations

PomA is the homologue of the MotA protein, which is believed to be the channel component essential for rotation of the H⁺-driven motor. PomA should have similar functions in the Na⁺-driven motor. To identify the residues of PomA essential for torque generation, we carried out random mutagenesis of the pomA gene by hydroxylamine. We isolated 37 non-motile mutants and identified 26 independent mutations in the pomA gene. Most of the mutants with single or double amino acid substitutions showed severe or strong dominance, and these mutant PomA proteins expressed from multicopy plasmids could be detected by immunoblotting. These results suggest that the mutant proteins which exhibit dominance are expressed and inserted into the motor but do not function properly, that is, they seem to have defects in torque generation. On the other hand, recessive and weakly dominant mutants could not be detected by immunoblotting. We also could not detect the wild-type level of PomA (Fig. 5a, b, lanes 1), so mutant proteins might be synthesized in very small amounts, although they do not significantly affect the torque generation when expressed in PomA⁺ cells. Therefore we mainly discuss the strongly dominant mutations in the subsequent sections.
Localization of the dominant mutations

The dominant mutations were localized to the putative third or fourth transmembrane segments (Fig. 6), which are highly conserved between PomA and MotA. These transmembrane segments might be more important for function than other regions of the protein. Most of the mutations introduced amino acids with charged or large side-chains. According to the tryptophan-scanning mutagenesis of MotA (Sharp et al., 1995b), helices 3 and 4 of MotA were more sensitive to bulky Trp substitutions than were helices 1 and 2, suggesting that helices 3 and 4 might be more fully surrounded by other protein segments that cannot accommodate the Trp side chain. Therefore, substitutions in TM3 or TM4 of PomA might affect conformation or interfere with the interaction between PomA and other channel components such as PomB, MotX and MotY, and consequently disrupt the arrangement of the channel complex, which would no longer function properly in the motor.

Several dominant double mutants were isolated and mapped to the cytoplasmic domain. M219I/M220N were mutated in residues that adjoin each other, suggesting that this region might be involved in protein–protein interaction or be important to protein conformation. It will be necessary to separate these mutations to determine which confers the Pom- phenotype, or whether both are needed.

Comparison between PomA and MotA

Most of the dominant mutations isolated in this study occurred in the residues conserved in MotA proteins. Especially we found five mutations in residues that also gave a dominant, swarm-deficient phenotype when mutated in motA in E. coli. The mutations G8S, G8D (TM1) and P199L (TM4) correspond to G6S, G6D and P222L, respectively, isolated in E. coli MotA (Blair & Berg, 1991). These residues are also highly conserved in MotA homologues (Fig. 2). They would not have a direct role in ion translocation but rather a structural role. Zhou & Blair (1997) focused on, and investigated, this proline residue (Pro222 in E. coli MotA) by intensive mutagenesis: they speculated that Pro222 might function to mediate conformational changes in MotA that couple the events occurring on the membrane and cytoplasmic domain during energy conversion. Pro151 of PomA is another Pro residue located in the putative third TM segment and is highly conserved among MotA homologues. Mutations of this residue were not isolated in this study, but according to the mutational analysis of the corresponding residue in E. coli MotA (Zhou & Blair, 1997), it would have a role similar to that of Pro199 in PomA. The G154R mutation was identical to a chromosomal mutation in PomA of VIO586 (Y. Asai, I. Kawagishi, E. Sockett & M. Homma, unpublished). This residue corresponds to Gly176 in E. coli MotA, and mutation G176S of MotA severely impaired motility (Blair & Berg, 1991).

There are no charged residues in the TM segments of PomA, although some polar residues exist there. These residues might be expected to interact with ions in an ion channel. Among the polar residues, dominant mutations were isolated in Thr158 (TM3) and Thr186 (TM4). Thr186 is highly conserved in MotA homologues, and might have an important role in ion translocation. Thr158 is also conserved in MotA of R. sphaeroides, and T158I mutations exhibited the strongest dominance (Fig. 4). In E. coli MotA, the non-motile dominant mutation A180V was isolated in the corresponding residue. So a protein carrying the T158I mutation might affect the ion translocation or impede the rotation of the motor, acting as a ‘brake’ because of its bulky side chain. In this study we could not isolate the Na⁺-motorspecific dominant mutations. This suggests that PomA has very similar profile to MotA as a channel component. In other words, PomA might mainly constitute the Na⁺ channel pore structure, and Na⁺-selectivity might not be determined by PomA alone.

Expression of mutant PomA

Some mutations of PomA affected the electrophoretic mobility of the protein. The T158I, G176E and R135Q/M169I proteins were detected at a position slightly above the wild-type protein on gels. This altered mobility might reflect differences in conformation or other modifications. In addition to the PomA bands (25 kDa), we observed bands at approximately 45 kDa in both wild-type and mutant PomA. We suggest that this band might represent a different conformation of PomA or an interaction of PomA with itself or with other proteins, which persist even in the presence of SDS (T. Yorimitsu, K. Sato, Y. Asai, I. Kawagishi & M. Homma, unpublished). The 45 kDa bands of G176E and R135Q/M169I were also found at the upper position, supporting this proposal. In some mutants, the 45 kDa bands were weaker. It will be necessary to examine more
systematically the conditions that affect the appearance of the 45 kDa band.

We think that the basic mechanism of torque generation in the flagellar motor will be common, independent of the coupling ion, H⁺ or Na⁺. On the other hand, it is not known how the ion selectivity of the motor is determined, or what is the function of the additional motor genes, motX and motY, specific for the Na⁺ motor. To answer these questions, it will be necessary to investigate interactions between the motor components. In the case of Na⁺-type F,F₁ ATPase of *Propionigenium modestum*, an H⁺-type *E. coli* (F₁-δ)/Na⁺-type *P. modestum* (F₁δ) hybrid ATPase showed that the F₁ part is exclusively responsible for the recognition of the coupling ion (Kaim & Dimroth, 1993, 1994). Such hybrid studies, for example *Rhodobacter* MotA expressed in a *pomA*-deleted strain, are now in progress in our laboratory. We hope that further analysis of the common structure between the Na⁺ system and H⁺ system will lead us to the essential points of the torque-generating mechanism.

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Random mutagenesis of the pomA gene


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