学位論文

Na^+ 駆動型べん毛モータータンパク質 PomA と PomB に関する研究

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Abstract

The bacterial flagellar motor is a molecular machine that converts ion flux across the membrane into flagellar rotation. The coupling ion is either a proton or a sodium ion. In this study, I purposed to reveal the mechanism of torque generation of a sodium driven motor. At first (Chapter 2), I tried cloning the genes of putative flagellar motor channel components. When I started this study, two genes, *motX* and *motY*, had been just identified in the sodium driven motor of marine bacteria, *Vibrio parahaemolyticus*(Vp) and *V. alginolyticus*(Va). They have no similarity to the genes for proton-driven motors, *motA* and *motB*, whose products constitute a proton channel. So, it had been thought that the force-generating units of H^+ and Na^+ motor were very different. Here we identified additional sodium motor genes, *pomA* and *pomB*, in *V. alginolyticus*, which have similarities to MotA and MotB, respectively. These results suggest that PomA and PomB may be sodium-conducting channel components of the sodium-driven motor, and that the motor part consists of the products of at least four genes, *pomA*, *pomB*, *motX* and *motY*.

PomA is thought to have four transmembrane regions and compose an ion channel with PomB. The large cytoplasmic loop of PomA interacts directly to the rotor part of the motor, FliG. To characterize the two periplasmic loop regions of PomA, which may have a role as an ion entrance for the channel or interaction site to the other components, I carried out cysteine-scanning mutagenesis (Chapter 3). Among of a series of substitutions, mutants which conferred a non-motile phenotype, and whose motility was inhibited by the thiol-modifying reagents were included. Furthermore, from the labeling profiles by biotin maleimide, we predicted more directly the membrane topology of $loop_{3-4}$.

Next, to compare and study the coupling mechanism of the sodium and proton ion flux for the force generation, I tried identifying the recognition sites of the coupling ions using MotA and MotB of the photosynthetic bacterium, *Rhodobacter sphaeroides* (Rs) (Chapter 4). RsMotA shows ca. 40 % identity to VaPomA and was expressed in the *pomA* mutant of *V. alginolyticus*. The cells who had a hybrid motor composed of MotA, PomB, MotX and MotY restored motility. This hybrid motor did not work in sodium-free buffer, and was inhibited by phenamil and amiloride, sodium motor-specific inhibitors, but not by a protonophore. This shows that the proton motor component, MotA, of *R. sphaeroides* can generate torque by coupling with the sodium ion flux in place of PomA of *V. alginolyticus*.

Based on the conclusion of Chapter 4 that A subunits do not decide the ion specificity, I examined whether B subunits are involved in the recognition of coupling ion (Chapter 5). MotB did not work with either MotA or PomA in the *pomAB* mutant of *V. alginolyticus*. Next, I constructed six chimeric proteins (MomB) which had N terminal regions of MotB and C terminal regions of PomB. Among of them, even the MomB proteins with entire trasnmembrane region derived from the H^+-type MotB protein gave rise to Na^+-type hybrid motor with MotA. The two chimeric proteins, of which the junction sites were in the transmembrane region, also formed sodium motors with PomA, but the resulting motors were changed for the affinity to Li^+. These results show that B subunits are the part of the ion channel of the motor and the channel part consisting of the transmembrane regions from the A and B subunits do not discriminate Na^+ and H^+.

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Abbreviations

CCCP	Carbonylcyanide m-chlorophenylhydrazone
CCW	Counterclockwise
CW	Clockwise
DCCD	Dicyclohexylcarbodiimide
DTNB	Dithionitrobenzoic acid
EMS	Ethyl methanesulfonate
HQNO	2-heptyl-4-hydroxyquinoline- *N*-oxide
Laf	Lateral flagella
LDM	Laser dark-field microscopy
Mpa^r	Motility resistant to phenamil
Mpa^s	Motility sensitive to phenamil
NEM	*N*-ethylmaleimide
PGB	Peptidoglycan binding domain
Pof	Polar flagella
Pom	Polar flagellar motility
Rif	Rifampicin
rps	Revolution per second
Rs	*Rhodobacter sphaeroides*
TM	Transmembrane
Va	*Vibrio alginolyticus*
3D	Three dimensional

Chapter I Introduction

Bacterial cells have two kinds of rotary machine, F_oF_1-ATPase and flagellar motor (Fig. I-1)(Khan, 1997). Both motor use the electrochemical potential of a specific ion (a proton or a sodium ion, depending on the organism) for the energy source of rotation. F_oF_1-ATPase exists in bacteria as well as mitochondria and chloroplasts, and produces ATP by the coupling the ion flux. Flagellar motor is only present in bacteria and converts the ion flux to the locomotive force of cell. These are all rotary motors to be known in nature.

In the case of F_oF_1-ATPase, the rotation of water-soluble F_1 part of enzyme was observed (Noji *et al.*, 1997). The F_1 part is composed of 3 α, 3 β, γ, δ and ε subunit, and the β subunits have ATPase activity (Fig. I-2a). On the other hand, the membrane-embedded F_o part consists of a, 2 b and 10-12 c subunits (Dimroth, 1994; Fillingame, 1997). It has been found that a hybrid enzyme composed of the F_o part from the sodium type and the F_1 part from the proton type are functional and show sodium type phenotype (Kaim & Dimroth, 1993; Kaim & Dimroth, 1994; Laubinger *et al.*, 1990). Thus, the F_0 part decides the coupling ion. It has been suggested that the a and c subunits are involved in the ion translocation. The c subunits include the conserved dicyclohexylcarbodiimide(DCCD)-reactive Glu residue, which is thought to be an ion binding site, and the mutations changing the coupling ion specificity have been found in the a and c subunits (Kaim *et al.*, 1998; Kaim *et al.*, 1997; Valiyaveetil & Fillingame, 1997). The proton-type a and c subunits of *E. coli* and the sodium-type subunits of *Propionigenium modestum* have 25 % identity, respectively (Kaim *et al.*, 1992). In the case of sodium type F_oF_1-ATPase c subunits, 5 residues which are involved in the ion recognition have

Fig. I-1. Major ionic cycle of a bacterial cell.

Fig. I-2. (a) The structure of F_0F_1-ATPase. (b) The membrane topology of *P. modestum* (sodium type) and *E. coli* (proton type) c subunits. White boxes mean transmembrane regions. (c) The binding model of the coupling ions in the sodium type c subunit. The figures of (a) and (b) are referred from Dimroth *et al.* (1999) and Zhang and Fillingame (1995), respectively. The figure of (c) is modified from Kaim *et al.* (1997).

been identified, and include the DCCD-reactive Glu65 residue, Ser66 and Gln32 (Fig. I-2c)(Kaim *et al.*, 1997). The corresponded residues in proton type c subunits are Asp61, Ala62 and Ile28, respectively (Fig. I-2b). The substitution Gln32 of sodium type to Ile abolished only the ability of Na^+ coupling. The substitution Ser66 to Ala abolished the ability of Na^+ and Li^+ coupling, retain H^+ coupling ability. From these results, it was proposed that the side chain of these residues coordinated of an ion. Recently, the 3D structure (Dmitriev *et al.*, 1999) and their conformational change between protonated and deprotonated form (Rastogi & Girvin, 1999) of *E.coli* c subunits were resolved. Those showed that the side chains of these residues faced each other.

Another rotary machine, bacterial flagella motors rotate helical flagella and propel cells toward to the favorable stimuli. The proton driven motors of *Escherichia coli* and *Salmonella typhimurium* have been extensively studied in terms of structure, genetics, biochemistry and biophysics (Berry & Armitage, 1999; Blair, 1995; DeRosier, 1998). The products of about 50 genes are involved in producing and controlling flagellar motors, and the working flagellum contains about 20 products of the genes. The helical flagella filament is connected to the hook by two junctional proteins. The hook functioning as a universal joint is connected to the rod acting as the drive shaft. The rod forms the basal body with four rings; L, P and MS ring. The L, P and MS ring penetrate the outer membrane, peptidoglycan and inner membrane, respectively. Moreover, the C ring exists on the cytoplasmic face of the MS ring. Rotational force is generated by interaction between elements of the rotor part, C ring, and the stator part, force-generating units in the cytoplasmic membrane (Jones & Aizawa, 1991). Multiple force-generating units are inferred to function independently because rotational speed changes in a series of equal increments after the component

Fig. I-3. The structure of flagellar motor.

proteins of the force-generating units is induced from regulatable plasmids. Sixteen or 8 steps were observed in fully functional H^+-driven motors of *E. coli* (Blair & Berg, 1988; Block & Berg, 1984). The C ring is composed of FliG, FliM and FliN (Macnab, 1995; Yamaguchi *et al.*, 1986), which is also called the switch complex. FliM interacts the chemotaxis protein phospho-CheY and determines the direction of flagellar rotation, counterclockwise (CCW) or clockwise (CW) (Mathews *et al.*, 1998; Parkinson & Kofoid, 1993; Welch *et al.*, 1993). On the other hand, the stator complex consists of MotA and MotB, which interact via their transmembrane regions and function as a proton conducting channel (Blair & Berg, 1990; Stolz & Berg, 1991). MotA has four transmembrane regions and a large cytoplasmic loop. It was proposed that two highly conserved proline residues (Pro173 and Pro222) at the cytoplasmic ends of transmembrane segments 3 and 4 play a role in coupling proton flow to motor rotation and in regulating the conformation and/or conformational changes of stator complex (Braun *et al.*, 1999). MotB has a single transmembrane region including an important Asp which seems to be involved in proton transfer (Sharp *et al.*, 1995b; Zhou *et al.*, 1998b). Moreover, a peptidoglycan binding motif (PGB) is conserved in the C-terminal region of MotB and plays a role of anchoring the motor to the cell wall (Chun & Parkinson, 1988; De Mot & Vanderleyden, 1994). The region between the transmembrane and peptidoglycan binding motif is thought to be a linker. This was also supported by a series of 10 amino acids deletion analysis in *Salmonella* MotB (Muramoto & Macnab, 1998).

It has been proposed that various charged residues of both rotor (FliG) and stator (MotA) proteins are important for motor function in *E. coli* (Lloyd & Blair, 1997; Zhou & Blair, 1997). In FliG, the residues Arg281, Asp288 and Asp289 are of primary importance for function. In MotA, the charged residues most important

Fig. I-4. Critical residues involved in the force generation of the flagellar motor of *E. coli*. FliG and MotA have charged residues essential for the motor rotation on the rotor-stator interface. Several combinations of charged-residue substitutions in FliG and MotA exhibit strong combined effects on the motility, suggesting that electrostatic interactions might occur between rotor (FliG) and stator (MotA). Solid lines indicate the primary electrostatic interactions, and broken lines show interactions that are of secondary importance. The segment containing residues 281 through 289 in FliG is postulated to form an α Helix, in which Arg281, Asp288 and Asp289 are close to each other on one surface. The other important residues involved in force generation of the motor (Pro173 and Pro222 in MotA and Asp32 in MotB) are also indicated. This figure is modified from Lloyd & Blair (1997), Zhou & Blair (1997), Zhou *et al.*, (1998a).

for the function are Arg90 and Glu98. Single charge-reversing replacements for those residues impaired the function severely. However, several combinations of FliG mutations with MotA mutations showed strong suppression (Zhou *et al.*, 1998a). This result suggests that these electrostatic interactions between rotor and stator are important for the motor rotation. Recently, the structure of the C-terminus of FliG was resolved (Lloyd *et al.*, 1999). Arg281, Asp288 and Asp289 are lined along a prominent ridge on the surface of protein.

The photosynthetic bacterium *Rhodobacter sphaeroides* has also proton driven motors and a single flagellum extending from the center of the cell body. The cell is motile under a wide range of growth conditions, pH 6 to 9 (Packer *et al.*, 1994) and at speeds of up to 100 μm/s (Poole *et al.*, 1988). The rotation of *R. sphaeroides* flagellum is unidirectional in the CW direction, and stops and restarts periodically (Armitage & Macnab, 1987). The flagella motor of *R. sphaeroides* is active in the absence of sodium ion, and is inhibited by the protonophore CCCP (Packer *et al.*, 1994). Amiloride, a specific inhibitor of the sodium-driven flagellar motor inhibits the motility, however, the effect has been shown to be non-specific. The motor genes, *fliG*, *fliM*, *fliN*, *motA* and *motB* were identified, which are similar to those of the other proton driven motors (Garcia *et al.*, 1998; Shah *et al.*, 1995; Shah & Sockett, 1995). The important residues involved in torque generation are conserved.

The marine bacteria, *Vibrio alginolyticus* and *V. parahaemolyticus,* have proton-driven lateral flagella as well as sodium-driven polar flagella working better for swimming in low-viscosity environments (Atsumi *et al.*, 1992b; Kawagishi *et al.*, 1995). The polar flagella extend from one pole of the cell body and single per cell. The diameter of the sheathed flagella filament is ca. 30 nm and is greater than that of unsheathed flagella in other species (Allen & Baumann, 1971; Follett, 1963).

Thus, the rotation speeds of the polar flagella of *V. alginolyticus* can be measured easily by laser dark-field microscopy (LDM). It has been shown that the rotation speeds of polar flagella are very fast, about 1,700 rps in 300 mM NaCl at 35 °C (Magariyama *et al.*, 1995; Magariyama *et al.*, 1994; Muramoto *et al.*, 1995). Moreover, the sodium driven motor has the other advantages for experimental analysis, since sodium-motive force can be manipulated more readily than proton-motive force (Imae, 1991; Imae & Atsumi, 1989; Yoshida *et al.*, 1990). Amiloride and its analogues, phenamil, benzamil and iodoamiloride which specifically and strongly inhibit the polar-flagella motors, are available and are thought to interact with a sodium channel of the flagellar motor (Atsumi *et al.*, 1992a; Atsumi *et al.*, 1990; Sugiyama *et al.*, 1988). Five to 9 steps were observed in Na^+-driven motors when motors were progressing slowed by a photoactivated inhibitor, iodoamiloride (Muramoto *et al.*, 1994).

The *motX* and *motY* genes, which seem to encode components of the force-generating units of the Na^+ motor, were isolated from *Vibrio parahaemolyticus* (McCarter, 1994a; McCarter, 1994b). However, the predicted products have no homology to MotA and MotB which are components of the force-generating units of H^+ motors and are thought to form a H^+ channel (Blair & Berg, 1990; Stolz & Berg, 1991). On the other hand, the C-terminal domains of MotY and MotB do share sequence similarities to peptidoglycan-interacting proteins (McCarter, 1994b). MotX is inferred to be a component of the Na^+ channel of the motor because overproduction of MotX was lethal to *E. coli* in proportion to the external Na^+ concentration, and this effect was suppressed by the addition of amiloride (McCarter, 1994a). Genes identical to *motX* and *motY* of Na^+ motor genes were also detected in *V. alginolyticus* (Okunishi *et al.*, 1996; unpublished data). So, it had been thought that the force-generating units of H^+ and Na^+ motor were very

different. MotX and MotY of the Na^+ motor, each of which has a putative single transmembrane region, were thought to form a channel component and/or a stator component, functionally homologous to the H^+ motor components of MotA and MotB. However, in this study, I isolated novel *pom* (polar flagella motility) genes whose predicted products are structurally homologous to the H^+ motor components, MotA and MotB (see chapter II). This shows that the channel components of the Na^+ motor are similar to those of the H^+ motor and that the sodium-driven motor consists of four components, PomA, PomB, MotX and MotY (Fig. II-4).

While I progressed with this study after I cloned the *pomA* and *pomB* genes, the other studies on the genes were carried. The mutations conferring resistance to phenamil, were found in both *pomA* and *pomB* in *V. alginolyticus*, and in their homologs of *V. parahaemolyticus* (Jaques *et al.*, 1999; Kojima *et al.*, 1999a). The mutations of both subunits are near the cytoplasmic ends of the putative transmembrane segments. This shows that phenamil interacts directly with the Na^+ motor components. Recently, it was reported that PomA and PomB of *Vibrio alginolyticus* were purified and reconstituted into proteoliposome, and that they showed $^{22}Na^+$ uptake activity into proteoliposome (Sato & Homma, 1999). This evidence supports strongly that PomA and PomB form a sodium conducting channel.

In this thesis, I purposed to reveal the mechanism of torque generation of a sodium driven motor and the mechanism of recognition of coupling ions. I started to try cloning the novel genes of putative flagellar motor channel components (chapter II). Furthermore, I investigated the function of periplasmic loop regions of PomA (chapter III) and compared the sodium type components to the proton type's in order to identify the binding site of the coupling ion (chapter IV and V).

Chapter II Cloning and characterization of sodium driven motor genes, homologous to proton type, *motA* and *motB*.

Until then, two sodium-driven motor genes, *motX* and *motY*, have been identified in *V. parahaemolyticus* and *V. alginolyticus*. The predicted products have no homology to MotA and MotB which are components of the force-generating units of H^+ motors. So, it had been thought that the force-generating units of H^+ and Na^+ motor were very different. In this chapter, I tried cloning novel *pom* genes using the *pom* (polar flagellar motility) mutants which have paralyzed polar flagella (Okunishi *et al.*, 1996).

Cloning of a novel *pom* gene required for polar flagellar rotation. Using a *pom* mutant (VIO586) as a recipient for shot-gun cloning, I cloned a *pom* gene in pYA101 and pYA201 by using *Hin*dIII and *Eco*RI, respectively. When these plasmids were introduced into VIO586 cells, the cells swarmed in the 0.3 % agar VPG plate (Fig. II-1a & II-1b). Cells with pYA201 could swarm in 5 hours, however cells with pYA101 could make only small swarms in 9 hours. pYA101 seemed not to contain the complete gene. Therefore, I used pYA201 for further analysis. The fragment did not complement *motX* and *motY* mutants of *V. parahaemolyticus* or a *motY* mutant of *V. alginolyticus* (data not shown) and the plasmid pIO3 carrying the *motY* gene of *V. alginolyticus* (Okunishi *et al.*, 1996) did not complement the *pom* mutant. So, this gene must be a novel one related to the sodium-driven motor. A 3.2 kb *Eco*RI fragment was subcloned, which still complemented the *pom* mutant, and the resulting plasmid was named pYA202. Next, I made restriction maps of pYA202 and pYA203 (which has the same fragment as pYA202 but in the opposite orientation), and constructed various deletion derivatives. Complementation assays using deletion derivative plasmids

Fig. II-1. Isolation of novel *pom* genes. (a), (b) Swarming abilities of VIO586 cells carrying various plasmids. Fresh colonies were inoculated in 0.3% agar VPG plate containing chloramphenicol and incubated at 30 °C for 5 hr (a) and for 9 hr (b). (c) Restriction map of plasmids. The swarm recovery of VIO586 by transformation of the plasmids in 0.3% agar VPG plate are shown on the right side of the map. The chromosomal DNA fragments were indicated by solid lines. CAT indicates the acetyl transferase gene from the vector pSU21. A plasmid (pYA2021) containing a 2.7 kb *Hin*dIII-*Eco*RI fragment made small swarms after a long incubation (-*). Probably the functional *pom* gene was formed by homologous recombination between the mutant *pom* gene on chromosome and a part of the *pom* gene on the plasmid. Restriction sites are shown as: E, *Eco*RI; H, *Hin*dIII; Sc, *Sac*I; Xb, *Xba*I.

revealed that the *pom* gene was located in the 1.45 kb *Xba*I-*Eco*RI fragment containing the *Hin*dIII site (Fig. II-1c). The plasmid containing the fragment is pYA2032. After sequencing the fragment (see below), I found that it also contained part of a second *pom* gene. To clone the whole gene, shot-gun cloning and screening were done using *Sac*I which cut out ca. 3.3 kb chromosomal fragment which hybridized to the 1.45 kb *Xba*I-*Eco*RI fragment (data not shown). Plasmid pHK2, containing the ca. 3.3 kb *Sac*I fragment, complemented the mutation of VIO586.

Nucleotide sequence of the *pom* genes. I determined the nucleotide sequence of the 2196 bp *Xba*I-*Sac*I fragment and identified two open reading frames (accession number AB004068 in DDBJ), which I named *pomA* and *pomB*; they encode proteins containing 253 and 314 amino acids, respectively. A consensus of ribosome binding sites was found at 12 and 11 bp upstream from the start codons of *pomA* and *pomB*, respectively (Fig. II-2a & II-2b). There are only 15 bp between the two genes (Fig. II-2b). Thirty-five bp downstream of the stop codon of *pomB*, there is a consensus of ρ-independent transcription termination site (Fig. II-2c). A potential promoter sequence of TAAA(N_{15})GTCGATAC, which is very similar to σ^{28} promoter consensus of TAAA(N_{15})GCCGATAA of *Salmonella typhimurium* and *Escherichia coli* (McCarter, 1995; Okunishi *et al.*, 1996), was found 67 bp upstream of the start codon (Fig. II-2a). Thus, the two genes seem to constitute a single transcription unit. A plasmid pHK3 (Fig. II-1c) containing only the *pomB* gene complemented the *pom* strains of NMB104, NMB152, and NMB161. This indicates that the three strains are *pomB* mutants and PomB is actually required for polar flagellar motility.

Comparison of PomA and PomB to MotA and MotB of H^+ motor. PomA and PomB protein were found to be homologous to the proton-driven motor

(a)

```
                                                                  -35
241  GGTAGTTAAGTGTAATTTGTGATCCATTAGCTAAGATTTTTCTAAAAATTCTGCGCGAGT
       -10                                                   SD
301  TGTCGATACAGGTAATAATCCGCCCAATGGCAGTATTTGAGTATAGCGTGAGGAGTGCTT

361  TGTGGATTTAGCAACCCTATTAGGTTTGATTGGTGGTTTTGCATTCGTCATTATGGCGAT
      V  D  L  A  T  L  L  G  L  I  G  G  F  A  F  V  I  M  A  M
```

(b)

```
                                                        SD
1081 CTACCTCAATGAAGGTAAACGTGCCCTTGAGATTGACGAGTAACTTGGAGAGTCGTGATG
      Y  L  N  E  G  K  R  A  L  E  I  D  E  *                M

1141 GATGATGAAGATAACAAATGCGATTGTCCGCCACCTGGCCTCCCGTTATGGATGGGGACA
     D  D  E  D  N  K  C  D  C  P  P  P  G  L  P  L  W  M  G  T
```

(c)

```
2041 CAAGGCGAGCCTCTCTACAGCGAAGAAGTGCCGGTAATTCAATAACTAGGCTTTAGTTTT
     Q  G  E  P  L  Y  S  E  E  V  P  V  I  Q  *

2101 AAGTCAGTAAAGAGATAAGAAAGCGAGCAGATTGCTCGCTTTTTTTGTTTTTCCAATCCG

2161 CTTGGGTGTATAATCTTGCGCCTTAAAAGTGAGCTC
```

Fig. II-2. The promoter region of *pomA* (a), the non-coding region between *pomA* and *pomB* (b), and the terminater region of *pomB* (c) in the 2,196 bp *Xba*I-*Sac*I fragment. The sequences enclosed by boxes indicate the predicted promoter region, Shine-Dalgano (SD) sequences and start codons. The complementary sequences are shown by arrows.

proteins, MotA and MotB, respectively, of *Rhodobacter sphaeroides*, *Bacillus subtilis* and *E. coli* (Fig. II-3). The hydropathy profiles of PomA and PomB are very similar to those of MotA and MotB of *E. coli* (data not shown), which have been shown to have four and one membrane-spanning helices, respectively. As with *motA* and *motB* in *E. coli* or *B. subtilis*, *pomA* and *pomB* are in the same operon and have a σ^{28} type promoter sequence upstream. In *E. coli*, *cheA* and *cheW* reside downstream of the *mot* genes in the same operon. In *V. alginolyticus* as in *R. sphaeroides*, the operon contains only the two motor genes. Upstream of *pomA*, I found genes homologous to *E. coli xseB* and *ispA*, which encode the exodeoxyribonuclease small subunit and the farnesyl diphosphate synthase (Fujisaki *et al.*, 1990), respectively. The other sodium motor genes, *motX* and *motY*, are localized elsewhere on the chromosome (McCarter, 1994a; McCarter, 1994b; Okunishi *et al.*, 1996).

Fig. II-3a shows the alignments of PomA to various MotA sequences. The four membrane-spanning regions, especially the 3rd and 4th, are more similar than the other regions and, in MotA, are thought to form the proton channel. It has been shown that non-motile or severe *mot* mutations are localized in the four membrane-spanning regions of MotA of *E. coli* (Blair & Berg, 1991). PomA is more similar to MotA of *B. subtilis* than to MotA of *E. coli*, even though the latter is phylogenetically closer to *V. alginolyticus*. PomA and *R. sphaeroides* MotA are extremely similar and contain the same number of residues (Shah & Sockett, 1995). It would be interesting to see whether these motor components are functionally interchangeable. For example, the channel components of proton type and sodium type F_0F_1 ATPases are similar and it has been shown that the substitution of only two amino acids changes the ion specificity of F_0F_1 ATPase (Kaim & Dimroth, 1995).

(a)

```
VaPomA     1:    MDLATLLGLIGGFAFVIMAMVLGGSIG---MFVDVTSILIVVGGSIFVVLMKFTMGQ
RsMotA     1:    MDIAAAIGLIGAIVMVVGSMIYAGGVA---PFVDIPSLVIVVAGTAFIVLAMKPLPV
BsProA     1:MKRFDYLTPVGFVLGTIIIVIGIISGSGVSGFRSFLDLTSFFIVTGGLCAAVFISFPPSE
BsMotA     1:    MDKTSLIGIILAFVALSVGMVLKG--VSFSALANPAAILIIIAGTISAVVIAFPTKE
VpLafT     1:      MQKFLGVLTILVCVFGGYMWAGG--KLGAIWQPAEFLIIIGAAAGSLIIGNPPHV
EcMotA     1:      MLILLGYLVVLGTVFGGYLMTGG--SLGALYQPAELVIIAGAGIGSFIVGNNGKA

VaPomA    55:FFGATKIAGKAFMFKAD-EP--EDLIAKIVEMADAARKGGFLALEEMEINNT--------
RsMotA    55:FLGHFKAMMKVFKPSRF-DM--NEVISTMVELSNLARKDGIMALEG-KAVPDA-------
BsProA    61:LKKAPSVLKQAFIRQED-NV--KDLVKTFVSLSDHARKHGLLSLDDQAREIKDP------
BsMotA    56:IKKVPTLFRVLFKENKQLTI--EELIPMFSEWAQLARREGLLALEASIEDVDDA------
VpLafT    54:LKEMRQQVPATIKGPTEEYEYYMELMALLNNLLETARSRGFKFLDSHIEAPEQSSIFLMY
EcMotA    54:IKGTLKALPLLFRRSKYTKAMYMDLLALLYRLMAKSRQMGMFSLERDIENPRESEIFASY

VaPomA   104:-----------FMQKGIDLLVDGHDADV-VRAALKKDIALTDERHTQGTGVFRAFGDVAP
RsMotA   104:-----------FFEKGLQLLVDGTDEAK-LVKQLKYEIKAMKARHEAYQGAVKAWIDIGP
BsProA   112:-----------FLKKGLLLAIDGWDEET-IRLVMDSEIAAMEERHRKGRRVFEKAGEFAP
BsMotA   108:-----------FLKNGLSMAVDGQSAEF-IRDIMTEEVEAMEDRHQAGAAIFTQAGTYAP
VpLafT   114:PLVSEDHRLISFITDNLRLMAMGQMSPHELEGLLEQEIEAIQNELLLPSRSLQRTAEALP
EcMotA   114:PRILADSVMLDFIVDYLRLIISGHMNTFEIEALMDEEIETHESEAEVPANSLALVGDSLP

VaPomA   152:AMGMIGTLVGLVAMLSNMDDPKA-IGPAMAVALLTTLYGAILSNMVFFPIADKLSLRRDQ
RsMotA   152:AMGMVGTLIGLVLMLGNMSDPKS-IGPAMAVALLTTLYGALMANVIFAPILNKLEGYSAD
BsProA   160:AWGMIGTLVGLVLMLKNLNDPHM-LGPNMAIALLTTLYGSLLANMVFNPIAAKLEEKTES
BsMotA   156:TLGVLGAVIGLIAALSHMDNTDE-LGHAISAAFVATLLGIFTGYVLWHPFANKLKRKSKQ
VpLafT   174:GFGILAAVGGIIITMQAIDGSIALIGYHVAAALVGTFIGIFGCYCGLDPLSNAMAQRVKR
EcMotA   174:AFGIVAAVMGVVHALGSADRPAAELGALIAHAMVGTFLGILLAYGFISPLATVLRQKSAE

VaPomA   211:ETLNRRLIMDGVLAIQDGQNPRVIDSYLKNYLNEGKRALEIDE
RsMotA   211:EVTYRELVIEGLRGIARGESARMIEDQMVCALDRKQQMKRKAA
BsProA   219:EIFIKQVMVEGIIGVQSGKNPRNLESQLVVFSSREEWQKQPKQVKTKKGSVHEA
BsMotA   215:EVKLREVMIEGVLSVLEGQAPKVIEQKLLMYLPAKDRLKFAEQGEAQNGEKKEEEA
VpLafT   234:NMTAFECVRATLVAYVAKKPTLLAIDAGRKHIQLDIKPTFNQMEKWLAEQEG
EcMotA   234:TSKMMQCVKVTLLSNLNGYAPPIAVEFGRKTLYSSERPSFIELEEHVRAVKNPQQQTTTE

EcMotA   294:EA
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Fig. II-3. Amino acid alignments of PomA (a) and PomB (b) against homologous proteins of various species. Dotted bars indicated membrane spanning regions of *E. coli* MotA and MotB. Abbreviations: VaPomA, *V. alginolyticus* PomA; RsMotA, *R. sphaeroides* MotA; BsProA, *B. subtilis* Hypothetical ProteinA; BsMotA, *B. subtilis* MotA; VpLafT, *V. parahaemolyticus* LafT; EcMotA, *E. coli* MotA; VaPomB, *V. alginolyticus* PomB; RsMotB, *R. sphaeroides* MotB; BsProB, *B. subtilis* Hypothetical ProteinB; BsMotB, *B. subtilis* MotB; VpLafU, *V. parahaemolyticus* LafU; EcMotB, *E. coli* MotB. White letters in blackboxes show identical residues.

(b)

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VaPomB     1 :                   MDDEDNKC-DCPPPGLPLWMGTFADLMSLLMCFFVLLLSFSEM
RsMotB     1 :   MSAKPKVIRFQPPVPDDDEGEDCPKCPPPGAPAWLATFADIATNLMAFFVLILGFAKF
BsProB     1 :    MKLRRERFERRNGSGKNSQSSS---------SWMVTFTDLITLILVFFILLFSMSQI
BsMotB     1 :          MARKKKKKHEDEHVDE---------SWLVPYADILTLLLALFIVLYASSSI
VpLafU     1 :   MQKQEHVVFKRAKAHGHDEPHGG---------AWKVAFADFMIALMALFLVLWVMQVV
EcMotB     1 :MKNQAHPIIVVKRRKAKSHGAAHG----------SWKIAYADFMTAMMAFFLVMWLISIS

VaPomB    43 :DVLKFKQIAGSMKFAFGVQNQLEVKDIPKGTSIIAQEFRPGRPEPTPIDVIMQQTMDITQ
RsMotB    59 :DEPSFSKMAGAMRETFGFHSIRDATSGNTMIDFGLPTADPDGAQPDEKSDTGGSEDGGDA
BsProB    49 :DLQKFKAAVDSIQKEGNG-------------------------------------LQPD
BsMotB    43 :DAAKFQMLSKSFNEVFTG----------------GTGVLDYSSVTPPENESDGIDEVKKE
VpLafU    50 :DKEERKAIVAHLHSSSVF--------------------DKSYGNPFDTSQSISPIDLAQD
EcMotB    51 :SPKELIQIAEYFRTPLAT---------------AVTGGDRISNSESPIPGGGDDYTQSQG

VaPomB   103 :QTLEFHEGESDRAGGTKRDEGKLTGGQSPATSTQNNESAEADMQQQQSKEMSQEMETLME
RsMotB   119 :AERVAEALKKALEDGKLQVRSDEGEVVIELSGEDGRQQAQSLARALAETAGLGPLPEPQT
BsProB    71 :QTSIEKKNTSPSDTKKQEDQQDQL-------------------LKKVNTYIKDNHLKAQM
BsMotB    87 :KEEKEKNKKEKEKAADQEELENVK--------------------SQVEKFIKDKKLEHQL
VpLafU    90 :SSVPSKHNSNHVVSSYFQGDGDGPEINSLVPGTFDTQEQLAALAKVIEEMTAQINAQGNV
EcMotB    96 :EVNKQPNIEELKKRMEQSRLRKLR----------------GDLDQLIESDPKLRALRPHL

VaPomB   163 :SIKKALEREIEQGAIEVENLGQQIVIRMREKGAFPEGSAFLQPKFRPLVRQIAELVKDVP
RsMotB   179 :TSSRGPSRRSGRRAPERVTGARAARWRRHGRCAAPVGAGRTRCAPAAQCARPRSGRGAGE
BsProB   112 :TAKRDERGVVLVLQEAVLF--------------DTGEAKVLKNAETLLHQIAVLLQTI-P
BsMotB   127 :ETKMTSEGLLITIKDSIF--------------FDSGKATIRKEDVPLAKEISNLLVINPP
VpLafU   150 :NVTVTPQGLRIVLQDDY------------KQHMFSRGGAELTPFFEDLLLALAPLFEQVT
EcMotB   140 :KIDLVQEGLRIQIIDSQ-----------NRPMFRTGSADVEPYMRDILRAIAPVLNGI-P

VaPomB   223 :GIVRVSGHTDNRPLDSELYR-SNWDLSSQRAVSVAQEMEKVRGFSHQRLRVRGMADTEPL
RsMotB   239 :GGADRRQGVREPRRGADPSLRLRRPHARCARGHGPDCRGHAQSRTHHHRDGSYRQCPVWA
BsProB   157 :NDIQVEGHTDSRNISTYRYP-SNWELSAARASGVIQYFTSKEKLPSKRFIAVGYADTKPV
BsMotB   173 :RNIIISGHTDNMPIKNSEFQ-SNWHLSVMRAVNFMGLLIENPKLDAKVFSAKGYGEYKPV
VpLafU   198 :NPLIISGHTDAIPFKKRFGRQSNWALSASRADVARKTLVEGGMPDDRVMQVTGMSDRALL
EcMotB   188 :NRISLSGHTDDFPYASGEKGYSNWELSADRANASRRELMVGGLDSGKVLRVVGMAATMRL

VaPomB   282 :LPNDSDDNRALNRRVEISIMQGEPLYSEEVPVIQ
RsMotB   299 :APSGTISRSPPGAPQAWCASLSPRAASIPDRSPR
BsProB   216 :KDNKTNEHMKENRRVEIVIKKSKTTSS
BsMotB   232 :ASNKTAEGRSKNRRVEVLILPRGAAETNEK
VpLafU   258 :NPDEPDSSE--NRRIELFILTTPAAKVLETLFGNQDDSELQKAKQKAEFNQPVIRQEVIR
EcMotB   248 :SDRGPDDAV--NRRISLLVLNKQAEQAILHENAESQNEPVSALEKPEVAPQVSVPTMPSA

VpLafU   316 :YSADAEKQEAKIQAL
EcMotB   306 :EPR
```

PomB consists of 314 amino acids and the N-terminal and the C-terminal regions are similar to those of MotB in other species (Fig. II-3b). In those C-terminal regions of *V. alginolyticus* and *E. coli*, the peptidoglycan-binding motif is highly conserved but *R. sphaeroides* does not have the motif (Shah *et al.*, 1995) (The sequence of *R. sphaeroides* MotB was corrected, and it was found that RsMotB has the motif in the C terminus as described below). The membrane-spanning region at the N terminus of MotB is thought to complex with the transmembrane regions of MotA and form a part of the proton channel (Sharp *et al.*, 1995b). The N-terminal regions of PomB and *R. sphaeroides* MotB are very similar (Shah *et al.*, 1995). These results suggest that PomB also has only one membrane-spanning region and interacts with the cell wall peptidoglycan via its C-terminal domain.

Flagellar motor proteins

Proton driven	Sodium driven		Function
E. coli	*V. alginolyticus*	*V. parahaemolyticus*	
MotA	PomA		Channel?
MotB	PomB		Stator/Channel?
	MotX	MotX	Channel?
	MotY	MotY	Stator?

Fig. II-4. Motor genes of membrane components for the proton-driven and sodium-driven type flagella and the predicted membrane topology of the four *pom* gene products.

Chapter III Cysteine-scanning mutagenesis of the periplasmic loop regions of PomA, a putative channel component of the sodium-driven flagellar motor.

On the basis of homologies to MotA and MotB of proton-driven motors, it is believed that PomA and PomB make a complex and function as a sodium channel (chapter II; Yorimitsu *et al.*, 1999). PomA and MotA are predicted to have two short loops in the periplasm (chapter II; Zhou *et al.*, 1995). The loop regions may help to maintain the arrangement of the membrane-spanning helices that form the ion channel, guide the coupling ion into the channel, or interact with the coupling ions, Na^+ and H^+, or with other proteins, such as PomB or MotB, respectively. In this chapter, I investigated the function of these loop regions of PomA by performing systematic cysteine-scanning mutagenesis and characterizing the motility of the Cys mutants and the binding effects of sulfhydryl reagents on the mutant's motility. Moreover, the combination of the Cys mutagenesis and the chemical labeling gave the topographical information on periplasmic loops of PomA.

Construction and effects on the swarming ability of the Cys substituted cells. Mutations were introduced into the $loop_{1-2}$ and $loop_{3-4}$ segments of PomA, defined as the regions from V21 to L36 and from S167 to A180, respectively, by using a PCR method (Fig. III-1). The mutations were confirmed by sequencing one or the other DNA strand of the *pomA* region. Sixteen mutations were made in $loop_{1-2}$ and 14 mutations were made in $loop_{3-4}$. In some cases, a silent mutation or a mutation in the non-coding region was also present, however, those mutations did not affect the phenotype of motility. In addition to the periplasmic loop regions, Cys substitutions were generated in the

Fig. III-1. Model of the membrane topology of PomA. Positions substituted by Cys are shown as large circles. The reactivity with biotin maleimide are indicated: solid black circle, strong reactivity; striped circle, quite week reactivity; open large circle, no reactivity. Relative swarm sizes in the mutants are indicated by the shaded circles. Where no shaded circle is given, swarm size was similar to that of the wild-type and was not affected by DTNB. Inhibition or lack of inhibition by DTNB is indicated by the + or - symbols, respectively, in the circles.

fourth transmembrane segment (T186C) and in the predicted cytoplasmic regions (D71C, D148C, and D202C). The four mutants are thought to be the references against the periplasmic mutants. Furthermore, the cytoplasmic charged residues might have an important role for the channel function.

The mutant plasmids were introduced into the *pomA* mutant strain (VIO586), and the motility of the transformed cells was examined by measuring swarm expansion rates in soft-agar plates with or without DTNB (Fig. III-2, Table III-1). It is known that DTNB, a sulfhydryl (SH) modifying reagent, is membrane impermeable and causes no significant damage to cellular functions. None of the substitutions in the $loop_{1-2}$ abolished swarming ability, although G23C, S25C, and D31C seemed to impair swarming. DTNB reduced the swarm rate of mutants containing the G23C, G24C, F29C, D31C, and T34C substitutions. The swarming ability of the F29C mutant was almost completely inhibited by DTNB. On the other hand, several Cys replacements in $loop_{3-4}$ severely impaired swarming. The M179C mutant did not form any swarm and the P172C mutant produced significant swarms only after prolonged incubation. In the presence of DTNB, swarm-expansion rates were reduced in the mutants M169C, D170C, K173C, I175C, G176C, and G180C. The I175C mutant seemed to be more affected by the SH-modifying reagent than the others.

The T186C substitution, which alters the putative fourth transmembrane segment, eliminated swarming. Residue T186 of PomA corresponds to T209 of *E. coli* MotA, which is a conserved polar residue that might form part of the proton channel (Sharp *et al.*, 1995a). The mutations D71C, D148C, and D202C, which are located in regions predicted to be the cytoplasm, did not affect swarming even in the presence of DTNB.

Effects of the Cys substitutions on the swimming speed of cells.

Fig. III-2. Swarm profiles of the Cys mutants. Fresh colonies were inoculated in triplicate into 0.25% agar-VPG plates containing kanamycin without (a) and with (b) 500 μM DTNB and incubated at 30 ˚C for 4 h. The mutations are indicated on the sides. The unlabeled non-swarming colonies contain only the vector plasmid (pSU41). pYA301 is a plasmid expressing the wild-type PomA.

Table III-1. Swarm size and swimming speed of the mutants

No.	swarm size[a]		swimming speed (μm/s)[b]		
	none	+DTNB	none	+ DTNB	+ NEM
none	+++	+++	82	82	77
V21C	+++	+++	76	76	77
L22C	+++	+++	67	69	67
G23C	++	+	45	31	25
G24C	+++	++	68	49	37
S25C	++	++	43	39	33
I26C	+++	+++	77	74	74
G27C	+++	+++	73	72	72
M28C	+++	+++	75	73	74
F29C	+++	±	62	50	48
V30C	+++	+++	82	79	80
D31C	++	+	32	17	16
V32C	+++	+++	81	80	78
T33C	+++	+++	64	57	48
S34C	+++	++	58	57	43
I35C	+++	+++	73	76	75
L36C	+++	+++	73	74	69
S167C	+++	+++	77	69	60
N168C	+++	+++	79	78	75
M169C	+	±	48	16	5
D170C	++	+	52	26	37
D171C	+	+	20	17	12
P172C	±	±	ND[c]	ND	ND
K173C	+	±	25	24	28
A174C	+++	+++	76	72	59
I175C	++	±	22	0	0
G176C	+++	++	40	25	17
P177C	+++	+++	34	30	34
A178C	+++	+++	50	23	13
M179C	-	-	0	—	—
A180C	+++	++	71	72	62
T186C	-	-	0	—	—
D71C	+++	+++	82	84	73
D148C	+++	+++	64	61	42
D202C	+++	+++	77	75	68

[a]The swarm size was classified into 5 types (+++, ++, +, ±, -) from Fig. III-2. The mutations whose swarm ability was detected by the prolonged incubation were shown by ±. [b]The average swimming speeds were obtained by measuring at least 20 swimming tracks of cells generated from the integrated video images. [c]ND, not determined. In the P172C mutant, the motile fraction was too low to measure the swimming speed.

NMB188 (PomA$^-$, Che$^-$) cells, which have a smooth swimming mutation to measure swimming speed more easily (Kojima *et al.*, 1999a), were transformed with the mutant plasmids. The transformed cells harvested in mid-log phase were measured in buffer containing 300 mM Na^+ with no SH-modifying reagent, 500 μM DTNB, or 200 μM NEM. In contrast to DTNB, NEM is membrane permeable and the reaction is irreversible. The effects of the mutations on swimming and swarming were correlated well with each other, with a few exceptions (Fig. III-2 and Table III-1). Such exceptions are not surprising because swarming ability is affected by growth and chemotactic behavior. Swarming was severely impaired by the F29C substitution in the presence of DTNB, but the swimming speed was not. Correspondingly, I found that Cys-29 is modified slowly by the SH-modifying reagents (Fig. III-3). In most of the mutants, swimming speed was dropped immediately after the addition of the SH-modifying reagents, but this was not true of F29C cells, which slowed down over the course of about 10 min after the addition of the SH-modifying reagents. Motility was restored to its original level in this mutant by the addition of a reducing agent, DTT. In contrast, cells of the I175C mutant were stopped immediately and completely by 500 μM DTNB or 200 μM NEM, and their swimming speeds were decreased with the concentration of the reagents (Fig. III-4). The slow reaction of Cys-29 is consistent with the following result the $loop_{1-2}$ region did not labelled by biotin maleimide whose molecular weight is larger than that of DTNB or NEM.

Detection of the mutant PomA proteins. The levels of PomA proteins were determined for *pomA* cells (NMB188) transformed with plasmids containing the mutant *pomA* genes that conferred impaired motility. Total cell proteins was separated by SDS-PAGE, and immunoblotting was performed by using an antibody raised against PomA peptides. I could detect the comparable amount of Cys

Fig. III-3. Time course of reaction of the F29C mutant with the SH-modifying reagent DTNB. Cells from a 1 ml culture of strain NMB188 containing a plasmid expressing the F29C protein were harvested in late logarithmic phase and suspended in 200 μl of TMN50. This suspension was diluted 10 fold by TMN50 with (■) or without (□) 500 μM DTNB. After various times, the diluted suspension was diluted 50 fold more into TMN300 buffer, and the swimming speed was measured within 1 min after the dilution. At 25 min, DTT was added to 1 mM final concentration, and the swimming speed was measured after 5 min.

Fig. III-4. Effect of varying the concentration of the SH-modifying reagents. NMB188 cells containing a plasmid expressing the I175C protein were harvested as described in the legend to Fig. III-3. The suspension was diluted 10 fold into TMN50 containing the indicated concentrations of the SH-modifying reagent DTNB (□) or NEM (●). At various times, the cell suspension was diluted 50 fold more with TMN300, and the swimming speed was measured within 1 minute after the dilution.

substituted PomA proteins to the wild-type PomA level (data not shown). Among all of the mutations tested, the amount of PomA was not significantly different in any mutant although the electrophoretic mobilities were slightly different for some mutant proteins (Fig. III-5). These minor changes in mobility and amount were not correlated with the severity of the effects of the mutations on motility.

Labeling with biotin maleimide. Generally, maleimide is thought not to bind to thiol groups buried in the hydrophobic interior of the membrane. To determine which residues is located at each boundaries between a periplasmic loop and a transmembrane region, biotin maleimide was reacted to the Cys-substituted PomA proteins. The results of labeling (a) and immunoblotting (b) were shown in Fig. III-5. Wild type PomA (pYA301) was not biotinylated, because it has no Cys. T186C, which is predicted to be in the interior of TM4, was not labeled, either. In $loop_{3\text{-}4}$, the residues from N168C to A178C were labeled, suggesting that these are exposed to the aqueous phase. P177C was reproducibly labeled weaker than the others. On the other hands, biotin maleimide bound to S167C quite weekly, and did not bind to M179C and A180C at all. It is possible that S167 and M179 are exsisting at the boundaries between TM3 and periplasmic $loop_{3\text{-}4}$, and between $loop_{3\text{-}4}$ and TM4, respectively. I could not detect the biotinylated PomA in any $loop_{1\text{-}2}$ substitutions (Fig. III-5a). The residues of $loop_{1\text{-}2}$ may not be exposed to the periplasm. Three Cys substitutions in the cytoplasmic region were also biotinylated to same extent with periplasmic mutants. This result shows that biotin maleimide can penetrate the membrane of *V. alginolyticus*.

Effect of SH-modifying reagents at different concentrations of sodium ion. The extracellular loop regions might interact with Na^+ as it enters the PomA channel. Therefore, the effect of Na^+ on the reaction rates of

Fig. III-5. Labeling PomA with biotin maleimide. Immunoprecipitated PomA proteins were separated by SDS-PAGE, transferred onto PVDF membrane and detected by chemiluminescence of HRP-conjugated streptavidine (a). PomA was detected with anti-PomA antibody and AP-conjugated second antibody (b). pSU41 is the vector plasmid (none). The pYA301 plasmid carries the wild-type (no Cys) *pomA* gene.

Fig. III-6. The effect of Na^+ on the reactions of the M169C and D170C mutant proteins with SH-modifying reagents. Cells from a 1 ml culture of the wild-type strain (a) and the M169C (b) and D170C (c) mutants were harvested in late logarithmic phase and suspended in 200 μl of TMN50. This suspension (10 μl) was diluted 10 fold by TMN300 with (●) or without 10 μM DTNB (○), and by sodium-free buffer (TMK300) with (■) or without 10 μM DTNB (□). After various times, the diluted suspension was diluted 50 fold more into TMN300, and the swimming speed was measured within 1 min after the dilution. At 15 min, DTT was added to 1 mM.

SH-modifying reagents was investigated with the M169C and D170C mutants (Fig. III-6). The D170C mutant showed a difference in the time course of inhibition by DTNB, when the Na^+ concentration was low or high (Panel c). The M169C mutant did not show any such effect. Inhibitions of the D170C and M169C mutants reached a maximum at 300 mM NaCl in 5 min and 15 min, respectively. Inhibition was relieved by the addition of 1 mM DTT (1,4'-dithiothreitol), and swimming speed was restored to the original level. The Na^+ flow may affect the reactivity of Cys-170 or may change the environment around the Cys residue.

Chapter IV Construction and characterization of hybrid motor with the H^+- and Na^+-driven components in *Vibrio* polar flagella.

As described in Introduction, F_0F_1-ATPase and bacterial flagellar motor have many common features. In F_0F_1-ATPase, the mechanism of the coupling-ion recognition have been studied well and proposed based on comparison of amino acid sequences between the c subunits of proton- and sodium-type (Zhang & Fillingame, 1995).

Ultimately, I thought that it might be possible to compare and study the coupling mechanisms of a proton or a sodium ion flux for force generation of flagellar motors like F_0F_1-ATPase. At first, I tried constructing hybrid motor between proton type and sodium type, and introducing the entire *R. sphaeroides motAB* genes into *pomA* or *pomB* mutants of *V. alginolyticus*, respectively. It is the reason to choose the motor genes of *Rhodobacter sphaeroides* as the proton type that the sequences of A and B subunit of *R. sphaeroides* are most similar to those of *V. alginolyticus*, respectively. *R. sphaeroides* MotA and *V. alginolyticus* PomA are 253 amino acids proteins, and they have ca. 40 % identity over the entire region (Fig. IV-1a). Moreover, the transmembrane regions of MotB and PomB have also high similarity (Fig. II-3b).

Introduction of *R. sphaeroides motA* and *motB* into the *pom* mutants of *V. alginolyticus*. As shown in Fig. IV-1b, in pYA301, pYA303 and pYA701, the *pomA*, *pomAB* and *Rhodobacter motA* genes were controlled under the *lac* promoter directly. pYA603 and pYA6031 include *Rhodobacter motAB* and *motA* genes under the native promoter. These plasmids were introduced into the *pomA* mutant, VIO586 and the *pomB* mutant, NMB161. Fig. IV-2a shows motility of the transformants. The transformed VIO586 was restored

(a)

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VaPomA  1:MDLATLLGLIGGFAFVIMAMVLGGSIGMFVDVTSILIVVGGSIFVVLMKFTM
RsmotA  1:MDIAAAIGLIGAIVMVVGSMIYAGGVAPFVDIPSLVIVVAGTAFIVLAMKPL
                TM1                                  TM2
VaPomA    GQFFGATKIAGKAFMFKADEPEDLIAKIVEMADAARKGGFLALEEMEINNTF
RsmotA    PVFLGHFKAMMKVFKPSRFDMNEVISTMVELSNLARKDGIMALEGKAVPDAF

                                                          ◆
VaPomA    MQKGIDLLVDGHDADVVRAALKKDIALTDERHTQGTGVFRAFGDVAPAMGMI
RsmotA    FEKGLQLLVDGTDEAKLVKQLKYEIKAMKARHEAYQGAVKAWIDIGPAMGMV
                                                          TM3
                                  ★
VaPomA    GTLVGLVAMLSNMDDPKAIGPAMAVALLTTLYGAILSNMVFFPIADKLSLRR
RsmotA    GTLIGLVLMLGNMSDPKSIGPAMAVALLTTLYGALMANVIFAPILNKLEGYS
                                  TM4
VaPomA    DQETLNRRLIMDGVLAIQDGQNPRVIDSYLKNYLNEGKRALEIDE    :253
RsmotA    ADEVTYRELVIEGLRGIARGESARMIEDQMVCALDRKQQMKRKAA    :253
```

(b)

Fig. IV-1. (a) Amino acid alignments of *Vibrio alginolyticus* PomA and *Rhodobacter sphaeroides* MotA. White letters in black boxes, diamond, and star show identical residues, mutation site of PomA mutant VIO586 (G154R) and threonine residue highly conserved among all species of MotA, respectively. Dotted bars indicate putative transmembrane regions. (b) Restriction map of plasmids. White boxes and the direction of arrow heads indicate the vector part of pSU41 and the direction of translation from *lac* promoter, respectively. Insertional fragments were indicated by solid lines. The bold lines of inserted fragments are the region of the native promoter. The open arrows show the coding regions of PomA, PomB, MotA and MotB. Abbreviations: VaPomA, *V. alginolyticus* PomA; RsMotA, *R. sphaeroides* MotA; B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; Sp, *Sph*I; X, *Xho*I; Sm, *Sma*I; Sl, *Sal*I.

to motility by pYA603, pYA6031 (data not shown) and pYA701, which contain the *motA* gene of *R. sphaeroides*, though the swarm size was smaller than that of cells transformed by pYA301, which contain the *pomA* gene. On the other hand, the transformed *pomB* mutants, NMB161 and NMB152 could not have motility restored.

I thought the possibility that the defective PomA of VIO586 might be suppressed by MotA, and identified the *pomA* mutation site of VIO586 by amplifying and sequencing the PCR fragment of the whole *pomAB* operon. Only the G154R mutation was found (diamond in Fig. IV-1a). If nonfunctional PomA proteins were expressed in VIO586, it was also possible that *R. sphaeroides* MotA could 'suppress', not complement, this PomA mutation. Thus, I constructed a *pomA* null mutant to examine the possibility.

The *pomA* deletion allele has been isolated by Kojima *et al.* (Kojima *et al.*, 1999b). Using this mutant *pomA* and suicide vector pKY704, I constructed a strain (NMB190) carrying the chromosomal deletion mutation of *pomA*. Even in this strain, *motA* of pYA701 could restore motility to the same extent as for VIO586 (Fig. IV-2b).

The threonine residue in the fourth transmembrane region (star in Fig. IV-1a) is conserved among sodium type PomA and all of the reported proton type MotAs. This residue corresponds to position 202 in *E. coli*, and 186 in *V. alginolyticus* and *R. sphaeroides*. It was proposed that a mutation like T202W in *E. coli* and T186C or T186I in *V. alginolyticus* would be non-functional (Kojima *et al.*, 1999b; Sharp *et al.*, 1995a). I introduced the T186C mutation into pYA701 and examined if the mutated *R. sphaeroides* MotA could restore motility (Fig. IV-2b). Wild-type MotA (pYA701) restored motility to both *pomA* mutants. In contrast, mutant MotA (pYA701-T186C) did not. Other non-functional amino acid substitutions at highly

Fig. IV-2. Swarming abilities of transformants. Fresh colonies were inoculated in 0.3 % agar VPG plates containing 100 μg/ml kanamycin and incubated at 30 °C for 5 hr. (a) The *pomA* mutant (VIO586) and the *pomB* mutant (NMB161) as the host strain were transformed with the plasmids, pYA301, pYA603, pYA701, pSU41, and pYA603, respectively. Introduced plasmids and the host strains are noted on the left side and right side, respectively. (b) The effect of T186C mutation of *R. sphaeroides* MotA. Two *pomA* mutants, VIO586 and NMB190 were transformed with the plasmids, pYA701 and pYA701-T186C.

conserved residues, T186I and P199L (corresponds to P222 in *E. coli*) (Blair & Berg, 1991; Kojima *et al.*, 1999b; Zhou & Blair, 1997), also did not restore motility (data not shown). These results suggested that *R. sphaeroides* MotA, which is proton-component, could work as a replacement for PomA in *V. alginolyticus*.

Detection of *R. sphaeroides* MotA. I tried to detect the *R. sphaeroides* proteins by immunoblotting using anti-RsMotA synthetic peptide antibody (Fig. IV-3a). A band with a molecular mass of 25 kDa was detected specifically from NMB190 harboring pYA701 (lane 3). The molecular weight of 25 kDa is almost equal to the predicted size of *R. sphaeroides* MotA, 27 kDa. On the other hand, in the case of anti-VaPomA antibody (Fig. IV-3b), no band appeared in NMB190 harboring pYA701 (lane 7), however, the *V. alginolyticus* PomA band could be detected in NMB190 harboring pYA301 (lane 6). From these results, I assessed that this anti-RsMotA antibody could detect specifically *R. sphaeroides* MotA. None of the nonfunctional mutant *R. sphaeroides* MotA proteins could be detected by our system (for example, T186I in lane 4). The mutant proteins might be degraded more rapidly than the wild type, or changed in this antigenicity.

Characterization of the hybrid motor between the H^+- and Na^+-type. NMB190 with pYA701 should have hybrid motors composed of H^+-type MotA and Na^+-type PomB, MotX and MotY. Which ions couple to the flagellar rotation in the motors? I examined this question by several experiments. In the first, I asked whether cells can swim in the Na^+-free buffer, TMK300 and is the swimming speed of cells dependent on the Na^+ concentration. As the results (Fig. IV-4a) show, the hybrid motor (pYA701/NMB190) could not rotate in Na^+-free buffer as the Na^+-type motor (pYA301/NMB190). Moreover, the swimming speed by the hybrid motor increases with the Na^+ concentration, although the maximum

Fig. IV-3. Detection of *R. sphaeroides* MotA protein by the anti-peptide antibody. NMB190 cells harboring each plasmids (lanes 1, 5, pSU41; lanes 2, 6, pYA301; lanes 3, 7, pYA701; lane 4, pYA701-T186I) were cultured till mid-log phase, harvested and suspended into distilled deionized water. Immunoblottings were done using anti-RsMotA antibody (a) and anti-PomA antibody (b).

Fig. IV-4. Swimming speed of NMB190 cells harboring pYA301 (open square) or pYA701 (filled circle). (a) The cells suspended in Tris-motility buffer were diluted 100 fold into the buffer containing various concentration of sodium ion, and the swimming speed was measured. (a') The low Na^+ concentration part of (a) was enlarged up. (b), (c) The swimming speed was measured at various concentration of the specific inhibitor of sodium motor, phenamil (b) or amiloride (c). The sodium concentration was 50 mM in all measurements.

speed was low and saturated at lower Na^+ concentration than that by the Na^+-type motor of pYA301/NMB190. Second, I asked whether the free swimming is inhibited by phenamil, an Na^+ driven motor specific inhibitor? The swimming speeds were measured in various concentration of phenamil (Fig. IV-4b). I found that pYA701/NMB190 cells were inhibited in motility strongly with the increase of the phenamil concentration, and stopped almost completely with 50 μM of phenamil. pYA301/NMB190 cells showed similar results. They were also inhibited in motility by amiloride (Fig. IV-4c). The motility inhibition of pYA701/NMB190 cells by amiloride was competitive with sodium ions as that of pYA301/NMB190 cells though their initial swimming speeds were different (Table IV-1). Finally, I tested a protonophore, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) effects (Table IV-2). In alkaline Tris motility buffer, TMN300 [pH8.5], YM19 (Pof^-Laf^+) cells could swim in the absence of 20 μM CCCP and stop completely in the presence of CCCP, because lateral flagella have proton-driven motors. On the other hand, pYA301/NMB190 and pYA701/NMB190 cells did not stop in the presence of 20 μM CCCP, but both cells stopped completely by the further addition of 20 μM 2-heptyl-4-hydroxyquinoline- *N*-oxide (HQNO), a specific inhibitor of the Na^+ pump (Tokuda & Unemoto, 1982). I concluded from these results that the hybrid motor of pYA701/NMB190 rotates the flagella using Na^+-motive force.

I tested the chemotaxis of pYA701/NMB190, because pYA701/NMB190 formed a very small swarm on semi-soid agar plate even though the swimming speed in liquid medium was not so slow. The tumble frequency of pYA30 1/NMB190 was increased from the basal level after 2 mM phenol was added as repellent (Table IV-3). By the further addition of 20 mM serine as attractant, the tumble frequency decreased to 0.40 sec^{-1} from 3.10 sec^{-1}. In contrast, pYA 701/NMB190 showed smooth-biased swimming as a basal condition and little

Table IV-1. Effect of Na^+ concentration on the inhibition of motility by amiloride

Na^+ conc. (mM)	Amiloride conc. (mM)	Swimming speed ($\mu m \cdot sec^{-1}$)	
		pYA301/NMB190	pYA701/NMB190
10	0	33	23
	0.8	6	2
50	0	54	28
	0.8	18	6
300	0	56	28
	0.8	27	12

Table IV-2. Effect of pH and CCCP on the swimming speed

Na^+ conc. (mM)	pH	CCCP conc. (μM)	Swimming speed ($\mu m \cdot sec^{-1}$)		
			pYA301/NMB190	pYA701/NMB190	YM19
300	7.5	0	63	28	27
		20	10	8	0
	8.5	0	64	26	20
		20	64 (0)[a]	23 (0)[a]	0
0	7.5	0	0	0	22

[a]In the parenthesis, the swimming speed was measured after HQNO (20 μM final) was furter added.

Table IV-3. Chemotaxis of NMB190 cells having pYA301 or pYA701

phenol conc. (mM)	serine conc. (mM)	Tumble frequency (sec^{-1})	
		pYA301/NMB190	pYA701/NMB190
0	0	1.81	0.20
2	0	3.10	0.25
2	20	0.40	0.05

response to either repellent or attractant.

Chapter V Coupling ion specificity of chimeras between H^+- and Na^+-driven motor proteins, MotB and PomB, in *Vibrio* polar flagella.

In the previous chapter, I found that the hybrid motor consisting of *R. sphaeroides* (Rs) MotA, *V. alginolyticus* (Va) PomB, MotX and MotY, can work as a sodium driven motor in *Vibrio* cells. That shows that the A subunits do not determine the ion specificity. The most potent candidate to decide ion selectivity seems to be the B subunits. The B subunits are thought to form ion channel with the A subunits, and include an important Asp residue, which plays a role of ion transfer. In this chapter, I tried to make clear whether the B subunits are involved in the coupling ion recognition. I constructed and characterized the motors, *i.e.* RsMotA or VaPomA and the chimeric B subunit between RsMotB and VaPomB.

Introduction of *R. sphaeroides motB* into the *pom* mutants of *V. alginolyticus*. While I progressed this experiments, I found that the published sequence of *RsmotB* gene contained some misreadings in the half of 3' region. I determined the nucleotide sequence of the whole *motB* gene again. In Fig. V-1a, I show the corrected amino acid sequence. It was found that RsMotB has a peptidoglycan binding motif in the C terminal region as same as all MotBs or PomB. The linker region of RsMotB between the transmembrane region and the peptidoglycan binding motif is longer than the B subunits from other species. The transmembrane region of the proton type *R. sphaeroides* MotB (RsMotB) is very similar to the transmembrane region of the sodium type PomB of *V. alginolyticus*.

As shown in Fig. V-1b, the *pomAB* and *RsmotAB* genes could be controlled from the *lac* promoter in pYA303, pYA3031, pYA703 and pYA7031. I put a *Spe*I site between the stop codon of the A subunit gene and the SD sequence for the B subunit gene of pYA303 and pYA703, to give pYA3031 and pYA7031,

(a)

```
                                                   Junction site 6          site 5
VaPomB    1:                    MDDEDNKC-DCPPPGLPL WMGTFADLMSLLMCFFVLLI
RsMotB    1:MSAKPKVIRFQPPVPDDDEGEDCPKCPPPGAPA WLATFADIATNLMAFFVLIL
                                                                    TM
                 site 4                          site 3
VaPomB   39:FSEMDVLKFKQIAGSMKFAFGVQNQLEVKDIPKGTSIIAQEFRPGRPEPTPID
RsMotB   55:FAKFDEPSFSKMAGAMRETFGFHSIRDATSGNTMIDFGLPTADPDGAQPDEKS

                site 2                          site 1
VaPomB   93:IMQQTMDITQQTLEFHEGESDRAGGTKRDEGKLTGGQSPATSTQNNESAEADM
RsMotB  109:TGGSEDGGDAAERVAEALKKALEDGKLQVRSDEGEVVIELSGEDGRQQAQSLA

VaPomB  147:QQQSKEMSQEMETLMESIKKALEREIEQGAIEVENLGQQIVIRMR--------
RsMotB  163:ALAETAGLGPLPEPQTTSQPRPEPKAGPAGPGEGTGAPPGPPVGGDTGAALRQ

                                                   Junction site 0
VaPomB  192:----------------------------------------EKGAFPEGSAFLQPKF
RsMotB  217:VRAELDALRLRNALDREVAEGLVKVEQTDGKVFVSLGAGGSFPSGSDDLTPDA

VaPomB  209:PLVRQIAELVKDVPGIVRVSGHTDNRPLDSELYR-SNWDLSSQRAVSVAQEME
RsMotB  271:AVMARIAEATRNPERTITVTGHTDNVPV-SGGAFRDNIALAAGRAASVVRELV

VaPomB  262:VRGFSHQRLRVRGMADTEPLLPNDSDDNRALNRRVEISIMQGEPLYSEEVPVI
RsMotB  324:SGSVDPGRITAVSRGEFDPVADNATEEGRAQNRRIEIEISYKD
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Fig. V-1(a). Amino acid alignments of *Vibrio alginolyticus* PomB and *Rhodobacter sphaeroides* MotB. White letters in black boxes and arrow heads show identical residues and junction site of chimeric protein, respectively. Dotted bars indicate putative transmembrane regions.

Fig. V-1(b). Restriction map of plasmids. White boxes and the direction of arrow heads indicate the vector part of pSU41 and the direction of translation from *lac* promoter, respectively. Insertional fragments were indicated by a black and grey bold line which are derived from VaPomB and RsMotB, respectively. The shaded part of pRED1 are the region of the native promoter. The open arrows show the coding regions of PomA, PomB, MotA and MotB. Abbreviations: VaPomB, *V. alginolyticus* PomB; RsMotB, *R. sphaeroides* MotB; H, *Hin*dIII; Xb, *Xba*I; B, *Bam*HI; Sc, *Sac*I; E, *Eco*RI; Sp, *Spe*I; Sh, *Sph*I; X, *Xho*I; Sm, *Sma*I; Sl, *Sal*I.

Fig. V-2. Swarming abilities of transformants. Fresh colonies were inoculated in 0.3 % agar VPG plates containing 100 mg/ml kanamycin and incubated at 30 ˚C for 5 hr. The *pomAB* mutant (NMB191) was used as the host strain. Each expressed proteins are noted around swarms.

respectively. I switched the *Spe*I-*Sac*I fragments of pYA3031 and pYA7031,to give pYA3032(*pomA* and *motB*) and pYA7032(*motA* and *pomB*). These plasmids were introduced into the *pomAB* mutant, NMB191. Fig. V-2a shows motility of the transformants. NMB191 was restored to motility by pYA303, pYA3031 and pYA7032. All of these plasmids contain the *pomB* gene. On the other hand, the cells transformed by pYA703 and pYA3032 did not have motility restored. The whole *motB* could not complement the *pomAB* defect of *Vibrio* polar flagella even when PomA or MotA was co-expressed with it. I tested whether the proteins were expressed in non swarming cells. The result was shown in Fig. V-3. In all of swarming cells as well as non swarming cells, the bands of A and B subunits were detected. For example, in NMB191 cells having pYA3032, the bands of PomA and MotB were detected (Fig. V-3a & V-3d). Thus, I concluded that non swarming cells also expressed both subunits, and that MotB could not work in the *Vibrio* cells.

Construction of chimeric proteins between MotB and PomB. I found that the whole MotB could not work in polar flagellar motors of *V. alginolyticus*. Therefore, I constructed two chimeric proteins between the N terminal region of PomB and the C terminal region of MotB, named PotB. The positions of each chimeric junction are shown in Fig. V-1a. Although most of PotB0 and PotB1 is derived from PomB, neither was functional with either MotA or PomA (data not shown). Accordingly, I constructed six chimeric proteins between the N terminal region of MotB and the C terminal region of PomB, named MomB. The swarming abilities of the transformed NMB191 cells co-expressing MotA with each MomB are shown in Fig. V-2b. Three chimeric proteins, MomB3, MomB5 and MomB6, expressed together with MotA could restore the motility of *ΔpomAB* mutant. The swarm size of the strains containing MomB5 and MomB6 are comparable to that of control cells expressing MotA with PomB, although MomB3 makes smaller swarm

Fig. V-3. Detection of each proteins by the anti-peptide antibody. NMB191 cells expressed each proteins were cultured to mid-log phase, harvested and suspended into distilled deionized water. Immunoblottings were done using the anti-VaPomA antibody, PomA91 (a), -VaPomB antibody, PomB93 (b), -RsMotA antibody, RsMotA253 (c) and -RsMotB antibody, RsMotB2 (d).

Table V-1. Characterization of chimeric proteins.

salt (300 mM)	pH	CCCP (μM)	Swimming speed (μm·sec-1)							
			PomAB (pYA303)	PomA+		MotA+			MotA +PomB (pYA7032)	YM19
				MomB5	MomB6	MomB3	MomB5	MomB6		
Na^+	8.5	0	80 (3.8)[a]	11 (0.9)	70 (4.7)	20 (2.3)	26 (2.5)	53 (3.9)	28 (2.0)	20 (1.8)
		20	64 (6.9)	8 (1.3)	51 (4.8)	14 (2.0)	20 (1.9)	42 (3.6)	27 (1.7)	0
	7.5	0	80 (3.7)	10 (1.0)	67 (7.9)	23 (2.2)	31 (5.5)	54 (1.7)	28 (1.7)	27 (1.4)
		20	10 (1.7)	3 (1.5)	6 (0.9)	3 (1.7)	5 (1.0)	5 (2.2)	9.9 (1.5)	0
K^+		0	0	0	0	0	0	0	0	22 (2.4)
Li^+		0	35 (4.5)	24 (2.9)	8.5 (1.2)	L.F.[b]	5.5 (0.7)	L.F.	4.4 (0.8)	24 (2.2)

[a]In the parenthesis, S. D. are shown. [b]L.F.; low motile fraction.

than the others. When these MomB proteins were expressed with PomA, MomB5 and MomB6 were also functional in NMB191 cells. MomB6 could make swarm as big ás the wild type PomB. However, MomB3 could not restore the motility anymore with PomA. The other chimeric proteins, MomB1, MomB2 and MomB4 did not make swarm with either of the A subunits.

Detection of chimeric MomB protein by immunoblotting. I tried to detect MomB proteins by immunoblotting using anti synthetic peptide antibody (Fig. V-3b, d). All MomB proteins could be recognized by both of the anti-RsMotB and -PomB antibodies. In immunoblotting results using each antibody, bands with a similar molecular mass to that of PomB or MotB were detected in NMB191 cells harboring most plasmids expressing MomB proteins. Exceptionally, the MomB2 protein showed the different mobility from the others. This difference in mobility was also observed in different experimental conditions, when co-expressed with the different A subunit, or when using a different antibody, either the RsMotB2 antibody which recognizes the N-terminus of MomB2, or the PomB93 antibody which recognizes the C-terminus of it. I prepared plasmids from cells expressing MomB2 with PomA or MotA, and checked the DNA sequence of the MomB region. However, I could not find any mutations. Thus, I assume that a structural change has occurred in the MomB2 protein.

Characterization of each MomB protein with PomA or MotA. I examined which ions are coupled to generate torque in the motile cells expressing the MomB proteins. As shown in Table V-1, I tested whether cells could swim in Na^+-free buffer, TMK300 containing 300 mM K^+, and whether their motility was affected by a protonophore, carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The results were that none of the functional motors could rotate in the Na^+-free buffer as well as the Na^+-type motor (pYA303/NMB191). Moreover, these motors

Fig. V-4. Swimming speed of NMB191 cells expressing PomAB(pYA303), MomB5 with PomA or MomB6 with PomA. The cells suspended in TMN50 were diluted 100 fold into the Tris motility buffers containing the various concentration of Na^+ (open square) or Li^+ (closed square) as coupling ions, and the swimming speed was measured.

and the motors of pYA303/NMB191 did not stop in TMN300 [pH8.5] with 20 μM CCCP, but stopped completely by the further addition of 20 μM 2-héptyl-4-hydroxyquinoline- *N*-oxide (HQNO), a specific inhibitor of the Na^+ pump (Tokuda & Unemoto, 1982). In alkaline Tris motility buffer, YM19 (Pof^-Laf^+) cells could swim in the absence of CCCP but stopped completely in the presence of 20 μM CCCP, because lateral flagella have proton-driven motors. I concluded from these results that all of the MomB-based motors rotated using Na^+-motive force. When I tried to measure the swimming speeds of the cells expressing each MomB, I could find a few motile cells expressing MomB1 and MomB2 with MotA in TMN300. However, both motile fractions were too low to measure the swimming speeds and to determine their coupling ions.

Li^+ coupling abilities of functional MomB. The wild type sodium driven motor can rotate partially by the Li^+ flux. The maximal swimming speed produced by Li^+ was only about 30 % of that by Na^+ ions (Imae, 1991; Liu *et al.*, 1990). To test the ion utility, I measured the swimming speeds of the MomB cells in 300 mM Li^+ (Table V-1). I could not measure its swimming speed for the *pomAB* strain expressing MomB3 or MomB6 with MotA in TML300, because the motile fractions and the swimming speeds were very low. The motors consisting of MomB5 or MomB6 with PomA showed an interesting character. The cells expressing MomB5 with PomA could swim in TML300 faster than in TMN300. The cells expressing MomB6 with PomA swam much slower than those expressing PomA/B in TML300, although it could swim as fast as those expressing PomA/B in TMN300. In these cells, I examined effect of salt concentration on the swimming speed(Fig. V-4). The cells did not swim without Na^+ or Li^+, and their swimming speeds increased with an increase in either salt concentration. Cells expressing

Fig. V-5. Competition of Na^+ with Li^+ for swimming speed. NMB191 cells expressing PomAB(pYA303), PomA+MomB6, RsMotA+PomB or RsMotA+MomB6 were measured swimming speed in the various concentration of Na^+ with 200 mM K^+(◆) and with 200 mM Li^+ (□). Total salt concentration was adjusted to 300 mM with K^+.

Fig. V-6. The swimming speed was measured at various concentration of the specific inhibitor of sodium motor, phenamil in TMN50. The results of MomB chimeras with RsMotA(upper panel) or with VaPomA(lower panel) were shown.

MomB5 with PomA swam better in the presence of Li^+ than Na^+ at the various concentrations. The maximal swimming speed of cells expressing PomA/B by Li^+ was approximately one third of that produced by Na^+ as reported before. On the other hand, the maximal speed of cells expressing MomB6 with PomA by Li^+ is one tenth of that produced by Na^+. Next, I examined the competitive effect of Li^+ against Na^+, because it is thought that Li^+ share the same binding site as Na^+. I measured the swimming speed in Tris motility buffer, which contains from 0 to 100 mM Na^+ and constant 200 mM K^+ or Li^+. The total salt concentration of every condition was constantly adjusted to 300 mM with K^+ (Fig. V-5). In the presence of Li^+, whose efficiency is lower than that of Na^+, the maximum speed of cells expressing PomA/B decreased to 60 $\mu m \cdot sec^{-1}$, although that was 80 $\mu m \cdot sec^{-1}$ in the absence of Li^+. It was shown that Na^+ and Li^+ compete for the same binding site. In the case of motors consisting of MomB6 with PomA, cells showed a similar result to cells expressing PomA/B without Li^+. However, the maximum speed in the presence of Li^+ decreased to 45 $\mu m \cdot sec^{-1}$. This decrease was more than that of cells expressing PomA/B. Cells expressing MomB6 with MotA gave similar results to those with PomA though the overall swimming speed is lower. These results may suggest that the affinity for Li^+ in MomB6 was changed, compared to that in PomB.

The resistance to inhibitors in cells expressing each MomB. If the ion specificity was changed in the motor composed of MomB and PomA, the effects of phenamil and amirolide, specific inhibitors of sodium-driven motor, may be changed. In Fig. V-6, the cells expressing MomB5 or MomB6 with either MotA or PomA showed a resistant phenotype to phenamil. It is noteworthy that cells expressing MomB5 with either MotA or PomA were hardly inhibited even by the

	co-expressed A subunit	
	PomA	MotA
PomB	W.T.	Na^+ type, Mpa^S
MotB	N.F.	N.F.
PotB0	N.F.	N.F.
PotB1	N.F.	N.F.
MomB1	N.F.	N.F.
MomB2	N.F.	N.F.
MomB3	N.F.	Na^+ type, Mpa^S
MomB4	N.F.	N.F.
MomB5	Na^+ type, Mpa^r, $Na^+ \downarrow$, $Na^+ < Li^+$	Na^+ type, Mpa^r
MomB6	Na^+ type, Mpa^r, $Li^+, \downarrow Na^+ >> Li^+$	Na^+ type, Mpa^r

Fig. V-7. The chimeric proteins and their features. White and grey boxes show regions which are derived from VaPomB and RsMotB, respectively. Abbreviations: TM; transmembrane region, PGB; peptidoglycan binding motif, W.T.; wild type, Mpa^S; motility sensitive to phenamil, N.F.; non functional, Mpa^r; motility resistant to phenamil, Na^+; motility coupling to sodium ion, Li^+; motility coupling to lithium ion, $\downarrow$; impaired motility.

highest concentration of phenamil I tested. The IC_{50} value, the concentration of a inhibitor required for 50 % inhibition of motility, for phenamil of cells expressing MomB3 with MotA was comparable to that of cells expressing PomA/B. This may show that MomB of with changed ion specificity has acquired resistance to phenamil. On the other hand, all the strains tested showed the same sensitivity phenotype to amiloride as the wild type(data not shown). The noteworthy features of all chimera were summarized in Fig. V-7.

Chapter VI Discussion

Cloning and characterization of sodium driven motor genes, homologous to proton type, *motA* and *motB*. (Chapter II)

Until I started this study, two sodium-driven motor genes, *motX* and *motY*, have been identified in *V. parahaemolyticus* and *V. alginolyticus*. Their products are both predicted to have one transmembrane region and not homologous to proton type components. However, I cloned two novel sodium-driven motor genes, *pomA* and *pomB*, which are homologous to the *motA* and *motB* genes of proton-driven motors. So, the sodium-driven motor consists of the products of four genes, *pomA*, *pomB*, *motX* and *motY* (Fig. II-4). The genes, *lafT* and *lafU*, of the lateral flagellar motor which is proton-driven have been isolated and the predicted products are shown to be homologous to the *motA* and *motB* product of *E. coli*, respectively (McCarter & Wright, 1993).

From the homology to the proton motor components and their properties (Garza *et al.*, 1996a; Nguyen & Saier, 1996; Sharp *et al.*, 1995a; Sharp *et al.*, 1995b; Zhou *et al.*, 1995), I expect that the four transmembrane regions of PomA and the one transmembrane region of PomB also make a complex and function as a sodium channel. An Asp residue in the transmembrane region of *E. coli* MotB has been proposed to act as the donor in H^+ transfer to a recipient near the cytoplasmic side of the protein (Sharp *et al.*, 1995b). The Asp is conserved in the PomB transmembrane region, and may be the donor in Na^+ transfer in that protein. It is also possible that a sodium channel is formed from the four *pom* products, PomA, PomB, MotX, and MotY because it has been suggested that MotX is the channel component of the motor and interacts with MotY (McCarter, 1994b). It is not

ruled out that MotX and MotY might form a different sodium channel.

In the proton driven motor, MotB is thought to function as a stator by interaction to peptidoglycan. PomB presumably functions in a similar way. In addition to PomB, the sodium-driven type motor apparently needs another component, MotY, which has a peptidoglycan binding motif but no further similarity to MotB. These two possible stators, PomB and MotY, are required for polar motility of *V. alginolyticus*.

Why does the sodium driven motor need two stators? And why dose it need four motor components, whereas the proton-driven motor only needs two? *Vibrio* has two types of flagella, polar and lateral. The polar flagellum is thought to function as a viscosity sensor (McCarter *et al.*, 1988) that regulates expression of lateral flagellar genes. It has shown that *Vibrio* cells sense a decrease in the rotation rate of (or the sodium influx through) the polar flagellar motor (Kawagishi *et al.*, 1996). Therefore, I speculate that MotX and MotY might sense the rotation rate or the sodium flux. Alternatively, they may be needed for the very fast rotation of the sodium-driven motor. It has been reported that its speed is ca. 600 rps in 50 mM NaCl at room temperature, and increase up to ca. 1,700 rps in 300 mM NaCl at 35°C (Magariyama *et al.*, 1995; Magariyama *et al.*, 1994; Muramoto *et al.*, 1995), whereas the speed of proton-driven motor is 200-300 rps at most in *S. typhimurium* or *E. coli* (Kudo *et al.*, 1990; Lowe *et al.*, 1987).

Cysteine-scanning mutagenesis of the periplasmic loop regions of PomA, a putative channel component of the sodium-driven flagellar motor. (Chapter III)

Cysteine or alanine scanning is known as a useful method for the functional analysis of various ion channels and transporters (Frillingos *et al.*, 1998; Kimura *et al.*, 1998; Kubo *et al.*, 1998; Perez-Garcia *et al.*, 1996). Applying this methods to investigate flagellar motor was expected to give us information about not only functional residues, but the structural prospects around each residues from the binding effects of SH-modifying reagents. Thirty-four different site-directed *pomA* mutations were made in this study.

Of the 34 mutants, only two were non-motile, M179C and T186C. Nine mutants showed sub-normal motility. These mutant PomA proteins were detected in amounts comparable to the wild-type protein. T186 is predicted to be in a transmembrane segment (TM4), and this Thr residue is conserved among MotA proteins of various species (Fig. II-3a). In *E. coli* MotA, the T209W substitution abolished motility. This result was interpreted to mean that this residue faces the inside of the ion channel (Sharp *et al.*, 1995a). M179 is predicted to be at the periplasmic end of TM4. This methionine residue is not conserved in all MotA proteins but appears always to be a non-polar residue, for example, Ile in *E. coli*. Many substitutions in $loop_{3-4}$ had significant effects on motility. The five successive Cys substitutions from M169C to K173C impaired motility. This effect may be related to the observation that the transmembrane segments TM3 and TM4 are more conserved in MotA proteins of various species (Fig. II-3a).

While I progressed this study, it was found that the D148Y mutation conferred a resistant phenotype to phenamil, which is a sodium channel inhibitor, for the motor rotation. D148 is suggested to be one of high-affinity phenamil binding sites (Kojima *et al.*, 1999a). This Asp was changed to various amino acid residues, and the D148C mutant was a phenamil resistant (Kojima *et al.*, 1999a).

From a set of *E. coli motB* missense mutations, extragenic suppressors were

isolated and some of these suppressors altered residues on the periplasmic surface of MotA (Garza *et al.*, 1996a). In that study, a model was proposed in which mutations affecting residues in or near the putative peptidoglycan binding region of MotB misalign the stator relative to the rotor. The suppressors in the MotA periplasmic loop regions may cause a compensating realignment to restore motor function. This proposal suggests that the periplasmic loop regions in MotA or PomA may be important for the overall structure of the motor complex.

Among the various thiol-group specific binding reagents, I chose DTNB and NEM. It is known that DTNB is membrane impermeable and the reaction is reversible by a reducing agent, and on the other hand, that NEM is membrane permeable and the reaction is irreversible. Both reagents had comparable effects on the motility of every mutants. The motility of the I175C mutant was impaired and further decreased by the SH-modifying reagents. Only I175C, among the mutants made in this study, lost motility completely when it was treated with the SH-modifying reagents. This residue seems to be exposed to the reagents and I speculate that the ion channel is plugged by the modification. The Cys-175 protein might prove useful for probing channel function by modification with reagents of different sizes.

I tested whether I could observe the difference of the SH-modifying reagents' accessibility with or without sodium ions using the M169C and D170C mutants, whose substituted residues are predicted to exist near the boundary of the TM3. The reaction of DTNB with Cys-170 was slower when the Na^+ concentration was high (Fig. III-6c). In contrast, Na^+ did not protect Cys-169 from modification. This result may suggest that Asp-170 faces to the channel pore and interacts with Na^+ or that Na^+ changes the structure of PomA around Asp-170. When this reactivity can be assessed directly by chemical modification under various

conditions, structural changes around Asp-170 might be clarified.

Generally, maleimide is known not to bind to a thiol in the hydrophobic interior of membrane, and I can use many derivatives, i.e. isotope-labeled, biotinylated, fluorescein-labeled, and so on, to detect directly the binding. Using biotin maleimide, I tried to determine the membrane topology of PomA and the boundaries of the transmembrane regions and periplasmic $loop_{3-4}$ are inferred to be around S167C for TM3, and A178C for TM4 (Fig. III-5). Those results are consistent with the membrane topology predicted from the hydrophobicity profiles (Fig. II-3a; Dean *et al.*, 1984). As noteworthy, in spite of swarm was inhibited by DTNB, the A180C protein was not biotinylated (Fig. III-5). Swimming speed of the A180C mutant was not inhibited by DTNB and NEM, and this is consistent with that A180C PomA protein was not biotinylated. I speculate that DTNB might take a very long time or need only a certain condition to bind to Cys-180 and the slight motility inhibition was observed only in the swarming plate. This may suggest that the Ala-180 residue exists in membrane and faces to a hydrophilic channel pore. This prospect is supported by that Thr-186, which is corresponded to the conserved polar residue for the channel in MotA (Sharp *et al.*, 1995a), locates on the same phase as Ala-180 in the helical wheel of TM4. None of the Cys substitutions in $loop_{1-2}$ was labeled with biotin maleimide. On the other hand, the motility of the F29C and D31C mutants was affected by SH-modifying reagents. I showed that the substitution of F29C reacted slowly with the SH-modifying reagent, DTNB (Fig. III-3). It has been shown that fluorescein maleimide can react with S24C in $loop_{1-2}$ of *E. coli* MotA in spheroplast (Zhou *et al.*, 1995). Though I tried to label F29C with a prolonged incubation or with spheroplast, I could not detect biotinylated PomA (data not shown). This $loop_{1-2}$ may be covered with other proteins, such as the motor proteins, PomB, MotX, or MotY. Alternatively, this loop may be

embedded into the pore region of the channel, as predicted in the loops of many ion channels (Catterall, 1995).

The substituted residues of the periplasmic $loop_{1-2}$ were not accessible for the SH-modifying reagents, and few Cys substitutions caused motility impairment. While, in $loop_{3-4}$, many Cys substitutions impaired motility and the $loop_{3-4}$ seems to be expose to the periplasm. The two periplasmic loops may have quite different roles for the channel complex of the motor proteins.

Construction and characterization of hybrid motor with the H^+- and Na^+-driven components in *Vibrio* polar flagella. (Chapter IV)

In the chapter II, I reported that two sodium driven motor components, PomA and PomB, are similar to proton motor components MotA and MotB and thought that PomA and PomB may function as a sodium channel. Thus, I hope that it might be possible to study the ion specificity by various chimeric proteins between PomA and MotA. However, surprisingly, I found that the whole *R. sphaeroides motA* gene could partially complement a *pomA* mutant of *V. alginolyticus*. This worked despite the different codon usages of *Rhodobacter* and *Vibrio* genes (which may account for the less than 100% complementation that was achieved). I had previously tested for motility restoration of *pomA* mutants by *E. coli* MotA, but it did not work (data not shown). It is probably because *E. coli* MotA have lower similarity to *Vibrio* PomA than *R. sphaeroides*'s and could not precisely interact with PomB. Thus, the lateral motor components of LafT and LafU of *Vibrio* seem not to be interchangeable with PomA and PomB, because they are highly similar to

E. coli MotA and MotB, but not PomA and PomB (McCarter & Wright, 1993). As shown in Fig. IV-1a, the similarity between PomA and *R. sphaeroides* MotA transmembrane regions is very high. The large cytoplasmic loop regions have poor similarity, although some important residues for torque generation were conserved, for example, two proline, arginine and aspartate residue proposed by Zhou *et al.* in *E. coli* MotA (Zhou & Blair, 1997). It has been also proposed that the cytoplasmic loop of *E. coli* MotA interacts with the rotor part directly (Zhou *et al.*, 1998a). So, I speculate that if the transmembrane regions could form a functional channel, the conformational change coupling with the ion flux could be transmitted to the rotor since some important residues were at least conserved. If this is true, a chimeric protein, which consists of the transmembrane regions of *R. sphaeroides* MotA and the cytoplasmic loop of *V. alginolyticus* PomA, might propel the cell at the high speed as the native motor.

Which ion flux, proton or sodium, was coupled to the force generation in this hybrid motor? I conclude that sodium ions were involved in the torque generation of the hybrid motor from the following reasons. *V. alginolyticus* has two types of flagella, polar and lateral, and the latter is a proton type and it is known that the mutants defective in polar flagella can swim in sodium-free buffer using the proton-driven Laf (Atsumi *et al.*, 1992b; Kawagishi *et al.*, 1995). However, the hybrid motor could not rotate in sodium-free motility buffer at pH7.5 in which condition *V. alginolyticus* can make a sufficient proton gradient. Phenamil and the analogs, which are potent inhibitor of the sodium channels of various organisms (Atsumi *et al.*, 1990), inhibit polar flagellar motors but not lateral ones. Especially, phenamil has been shown to be more specific inhibitor for the motor function because target sites of phenamil are found in the sodium motor proteins, PomA and PomB (Jaques *et al.*, 1999; Kojima *et al.*, 1999a). Both the hybrid and native

motors were inhibited, severely dependent on the concentration of phenamil (Fig. IV-4b) and amiloride (Fig. IV-4c). The motility inhibition of the hybrid motors by amiloride was also competitive with sodium ions (Table IV-1). This shows that the effect of amiloride is specific in the hybrid motor. Moreover, it has been shown that the sodium-driven polar flagella can rotate even though proton motive force is abolished by CCCP, because *Vibrio* has respiration-coupled Na^+ pumps only active in an alkaline pH range (Atsumi *et al.*, 1992b; Kawagishi *et al.*, 1995). Either the hybrid motor in pYA701/NMB190 or the sodium motor in pYA301/NMB190 did not stop in the presence of 20 μM CCCP under the alkaline condition (Table IV-2). These results suggest that the hybrid motor is not depend on the proton motive force.

It has been shown that chemotactic signals are transduced to the C-ring of the flagellar rotor by phospho-CheY and the binding of phospho-CheY changes the rotor conformation, although little is known how the direction is determined. As shown in Table IV-3, pYA701/NMB190 cells change rarely the direction, compared with pYA301/NMB190 cells. To explain this phenotype, I suggest that the MotA cytoplasmic loop, interacts poorly with the C-ring in hybrid motors because it has poor similarity to the PomA cytoplasmic loop and this imperfect rotor-stator interaction is enough to generate torque, but not to change the direction of flagellar rotation properly. It was reported that a single mutation in the *E. coli* MotA periplasmic loop resulted in a CW biased phenotype (Garza *et al.*, 1996a) and the mutation in periplasmic space is supposed to affect the structure of the cytoplasmic loop and change the interaction with the switch complex of the rotor.

Coupling ion specificity of chimeras between H^+- and Na^+-driven motor

proteins, MotB and PomB, in *Vibrio* polar flagella. (Chapter V)

In chapter IV, I found that a hybrid motor consisting of *Rhodobacter sphaeroides* MotA, *Vibrio alginolyticus* PomB, MotX and MotY, can work as a sodium driven motor in *Vibrio* cells. This shows that the A subunits do not determine the ion specificity and raises the question: Which components decide the ion selectivity? The most potent candidate seems to be the B subunits. The PomB proteins are showed to form a sodium channel together with the PomA proteins (Sato & Homma, 1999), and contain a conserved Asp residue which is located in the transmembrane region and seems to be essential for the ion transfer. In chapter V, I examined whether B subunits are involved in the ion specificity of the motor.

Initially, I constructed plasmids encoding various combinations of A and B subunits of the proton type (*R. sphaeroides*) or the sodium type motors(*V. alginolyticus*), *i.e.* MotA and PomB, or PomA and MotB. These plasmids were introduced into the *pomAB* mutant of *Vibrio* cells. As shown in Fig. V-2a, MotB could not work with either MotA or PomA in the *pomAB* mutant. In spite of the higher GC content of the *motB* gene compaired to the *Vibrio pomB* gene, the MotB protein seemed to be sufficiently expressed. I speculate that the peptidoglycan binding domain of MotB can not associate with the *Vibrio* peptidoglycan layer. This speculation was supported by the evidence that the chimeric proteins, PotB, between the N-terminal region of PomB and the C-terminal region of MotB, did not function at all(data not shown) and that the mutation of the highly conserved Ser residue in the peptidoglycan binding domain caused a defect of the PomB function in *Vibrio* (NMB161) (data not shown).

Next, I constructed the chimeric proteins, named MomB, consisting of N-terminal regions of MotB and C-terminal regions of PomB. The features of all

chimera were shown in Fig. V-7. Among of them, MomB1, MomB2, MomB3 and MomB4 have junction sites in a nonconserved 'linker' region wich connects the transmembrane region and the peptidoglycan binding motif. The other chimeric proteins, MomB5 and MomB6, have junction points within the transmembrane regions which are highly conserved. These six MomB proteins were expressed with either MotA or PomA in the *pomAB* mutant. Out of the MomB chimeras, MomB5 and MomB6 functioned as sodium-driven motor components. However, the specificities for Na^+ and Li^+ were changed in the motors with MomB5 and MomB6. The MomB6-containing motor was driven by Na^+ as effectively as the wild type motor but the motility driven by Li^+ was severely impaired. The MomB5-containin g motor was severely impaired for Na^+-driven rotation but was coupled to Li^+ more effectively than to Na^+. Structural changes to the channel consisting of the transmembrane regions of the A and B subunits may affect the efficiency of the ion transfer and/or the energy coupling for Li^+ or Na^+. I speculate that the effects on ion specificity for motility may correlate to the ion size because the MomB5 whwhose transmembrane region derived mostly from the proton-type protein MotB, works better with the smaller ion Li^+ and because MomB6 whose transmembrane region derived mostly from the sodium-type protein PomB, works better with the larger ion Na^+.

MomB3 retains the region of the sodium-type B subunit from the linker region to the C terminus. MomB3 was functional in a *pomAB* strain with MotA, though the swimming ability was poor, but MomB3 did not function with PomA. This linker region of the B subunit is thought not to be involved in the ion conducting activity. However, the linker region might be required for the precise arrangement of the transmembrane regions. Namely, the linker regions of the proton and sodium type subunit might force the transmembrane regions to form small and

large pores which accomodate protons and sodium ions, respectively. MomB1, MomB2, and MomB4 expressed with either PomA or MotA were non functional. The parts of the linker between site 3 and 4 region from MotB and PomB might compete with each other. Garza *et al.* (Garza *et al.*, 1996b) also suggested that in *E. coli*, the MotA periplasmic region interacts with the linker region of MotB, or that this region helps the transmembrane domain positioning properly to interact with the transmembrane regions of MotA.

'Where' and 'How' do the motor proteins recognize the coupling ion? Many mutations that alter the ion specificity are known about in various ion channels and transporters (Hama & Wilson, 1993; Heginbotham *et al.*, 1994; Kellenberger *et al.*, 1999). However, it is almost unclear how these residues recognize the different ions. An important exeption is the KcsA potassium channel, for which the crystal structure has been solved and the mechanism of ion recognition has been revealed (Doyle *et al.*, 1998). Mutations at some residues in the pore region change the ability of the channel to discriminate K^+ from Na^+(Heginbotham *et al.*, 1994). The side chains of these residues face the out side of the pore, and K^+ ions interact with the oxygen atoms of these peptide backbones. The selective filter formed by four oxygen atoms fits and stabilizes a dehydrated K^+ electrostatically. However, smaller Na^+ ions can not fit to the filter and therefore fail to overcome electrostatic destabilization in the pore. By this analogy, the A and B subunits may form a selective pore for ion recognition. The selective pore may be modulated by the other motor proteins, MotX and/or MotY. Alternatively, MotX and/or MotY might recognize the ions and pass the ions to the PomA-PomB complex.

Chapter VII Concluding remarks

In this study, I identified the novel sodium motor genes, the *pomA* and *pomB* genes, homologous to proton type *motA* and *motB*. This finding opened the way to compare and study the mechanism of ion recognition and torque generation. I revealed the functional importance of the two periplasmic loops of the A subunit and the linker region of the B subunit. Moreover, I showed the first evidence that the ion specificity was changed by the change of the motor component, the B subunit. To understand of the mechanism of torque generation in bacterial flagellar motor, MotX and MotY in addition to PomA and PomB should be analyzed more because their function is almost unknown. Furthermore, the mechanism would not be resolved without the study of the rotor component, FliG, which interact with the stator component, PomA and which might have the binding site of the coupling ions.

Chapter VIII Materials and methods

Bacterial strains, plasmids, growth conditions and media. The strains and plasmids used are shown in Table VIII-1 and VIII-2. *V. alginolyticus* cells were cultured at 30 °C in VC medium (0.5 % Polypepton, 0.5 % yeast extract, 0.4 % K_2HPO_4, 3 % NaCl, 0.2 % glucose) or VPG medium (1 % Polypepton, 0.4 % K_2HPO_4, 3 % NaCl, 0.5 % glycerol). When necessary, chloramphenicol and kanamycin were added to the final concentration of 2.5 and 100 μg/ml, respectively. *E. coli* cells were cultured at 37 °C in LB medium (1 % Bactotryptone, 0.5 % yeast extract, 0.5 % NaCl). For *E. coli*, chloramphenicol and kanamycin were added to the final concentration of 25 and 50 μg/ml, respectively. The swimming speed was measured in Tris-motility buffers, TMN300 (300 mM NaCl, 50 mM Tris-HCl [pH7.5], 5 mM $MgCl_2$, 5 mM glucose), TMK300 (300 mM KCl, 50 mM Tris-HCl [pH7.5], 5 mM $MgCl_2$, 5 mM glucose) or mixtures of TMN300 and TMK300 to vary the sodium concentration.

Isolation and construction of *pom* mutants. From the lateral flagella-defective (Pof^+ Laf^-) mutant VIO5 (Okunishi *et al.*, 1996), VIO586, NMB201 and NMB205 (Kojima *et al.*, 1997), swarm-deficient mutants were isolated using ethyl methanesulfonate (EMS), as described previously (Okunishi *et al.*, 1996).

The *pomA* null mutant mutated on the chromosome was constructed as follows. The 0.4 kb *Hin*dIII fragment of a mutant *pomA* gene (allele number 2 in the reference Kojima *et al.*, 1999b) from pYA301-M2, which has 211 bp deletion in the gene, was replaced with the 0.6 kb *Hin*dIII fragment of *pomA* in pYA303 and the resultant plasmid was named pYA303-M2. The *Xba*I-*Sac*I fragment in pYA303-M2 was moved to a suicide vector pKY704 (Xu *et al.*, 1994). The

Table VIII-1. Bacterial strains

Strain	Genotype or description	Reference or source
V. alginolyticus strains		
138-2	Wild type	Tokuda *et al.*, 1981
VIK4	138-2 *rif* (Rif^r Pof$^+$ Laf$^+$)	Okunishi *et al.*, 1996
VIO5	VIK4 *laf* (Pof$^+$ Laf$^-$)	Okunishi *et al.*, 1996
VIO586	VIO5 *pomA* (Laf$^-$ Pom$^-$)	This work
NMB152	NMB201 *pomB* (Rif^r Laf$^-$ Pom$^-$ Mpa^r)	This work
NMB161	NMB201 *pomB* (Rif^r Laf$^-$ Pom$^-$ Mpa^r)	Kojima *et al.*, 1999a
NMB188	VIO586 *pomA* (Laf$^-$ Pom$^-$ Che$^-$)	Kojima *et al.*, 1999a
NMB190	VIO5 Δ*pomA* (211 bp deletion) (Rif^r Laf$^-$ Pom$^-$)	This work
NMB191	VIO5 Δ*pomAB* (0.94-bp *Dra*I-*Hpa*I fragment deletion) (Rif^r Laf$^-$ Pom$^-$)	This work
YM19	138-2 *pof* (Pof$^-$)	Kawagishi *et al.*, 1995
V. parahaemolyticus strains		
LM4170	*lafX313 motX118* (Laf$^-$ Pom$^-$)	McCarter, 1994a
LM4171	*lafX313 motY141* (Laf$^-$ Pom$^-$)	McCarter, 1994b
E. coli strains		
DH5a	F$^-$ λ$^-$ *recA1 hsdR17 endA1 supE44 thi-1 relA1 gyrA96* Δ(*argF-lacZYA*)U169 *f* 80d*lacZ*ΔM15	Grant *et al.*, 1990
XL1-Blue	*recA1 hsdR17 endA1 supE44* Δ(*lac-proAB*) {F'::Tn*10 proAB lacI* ZΔM15}	Stratagene
SM10 l *pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ *pir*RK6	Miller & Mekalanos, 1988

Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Rif^r, rifampicin resistant

Table VIII-2. Plasmids

Plasmid	Genotype or description	Reference or source
pSU21	*cat* (Cm^r) P_{lac} *lacZa*	Bartolome et al., 1991
pSU41	*kan* (Km^r) P_{lac} *lacZa* (MCS same as that in pSU21)	Bartolome et al., 1991
pKY704	suicide vector (Cm^r)	Xu *et al.*, 1994
pIO3	pSU21 0.4-kb and 6.0-kb *Hin*dIII fragment (*motY*$^+$)	Okunishi *et al.*, 1996
pYA101	pSU21 5.0-kb and 3.6-kb *Hin*dIII fragment	This work
pYA201	pSU21 5.2-kb and 3.3-kb *Eco*RI fragment (*pomA*$^+$)	This work
pYA202	pSU21 3.3-kb *Eco*RI fragment (*pomA*$^+$)	This work
pYA2021	pSU21 2.7-kb *Eco*RI-*Hin*dIII fragment (*pomA*$^-$)	This work
pYA2022	pSU21 1.9-kb *Eco*RI-*Xba*I fragment (*pomA*$^-$)	This work
pYA2023	pSU21 1.6-kb *Eco*RI-*Sac*I fragment (*pomA*$^-$)	This work
pYA203	pSU21 3.3-kb *Eco*RI fragment (*pomA*$^+$) (include the same fragment as pYA202, but in the opposite orientation)	This work
pYA2031	pSU21 2-kb *Sac*I-*Eco*RI fragment (*pomA*$^+$)	This work
pYA2032	pSU21 1.45-kb *Xba*I-*Eco*RI fragment (*pomA*$^+$)	This work
pHK2	pSU21 3.3-kb *Sac*I fragment (*pomAB*$^+$)	This work
pHK3	pSU21 1.2-kb *Hin*dIII-*Sac*I fragment (*pomA*$^-$*B*$^+$)	This work
pYA301	pSU41 0.8-kb *Bam*HI-*Bam*HI fragment (*pomA*$^+$)	Kojima *et al.*, 1999a
pMK201-M2	pSU21 0.6-kb *Bam*HI-*Bam*HI fragmnt (*pomA*$^-$) 211 bp deletion in *pomA*	Kojima *et al.*, 1999b
pYA303	pSU41 1.9-kb *Bam*HI-*Sac*I fragment (*pomAB*$^+$)	Kojima *et al.*, 1999a
pRED1	pUC19 2.5-kb *Sph*I-*Sal*I fragment (*motAB*$^+$)	Shah & Sockett, 1995
pYA603	pSU41 2.5-kb *Hin*dIII-*Eco*RI fragment from pRED1 (native promoter region and *motAB*$^+$)	This work
pYA6031	pSU41 1.6-kb *Hin*dIII-*Sma*I fragment (native promoter and *motA*$^+$)	This work
pYA701	pSU41 0.8-kb *Bam*HI-*Eco*RI fragment (*motA*$^+$)	This work
pYA303-M2	pSU41 1.7-kb *Bam*HI-*Sac*I fragmnt (*pomA*$^-$*B*$^+$) 211 bp deletion in *pomA*	This work
pYA801	pKY704 1.7-kb *Xba*I-*Sac*I fragment (*pomA*$^-$*B*$^+$) from pYA303-M2	This work
pYA3031	pSU41 0.8-kb *Bam*HI-*Spe*I fragment (*pomA*$^+$) and 1.1-kb *Spe*I-*Sac*I fragment (*pomB*$^+$)	This work
pYA3032	pSU41 0.8-kb *Bam*HI-*Spe*I fragment (*pomA*$^+$) and 1.1-kb *Spe*I-*Sac*I fragment (*motB*$^+$)	This work
pYA703	pSU41 1.9-kb *Bam*HI-*Sac*I fragment (*motAB*$^+$)	This work
pYA7031	pSU41 0.8-kb *Bam*HI-*Spe*I fragment (*motA*$^+$) and 1.1-kb *Spe*I-*Sac*I fragment (*motB*$^+$)	This work
pYA7032	pSU41 0.8-kb *Bam*HI-*Spe*I fragment (*motA*$^+$) and 1.1-kb *Spe*I-*Sac*I fragment (*pomB*$^+$)	This work

Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; MCS, multicloning site; P_{lac}, *lac* promoter

resultant suicide plasmid (pYA801) was transformed into *E. coli*, SM10λ*pir* (Miller & Mekalanos, 1988) and conjugal transfer was done to introduce pYA801 into *Vibrio alginolyticus* VIO5. The suicide plasmids are expected to integrate into the chromosomal *pomA* gene. Transconjugants which showed chloramphenicol resistant were inoculated on a semi-solid agar VPG plate to select motility mutants. Three Pom^- colonies were found in 100 chloramphenicol resistant transconjugants. Next, these Cm^rPom^- cells were cultured in liquid broth without antibiotics, inoculated into new broth everyday and single colonies were isolated on chloramphenicol-free VC hard agar plate. On the fourth day, I found one Cm^sPom^- colony. I confirmed the deletion mutation in the chromosomal *pomA* gene by the amplified fragment size of PCR and the complementation test with pYA301 and pYA303. This mutant was named NMB190 (Δ*pomA*).

***pom* gene cloning.** Plasmid pSU21 (Bartolomé *et al.*, 1991) and chromosomal DNA from *V. alginolyticus* strain VIO5 were digested with *Eco*RI or *Hin*dIII and ligated. These DNA libraries were transferred into a *pom* mutant (VIO586) cells by electroporation and selected as described previously (Okunishi *et al.*, 1996). To test the motility, cells were incubated at 30°C on 0.3 % agar VPG plates containing chloramphenicol.

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 (Kusatsu, Japan) and New England Biolabs (Beverly, Mass). Nucleotide sequences were determined by using the dye terminator cycle sequencing kit (Perkin Elmer Co.) and an ABI PRISM sequencer Model377 (PE Applied Biosystems), or by dideoxy chain termination method using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH) and [α-

^{32}P]dCTP (3,000 μCi/mmol; Amersham Japan, Tokyo) used for radioactive labeling.

Plasmid construction using PCR amplifying fragment. The plasmids, pYA301, pYA303, pYA701 and pYA703 were constructed using PCR amplifying fragments which have the cutting sites of restriction endonucleases the fragment ends. All of the *pom* or *mot* genes do not contain the native promoter in these plasmids, and are transcribed from the *lac* promoter. The construction of pYA301 and pYA303 are described previously (Kojima *et al.*, 1999a). pYA701 and pYA703 have the PCR amplified *motA* and *motAB* fragment from 40 bp upstream of the start codon of *motA* to 3 bp downstream of the stop codon of *motA* and 8 bp downstream of the stop codon of *motB*, respectively. The PCR amplification was done as described previously (Kojima *et al.*, 1999a). In the reaction, we used the end primers, RsmotA.B1 and RsMotA.E2 for pYA701 or RsmotB.Z3 for pYA703, and pRED1(Shah & Sockett, 1995) as templates. RsmotA.B1 has a *Bam*HI site and is homologous to the sense strand of the upstream *motA* gene, 5'-GCGGGATCCTGCCGCTCCGGACCTGGATGA-3'. RsmotA.E2 including a *Eco*RI site is complementary to the antisense downstream of the *motA* gene, 5'-CTCGAATTCGCCTCACGCCGCCTTGCGCT-3'. RsmotA.Z3 including a *Sac*I and *Eco*RI site is complementary to the antisense downstream of the *motB* gene, 5'-CGGAATTCGAGCTCGGACAAGGTCAGTCCT-3'. This PCR amplified fragment was inserted into pSU41 between the *Bam*HI site and the *Eco*RI site.

Site-directed mutagenesis. Site directed mutagenesis was performed by a two-step polymerase chain reaction as described previously (Kojima *et al.*, 1999a). For cystein scanning of the *pomA* gene, PomA-B1 and PomA-B2 or PomA-E2 as end primers, a pair of the mutant primers, and pYA2032 as the template were used. For the introduction of a mutation into *RsmotA* gene, RsmotA.B1, RsmotA.E2,

pYA701 and pair of the mutant primers were used. The mutant primers have a 1-3 base mismatch to the original sequence at the mutation site. The amplified mutant fragments were cloned into the plasmid vector pSU41 (Bartolomé *et al.*, 1991).

To introduce a *Spe*I site between the stop codon of the A subunit and SD sequence of the B subunit in pYA703 or pYA303, I used the pairs of mutant primers, RsmotA.spe1 and RsmotA.spe2 or VapomA.spe1 and VapomA.spe2. The sequences of the sense strand of RsmotA.speI and VapomA.speI is 5'-GGCGGCGTGACTAGTGAGGCCAGGCCATG-3' and 5'-ACGAGTAACTAGTGGAGAGTCG-3', respectively. I also constructed *momB* genes using the two-step PCR. The sense-strand sequences of the mutant primers were shown as follow: MomB1; 5'-AGAAGGCGCTCGCTGGTGGCACAAAACGT-3', MomB2; 5'-AAGTCGGAC ACGATGCAGCAAACCATG-3', MomB3; 5'-TTCCATTCGATCCTTGAAGTGAAAGAC-3', MomB4; 5'-GCTTCGCGAAGATGGATGTACTGAAGTTC-3', MomB5; 5'-ATGGCCTTCTTCGTTCTTCTGCTCTCG-3', MomB6; 5'-CTTGCGACCTTTG CAGATTTGATGTCG-3'.

Transformation of the *Vibrio* cells. Transformation of *Vibrio* cells was carried out by electroporation as described previously (Kawagishi *et al.*, 1994). The cells were subjected to osmotic shock and washed thoroughly with 20 mM $MgSO_4$. Electroporation was carried out with the Gene Pulser electroporation apparatus (Japan Bio-Rad Laboratories, Tokyo, Japan) at an electric field strength between 5.0 and 7.5 kV/cm.

Measurement of swimming speed. An overnight culture in VC medium was inoculated into fresh VPG medium at a 100-fold dilution and grown at 30 °C to exponential phase. Cells were harvested by centrifugation and suspended in TMN50

(50 mM NaCl, 250 mM KCl, 50 mM Tris-HCl [pH7.5], 5 mM $MgCl_2$, 5 mM glucose). The cell suspension was diluted about 100-fold into Tris motility buffers containing various concentrations of NaCl, and 20 mM serine to suppress the directional change of swimming for Che^+ strains. Motility of the cells was observed under a dark-field microscope and recorded on videotape. Swimming speed was determined as described previously (Atsumi *et al.*, 1996).

Behavioral assay. Tumbling frequency was determined as described previously (Homma *et al.*, 1996). Cell suspension as prepared for measurement of swimming speed was diluted about 100-fold into TMN50 and chemoeffectors (phenol and serine) were added. Within 1 min of addition, cell motility was observed under a dark-field microscope and recorded on videotape. Each frame (30 frames in 1 s) on the videotapes was played back on a monitor and the number of times the cell changed direction in 1 s was noted. At least 20 cells were measured for any given conditions.

Immunoblotting. Preparation of cells and immunoblotting were done as described previously (Yorimitsu *et al.*, 1999). For the first antibody of immunoblotting, we used anti-peptide antibody against each flagellar motor components. For the second antibody, we used an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Kirkegaad & Perry Laboratories, Gaithersburg, MD). The method for detection of the second antibody has been described (Yorimitsu *et al.*, 1999).

Anti flagellar motor component antibodies. PomA91, PomB93 and PomA1312 antibody were generated against three synthetic peptides (one for PomA1312) designed for the different regions of PomA or PomB (Yorimitsu *et al.*, 1999). The production and affinity purification of anti-RsMotA (RsMotA253) or -RsMotB (RsMotB2) antibody were carried out by Sawady technology (Tokyo,

Japan). The peptide fragment synthesized artificially is NH_2-CIEDQMVCALDRKQQMKRKAA-COOH which is located in the C terminus of MotA, or NH_2-SAKPKVIRFQPPVPDDDEGEC-COOH which is located in the N terminus of RsMotB. A cysteine was added to the terminus and the synthesized fragment was conjugated to KLH by it. Rabbits were immunized by the conjugated KLH.

Labeling with and detection of biotin maleimide. Cell suspensions were prepared for the same procedure of measurements of swimming speed as above. For labeling, the cells suspended in TMN50 were preincubated with 10 mM DTT at room temperature for 30 min and harvested by centrifugation. The pellet was resuspended in TMN50 and incubated with 0.2 mM biotin maleimide (Biotin-PE-maleimide; Dojin Corp., Kumamoto, Japan) for 10 min. The cells washed once with TMN50 were solubilized and immunoprecipitated with the anti-PomA (PomA1312) antibody, as described previously (Yorimitsu *et al.*, 1999). The immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membrane as described above. Biotinylated proteins were detected using streptavidin-conjugated horseradish peroxidase and chemiluminescence (Amersham Corp.)

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Notes

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1. Asai, Y., S. Kojima, H. Kato, N. Nishioka, I. Kawagishi, and M. Homma. (1997) Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium. *J Bacteriol.* **179**, 5104-5110.

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3. Asai, Y., I. Kawagishi, R. E. Sockett and M. Homma. (1999) Hybrid motor with the H^+- and Na^+-driven components can rotate *Vibrio* polar flagella using sodium ions. *J Bacteriol.* **181**, 6332-6338.

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