Na\(^+\)-driven Flagellar Motor Resistant to Phenamil, an Amiloride Analog, Caused by Mutations in Putative Channel Components

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The rotation of the Na\(^+\)-driven flagellar motor is specifically and strongly inhibited by phenamil, an amiloride analog. Here, we provide the first evidence that phenamil interacts directly with the Na\(^+\)-channel components (PomA and PomB) of the motor. The alterations in Mpar (motility resistant to phenamil) strains were mapped to the pomA and/or pomB genes. We cloned and sequenced pomA and pomB from two Mpar strains, NMB205 and NMB201, and found a substitution in pomA (Asp148 to Tyr; NMB205) and in pomB (Pro16 to Ser; NMB201). Both residues are predicted to be near the cytoplasmic ends of the putative transmembrane segments. Mutational analyses at PomA-Asp148 and PomB-Pro16 suggest that a certain structural change around these residues affects the sensitivity of the motor to phenamil. Co-expression of the PomA D148Y and PomB P16S proteins resulted in an Mpar phenotype which seemed to be less sensitive to phenamil than either of the single mutants, although motility was more severely impaired in the absence of inhibitors. These results support the idea that PomA and PomB interact with each other and suggest that multiple residues, including Asp148 of PomA and Pro16 of PomB, constitute a high-affinity phenamil-binding site at the inner face of the PomA/PomB channel complex.

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Introduction

Bacterial flagellar motors are very tiny molecular engines driven by electrochemical gradients of a specific ion, either H\(^+\) or Na\(^+\), across the cytoplasmic membrane (Blair, 1995; Imae & Atsumi, 1989). These motors propel the cells by rotating helical flagellar filaments, which function as propellers. A motor has several functionally independent force-generating units which are directly responsible for the mecanochemical coupling in the motor (Block & Berg, 1984; Blair & Berg, 1988; Muramoto et al., 1994). In the H\(^+\)-driven motor, two integral membrane proteins, MotA and MotB, are thought to be the components of the force-generating units (Dean et al., 1984; Stader et al., 1986). MotA and MotB have four and one transmembrane segments, respectively (Zhou et al., 1995; Chun & Parkinson, 1988), and it is inferred that they together form a proton-conducting channel (Blair & Berg, 1990; Stolz & Berg, 1991; Garza et al., 1995; Sharp et al., 1995b). MotB has a long extracellular domain that is believed to attach to the peptidoglycan layer (Chun & Parkinson, 1988; Blair et al., 1991; De Mot & Vanderleyden, 1994). It has therefore been suggested that the MotA/MotB channel complex is the stator, anchored to the cell wall by the peptidoglycan-binding domain of MotB.

Some critical residues of MotA and MotB involved in torque generation have been reported. Random mutagenesis of motA and motB revealed that many mutations in the membrane domains of MotA or MotB abolished their functions (Blair & Berg, 1991; Blair et al., 1991). Especially, Asp32 of...
Motility genes (Okunishi et al., 1998b). On the other hand, cytoplasmic domains of MotA might interact with the rotor, especially with the switch proteins (FliG, FliM, FliN) that function to control the direction of the rotation of the motor, and which are all located on the cytoplasmic side of the membrane and constitute the C-ring (Irikura et al., 1993; Sockey et al., 1992; Francis et al., 1994). Among these switch proteins, it has been shown that FliG is most directly involved in torque generation, and might be associated with MotA (Tan et al., 1996; Lloyd et al., 1996). A recent report suggested that three charged residues of FliG, Arg281, Asp288, Asp289, predicted to be on one surface, play important roles in torque generation (Lloyd & Blair, 1997). Moreover, it was suggested that two cytoplasmic charged residues of MotA, Arg90 and Glu98, are involved in torque generation (Zhou & Blair, 1997). It is speculated that these charged residues of FliG and MotA might engage in electrostatic interactions with each other and that these interactions are important for motor function (Zhou et al., 1998a).

The Na⁺-driven motor has some characteristics distinct from the H⁺-driven type. The Na⁺-driven polar flagellar motor of Vibrio alginolyticus rotates stably and remarkably fast, up to 1700 r.p.s. (Magariyama et al., 1994; Muramoto et al., 1995), as measured by laser dark-field microscopy (LDM) (Kudo et al., 1990). Four transmembrane proteins essential for torque generation, PomA, PomB, MotX, and MotY (Figure 1), were recently identified in the Na⁺-driven polar flagellar motor of V. alginolyticus (Okunishi et al., 1996; Asai et al., 1997). MotX and MotY, which were first identified in V. parahaemolyticus (McCarter, 1994a,b), were found to associate with the amiloride-binding site by low structural similarity, phenamil might also be able to associate with the amiloride-binding site by low affinity. Previously, we isolated motility mutants resistant to phenamil (Mpa'), whose motors rotate more stably than wild-type in the presence of phenamil (Kojima et al., 1997). This steadier rotation of the Mpa' motors can be explained by the rapid dissociation of phenamil from the high-affinity binding site in the Na⁺ channel of Mpa' motor as compared with the wild-type. Therefore, these Mpa' mutations presumably affect genes encoding components of the force-generating units, such as pomA, pomB, motX and motY. Here, we have mapped the Mpa' mutations to specific sites in PomA and PomB, the putative Na⁺ channel-forming proteins. The notable importance of these sites in torque generation is discussed.

Results

The Mpa' mutation of strain NMB201 is in pomA and/or pomB

On the basis of evidence presented in the previous study of Mpa' strains (Kojima et al., 1997), we expected that the Mpa' mutations would be found in the genes coding the channel components of the Na⁺-driven motor, such as the pom (polar flagellar motility) genes (Okunishi et al., 1996; Figure 1). We therefore sought to analyze the pom genes of the Mpa' strains NMB201 and NMB205 by using the pom mutants with paralyzed polar
flagella derived from them. Such a Pom\textsuperscript{-} Mpa\textsuperscript{+} double mutant was isolated firstly from NMB201, and named NMB161. Motility was restored to this strain when the plasmid pHK2, harboring wild-type pomA and pomB genes, was introduced. Next, sensitivity to phenamil was examined in the transformed cells (NMB161/pHK2). As shown in Figure 2, motility of the NMB161/pHK2 cells was completely inhibited by 50 μM phenamil, as is observed in the PomA\textsuperscript{-} strain VIO586 which harbored pHK2 (wild-type control), suggesting that the Mpa\textsuperscript{+} phenotype of NMB161 was suppressed by the wild-type pomA and/or pomB gene. This suggests that the Mpa\textsuperscript{+} mutation of NMB201 (and NMB161) is in the pomA or pomB gene.

The PomA mutation D148Y confers phenamil resistance

Since NMB201 and NMB205 show very similar Mpa\textsuperscript{+} phenotypes (Kojima et al., 1997), we tried to clone fragments containing the pomA and pomB genes from NMB201 and NMB205 by shot-gun cloning. Fortunately, a 2.9 kb SacI fragment containing pomA and pomB derived from NMB205 was firstly isolated by the complementation of the pomA mutant VIO586, so we advanced the analysis of Mpa\textsuperscript{+} mutation of NMB205. Then, from this SacI fragment we subcloned the pomA and pomB genes into the plasmids pSK502 and pSK602, respectively (Figure 3(a)). We measured swimming speeds in various concentrations of phenamil of the VIO586 (PomA\textsuperscript{-}) or NMB104 (PomB\textsuperscript{-}) cells containing pSK502 or pSK602, respectively (Figure 3(b)). Motility of the wild-type control strains, VIO586/pSK1(pomA\textsuperscript{+}) and NMB104/pHK4(pomB\textsuperscript{+}), were completely inhibited by 50 μM phenamil. On the other hand, cells of the VIO586/pSK502 strain, which harbored pomA derived from the mutant NMB205, showed phenamil-resistant motility, indicating that the Mpa\textsuperscript{+} mutation in NMB205 was in the pomA gene.

Nucleotide sequencing of the fragments in pSK502 and pSK602 was carried out to find the mutations. A single-base substitution (G442 to T) was detected in pomA, which is predicted to cause the substitution D148Y. No nucleotide changes were detected in pomB. Based on the membrane
topology of *E. coli* MotA (Zhou et al., 1995), the residue Asp148 of PomA should be located near the cytoplasmic end of the third transmembrane segment (Figure 3(c)). Asp148 is conserved in MotA of *E. coli* and *Rhodobacter sphaeroides* (Asai et al., 1997), indicating that this residue is not specific to Na\(^+\)-motors. When pSK502 was introduced into the Mpa\(^+\) (wild-type) strain, the transformants showed an Mpa\(^+\) phenotype.

**Mutational analysis of PomA Asp148**

To probe the requirements for function and for phenamil resistance at position 148, we replaced residue Asp148 of PomA by Tyr (the Mpa\(^+\) mutation of NMB205), Phe, Cys, Asn and Arg. To measure swimming speed more easily, we isolated the strain NMB188 (PomA\(^-\) Che\(^-\); see Materials and Methods), and used it for host. NMB188 cells harbored the plasmids containing pomA which swim smoothly and do not change the swimming direction at all (che phenotype). The swimming speeds of NMB188 (PomA\(^-\) Che\(^-\)) cells containing the mutant pomA genes were measured in the presence of various concentrations of phenamil (Figure 4). In the absence of phenamil, NMB188 cells containing wild-type PomA swam at about 53 μm/second. The PomA mutations D148Y, D148F, D148C, and D148N reduced swimming speeds to about 50-65% of this value. The D148R mutation impaired motility more severely. Motility of the wild-type strain was completely inhibited at 50 μM phenamil, but the D148Y, D148F and D148C mutants could be motile in that condition. The mutation D148N gave an Mpa\(^+\) phenotype comparable to wild-type. The concentrations of phenamil required for 50% inhibition of motility (IC\(_{50}\)) were obtained from the graph; 1.3 μM for wild-type, 8.7 μM for D148Y, 8.7 μM for D148F, 3.3 μM for D148C, 1.3 μM for D148N and 8.7 μM for D148R. D148R mutant cells could not swim at 50 μM phenamil, but the IC\(_{50}\) value is comparable with the phenamil-resistant mutants. So D148R mutation might confer the resistance to phenamil. These results suggest that a negative charge at residue 148 is not essential for sensitivity to phenamil, and that both large (Y and F) and small (C) side-chains at this position can weaken the interaction with phenamil.

We also examined the sensitivity of the Asp148 mutants to amiloride (Figure 5). Mutants that were significantly resistant to phenamil (Y, F and C) were also slightly resistant to amiloride, as compared with the wild-type. The IC\(_{50}\) values for amiloride were about 0.5 mM for wild-type, 1.5 mM for D148Y, 1.2 mM for D148F, 1.0 mM for D148C, 0.5 mM for D148N and 0.7 mM for D148R.

**Figure 4.** Phenamil resistance of PomA Asp148 mutants. Swimming speeds were measured in TMN medium containing various concentrations of phenamil as indicated in Materials and Methods, for the strain NMB188 (PomA\(^-\) Che\(^-\)) expressing the mutant PomA proteins D148F, D148C, D148N, D148R, and D148Y. Filled (●) and open (○) symbols indicate speeds of the mutants and wild-type, respectively.

**Figure 5.** Sensitivity to amiloride of PomA Asp148 mutants. The experimental conditions and symbols are as indicated for Figure 4, except the TMN medium contained the indicated concentrations of amiloride.
Another Mpa' mutation is in the pomB gene

As described above, we assumed that the Mpa' mutation in the strain NMB201 was likely to be in pomA or pomB, so we cloned these genes from NMB201 by using PCR, making plasmids pSK5021 (pomA') and pSK6021 (pomB'), respectively. These plasmids were introduced into strain VIO586 (PomA') or strain NMB104 (PomB'), and swimming speeds in various concentrations of phenamil were measured. NMB104/pSK6021 cells, carrying the pomB gene derived from NMB201, showed motility resistant to phenamil (Figure 6(a)). VIO586/pSK5021 cells, carrying the pomA gene derived from NMB201, showed the Mpa' phenotype (data not shown). The Mpa' mutation in NMB201 is therefore in pomB. DNA sequence analysis revealed two base changes in the pomB gene from NMB201. One caused the substitution P16S (C46 to T), whereas the other was silent (T834 to A; T278T). This silent mutation might have occurred during the PCR cloning. We removed this silent mutation from pSK6021 by replacing an EcoRI fragment from nucleotides at position 317 to the end of the pomB with that of wild-type pomB, derived from plasmid pHK4. NMB104 cells harboring the resulting plasmid, pSK603-P16S, also showed the Mpa' phenotype. We also introduced the P16S mutation into wild-type pomB by site-directed mutagenesis, and confirmed Mpa' phenotype. Hence, we concluded that the P16S mutation caused the phenamil resistance. Based on the membrane topology of E. coli MotB (Chun & Parkinson, 1988), residue Pro16 of PomB should be located in or near the cytoplasmic end of the transmembrane segment (Figure 6(b)). When pSK6021 was introduced into the Mpa' strain VIO5, the transformed cells showed an Mpa' phenotype.

Mutational analysis of PomB Pro16

To prove the requirements for function and phenamil resistance at position 16 of PomB, we carried out four replacements, with Phe, Tyr, Cys, and Ala. Plasmids harboring the P16C, P16F or P16Y mutations proved difficult to introduce into strain NMB104 (PomB'). Because this might be caused by the presence of large amounts of mutant PomB proteins, which might affect cell growth (mutant pomB fragments were directly connected to the lac promoter), we co-transformed cells with the mutant plasmids and pMMB206, which contains the lacIq gene. By this procedure, we succeeded in introducing wild-type as well as mutant pomB genes into the cells. Expression of the pomB genes could then be induced by the addition of 1 mM IPTG.

Swimming speeds of the pomB mutants were measured in various concentrations of phenamil (Figure 7). All of the mutations that introduced small side-chains (S, C and A) conferred resistance to phenamil. In the absence of phenamil, the swimming speeds of the P16S and P16A mutants were similar to that of wild-type cells, whereas the P16C mutation decreased swimming speed to 60% of wild-type. By contrast, the motilities of the large side-chain mutants (P16F, P16Y) were severely impaired even in the absence of phenamil, and the P16Y but not the P16F mutant, could swim at 50 μM phenamil. The IC50 values for phenamil were obtained from the graph: 1.3 μM in the wild-type, 8.3 μM in the P16S, 25 μM in the P16C, 8.7 μM in the P16A, 50 μM in the P16Y and 18 μM in the P16F. P16F cells could not swim at 50 μM phenamil, but the IC50 value is comparable with the phenamil-resistant mutants. So P16F mutation might confer the resistance to phenamil. These results suggest that replacement of Pro16 with

Figure 6. The mutation of another Mpa' strain, NMB201, maps to the pomB gene. (a) Cells containing pomB derived from the Mpa' strain NMB201 showed phenamil-resistant motility. The pomB gene of this strain, cloned by using PCR, was introduced into the pomB mutant (NMB104). Swimming speeds of these cells were measured as described in Materials and Methods. (b) The predicted transmembrane region of PomB. The Mpa' mutation of PomB P16S is located near the cytoplasmic end of the membrane segment of PomB. The topology of PomB was predicted by the hydropathy profile of PomB and the topology of the MotB protein of E. coli (Chun & Parkinson, 1988).
both large and small residues can cause structural changes that result in phenamil-resistant motility, and that the large side-chains at this position can greatly impair motor function.

We also examined sensitivity to amiloride in the PomB Pro16 mutants. As was observed in the PomA Asp148 mutants, the Pro16 mutants that showed significant resistance to phenamil (S, C, A, Y and F) also exhibited some resistance to amiloride (data not shown). The IC₅₀ for amiloride was about 0.5 mM in the wild-type, 1.4 mM in the P16S, 1.2 mM in the P16C, 1.2 mM in the P16A, 1.2 mM in the P16Y and 1.0 mM in the P16F.

**PomA D148Y/PomB P16S double mutant**

To examine the combined effect of the two independently identified Mpa' mutations in pomA and pomB, we constructed a plasmid containing both PomA D148Y and PomB P16S mutations (pYA303-D148Y/P16S), and introduced it into the PomA⁻ Che⁻ strain NMB188. In the absence of phenamil, motility of the double mutant was severely impaired. As the concentration of phenamil in the medium increased, however, the motility was inhibited very little. At 50 μM phenamil, the double mutant swam at 11 μm/second, a rate comparable with the D148Y and P16S single mutants (Figure 8). The double mutant exhibited resistance to amiloride similar to that of the P16Y mutant. When the double mutant D148Y/P16S was expressed in NMB104 (PomB⁻), similar results were observed. These results suggest that the double mutant is less sensitive to phenamil than either of the single mutants; although swimming is poorer in the absence of phenamil, phenamil seems to have practically no effect on this.

**Discussion**

Since phenamil specifically inhibits the rotation of the Na⁺-driven flagellar motor, the site of interaction with phenamil might be involved, directly or indirectly, in force-generation in the motor. Direct evidence that phenamil interacts with the force-generating units in the motor has not been reported. Previously, we isolated motility mutants resistant to phenamil (Mpa' mutants), and speculated that mutations occurred in a high-affinity phenamil-binding site (Kojima et al., 1997). Here, we showed that two Mpa' mutations are located in pomA and pomB, whose products are putative channel components in the motor. This is the first molecular evidence that phenamil directly interacts with the Na⁺ channel components essential for rotation of the motor.

Two Mpa' mutations were identified in pomA (D148Y) and pomB (P16S), respectively. This result provides evidence that both PomA and PomB contribute to phenamil sensitivity, essential for force-generation in the Na⁺ motor. Moreover, both of the mutated residues are predicted to be located near the cytoplasmic ends of transmembrane segments. This suggests a novel possibility...
that the high-affinity phenamil-binding site might be located on the cytoplasmic face of the motor. In order to gain more insight into the phenamil interaction site, we carried out site-directed mutagenesis of residues Asp148 in PomA and Pro16 in PomB. In the case of PomA, the negative charge of Asp148 did not prove of primary importance for the resistant phenotype, whereas changes in the size of the side-chain, either to larger (Tyr and Phe) or smaller (Cys), gave significant phenamil resistance. Mutational analysis of residues Pro16 in PomB showed similar results, in that substitution with either small side-chains (Ser, Cys and Ala) or large side-chains (Tyr and Phe) gave significant phenamil resistance. These Mpa\textsuperscript{R} mutations in PomA and PomB also conferred some amiloride resistance. In addition, the competition of amiloride with Na\textsuperscript{+} in the medium was significantly decreased in these Mpa\textsuperscript{R} mutants (data not shown). This may indicate that the amiloride-binding site is affected by the Mpa\textsuperscript{R} mutations, consistent with previous results (Kojima et al., 1997). The motility of some mutants, especially the PomA D148R, PomB P16F and P16Y mutants, was impaired, suggesting that these mutant residues may affect the structure of the force-generating unit or may prevent ion flux. Interestingly, the pomB genes with Mpa\textsuperscript{R} mutation, which showed the significant impaired motility (P16C, P16F and P16Y) were difficult to introduce into cells unless the pomB expression on the plasmids was regulated. We think that the large amounts of the mutant PomB proteins by themselves, or the ion leakage by the mutant proteins might give a toxic effect.

In the case of the H\textsuperscript{+}-motor, it was suggested that MotA and MotB form a complex that functions as a H\textsuperscript{+} channel (Stolz & Berg, 1991; Garza et al., 1995, 1996a,b; Sharp et al., 1995a,b). We assumed that PomA and PomB, which are homologous to MotA and MotB, respectively, form the Na\textsuperscript{+} channel complex in the motor (Figure 9). Based on this assumption, it is conceivable that residues Asp148 of PomA and Pro16 of PomB might be located near each other and may form a high-affinity binding site for phenamil at the inner face of the PomA/PomB channel complex. If so, the observation that at 50 \mu M phenamil the D148Y/P16S double mutant exhibited an Mpa\textsuperscript{R} phenotype comparable to each single mutation suggests the possibility that multiple residues, including Asp148 of PomA and Pro16 of PomB, constitute a high-affinity phenamil-binding site. On the other hand, the D148Y/P16S double mutation showed a synergistic effect, impairing motor function, much more severely than the individual mutations, even in the absence of inhibitors. This is consistent with the proposal that PomA and PomB function together to form a channel complex. Muramoto et al. (1996) suggested that binding of phenamil to the motor might induce additional drag on the motor, in addition to blocking Na\textsuperscript{+} influx. Strong direct interaction of phenamil with an internal binding site on the PomA/PomB channel complex might induce such function in the motor. From the present results, however, we cannot conclude that phenamil directly interacts with these residues.

The amiloride-binding site might also be located at the inner side of the channel complex, because Mpa\textsuperscript{R} mutations in PomA and PomB conferred some amiloride resistance and an internal Na\textsuperscript{+} binding site was suggested by Yoshida et al. (1990). In such a model, in order to reach the binding site, phenamil and amiloride would have to go through the channel pore, or cross the lipid bilayer to reach the cytoplasmic side (Figure 9). To discuss the precise mechanism for amiloride interaction, we have been trying to isolate motility mutants resistant to amiloride.

Amiloride-binding sites have been studied in eukaryotic sodium channels and transporters. In the eukaryotic epithelial sodium channel (ENaC), amiloride is effective only from the external side of the lipid bilayer (Ismailov et al., 1995). Amiloride-resistant mutations were identified at external or transmembrane region of ENaC, and also in the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. Mutations that cause a large decrease in $K_i$ for amiloride were found to occur in the second transmembrane segment of the $\alpha$ subunit of rat ENaC (Waldmann et al., 1995), and in residues of a hydrophobic region termed the pre-M2 domain that immediately precedes the second membrane domains of several different ENaC proteins (Schild et al., 1997; Fuller et al., 1997). Ismailov et al. (1997) showed that
deletion of a putative amiloride binding domain "WYRFHY" within the extracellular domain of zrENaC, whose existence was suggested by experiments using an anti-amiloride monoclonal antibody (Lin et al., 1994), caused a large decrease in amiloride sensitivity. In addition, they suggested that the His residue in this tract might interact primarily with the Cl atom on the pyrazine ring moiety of amiloride, to stabilize the binding. In the case of the eukaryotic Na⁺/H⁺ exchanger and prokaryotic Na⁺/H⁺ antiporter, residues within the putative second (Kuroda et al., 1997), fourth (Counillon et al., 1993, 1997) and ninth transmembrane domains (Orlowski & Kandasamy, 1996) are involved in amiloride binding. This putative amiloride-binding site in the fourth transmembrane domain contains a sequence, VFF, which is also found in PomA at the cytoplasmic end of the putative fourth transmembrane domain. In the case of the transporter, replacing these F residues with Y decreased the sensitivity to amiloride. However, PomA F197Y or PomA F198Y mutants showed an Mpas phenotype comparable with the wild-type strain (data not shown), so the VFF residues in PomA seem not to be involved in sensitivity to amiloride or phenamil.

The Na⁺ motor of V. alginolyticus has other putative channel components, MotX and MotY (McCarter, 1994a,b; Okunishi et al., 1996). MotX in particular has been suggested to be a target of amiloride. We speculate that the native Na⁺ channel complex in the motor would consist of PomA, PomB, MotX and MotY, and function only when these four proteins assemble properly. Identifying more mutations resistant to phenamil will help in understanding the Na⁺ channel complex essential for torque generation.

**Materials and Methods**

**Bacterial strains, plasmids, growth conditions, and media**

The strains and plasmids used are described in Table 1. V. alginolyticus cells were cultured at 30°C in VC medium (0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.4% (w/v) K₂HPO₄, 3% (w/v) NaCl, 0.2% (w/v) glucose). For swimming speed analysis, cells were cultured to late logarithmic phase at 30°C in VPG medium (1% polypeptone, 0.4% K₂HPO₄, 3% NaCl, 0.5% (w/v) glycerol), and the swimming speed was measured in the TMN medium containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM glucose, 50 mM NaCl, 250 mM KCl. For the induction of the pon genes in plasmids, cells were grown in VC medium containing 1 mM IPTG (isopropyl-1-thio-β-D-galactoside). E. coli cells were cultured at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). When necessary, chloramphenicol and kanamycin were added to final concentrations of 2.5 µg/ml and 100 µg/ml for *Vibrio* cells, or 25 µg/ml and 50 µg/ml for *E. coli* cells, respectively.

**Table 1. Bacterial strains and plasmids**

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</tbody>
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* Cm⁺, chloramphenicol-resistant; Km⁺, kanamycin-resistant; MCS, multicloning site; Pₐ₃, lac promoter; Pᵢ₃-myc, tandemly located lac and lac promoters; Rif⁺, rifampicin-resistant.

* The 1.2 kb HindIII-Sacl fragments contain 3′-terminal 155 bp of pomA and full-length pomB.
Chemicals
Amiloride was purchased from Sigma Chemical Co. Phenamil was synthesized by the method of Cragoe et al. (1967).

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

pom gene cloning
Plasmid pSU21 (Bartolomé et al., 1991) and chromosomal DNA from V. alginolyticus Mpa strain NMB205 (Kojima et al., 1997) were digested with SaeI and ligated. These DNA libraries were introduced into cells of the pomA mutant (VIO856) by electroporation and they were incubated at 30 °C on 0.25% agar VC plates containing chloramphenicol. To isolate the pom genes from the Mpa strain NMB201 (Kojima et al., 1997), PCR amplification was carried out with the Takara Ex Taq kit (Takara shuzo) and Thermal Cycler (Perkin-Elmer Japan). PCR primers complementary to each end of pomA were synthesized. The sense-primer (designated pomA-B1) was 5'-dCTCGGATCCGCGGATCAGT ATTGTGA-3', and generated a BamHI site. The antisense-primer (designated pomA-E2) was 5'-dCTCGGATCCAGTACTTCCGCGGATCAGTATTGTGA-3', and generated an EcoRI site. PCR primers complementary to each end of pomB were also synthesized: The sense-primer (designated pomB-A) was 5'-dATGGGATCCGGATCCGCGGATCAGT ATTGTGA-3', and generated a BamHI site. The antisense-primer (designated pomB-Z) was 5'-dATGGGATCCGGATCCGCGGATCAGT ATTGTGA-3', and contained both a SacI site and a BamHI site.

Site-directed mutagenesis
A two-step PCR method was used to introduce mutations (White, 1993). We synthesized pairs of mutant primers that were homologous to either the sense or antisense strands of pomA or pomB, with the exception of a one to three base mismatch at the site of mutation. In the first step, two PCR reactions were carried out separately, one using an antisense-mutant primer and wild-type sense-primer, and the other, a mutant-sense primer and wild-type antisense-primer. Each of the amplified fragments was separated from the remaining primers by polyacrylamide gel electrophoresis. In the second step, these fragments were mixed together, then the full target genes were amplified by using the appropriate end primers. PCR amplification was carried out by using the Takara Ex Taq kit (Takara shuzo) and Thermal Cycler (Perkin-Elmer Japan). PCR reactions were repeated for 25 cycles of 30 seconds at 94 °C, 60 seconds at 60 °C, and 60 seconds at 72 °C. Amplified mutant genes containing the full-length genes were digested with BamHI and EcoRI for pomA, or with BamHI and SacI for pomB, then ligated into the kanamycin-resistance vector pSU41 (Bartolomé et al., 1991). The resultant plasmids carrying mutant pomA or pomB genes have the same length of fragments as pYA301 (pomA+), and pSK603 (pomB+), respectively. For the construction of the PomA-D148Y /PomB-P16S double mutant, 1.2 kb HindIII-Sacl fragment containing the 3'-terminal 155 bp of pomA and full-length pomB which has the PomB-P16S mutation was amplified, digested with HindIII and SacI, and ligated into pSU41 (named pHK4-P16S). Then the HindIII fragment from pYA301-D148Y was inserted into the HindIII site of pHK4-P16S to make full-length pomA and pomB containing two mutations. The resultant plasmid has the same length of fragment as pYA303 (pomA+B+), so it was named pYA303-D148Y/P16S.

Isolation of PomA-Chε double mutant
In order to analyze the swimming speeds of pomA mutants easily, we isolated a pomA che double mutant. The plasmid pSK1-Δ28 (Asai et al., 1997) was introduced into strain VIO856 (PomA+), and EMS mutagenesis was carried out on the transformants (Asai et al., 1997). A che mutant was selected as described (Hommia et al., 1996). The plasmid pSK1-Δ28 was removed from this che mutant by growing in VC medium without kanamycin, and a kanamycin-sensitive clone was selected. The isolated pomA che mutant, named NMB188, could swim with a Che+ phenotype and the same swimming speed as the wild-type strain when a plasmid containing pomA was introduced.

Electroporation
Transformation of Vibrio cells by electroporation was carried out as described (Kawagishi et al., 1994) with the Gene Pulser (Japan Bio-Rad Laboratories, Tokyo) at an electric field strength between 5.0 and 7.5 kV/cm.

Measurement of swimming speed
Cells were harvested at late logarithmic phase, and resuspended in TMN medium. Then a small amount of the cell suspension was diluted about 10-fold into TMN medium with or without the inhibitors, and motility of the cells was observed at room temperature under a dark-field microscope and recorded on video tape. Swimming speed was determined as described (Atsumi et al., 1996). The average swimming speed was obtained by measuring more than 20 swimming tracks.

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References


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