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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 Mpa^r (motility resistant to phenamil) strains were mapped to the *pomA* and/or *pomB* genes. We cloned and sequenced *pomA* and *pomB* from two Mpa^r 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 Mpa^r 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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Keywords: flagella; phenamil; amiloride; motor; sodium channel

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 mechanochemical 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 extracellar 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

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MotB, a unique acidic residue in the membrane segment of MotB, is speculated to function in conveying protons. This Asp residue is well conserved among species. Recently 15 different substitutions were studied at this position; only the conservative replacement D32E mutant retained any function (Zhou *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; Sockett *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 (Tang *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), each have a single putative transmembrane segment, and PomA and PomB have four and one transmembrane segments, respectively. Because of sequence similarity to MotA and MotB, PomA and PomB are thought to be the Na^+ channel components. MotX is also inferred to be a Na^+ channel component of the motor because overproduction of MotX is lethal in *Escherichia coli* in proportion to the external Na^+ concentration, and because this lethality is suppressed by the addition of amiloride, which is known to be a potent inhibitor of Na^+ channels (Cragoe *et al*., 1992) and of Na^+-driven flagellar motors (Sugiyama *et al*., 1988). Now, residues in these components that might play important roles in torque generation are being intensively investigated.

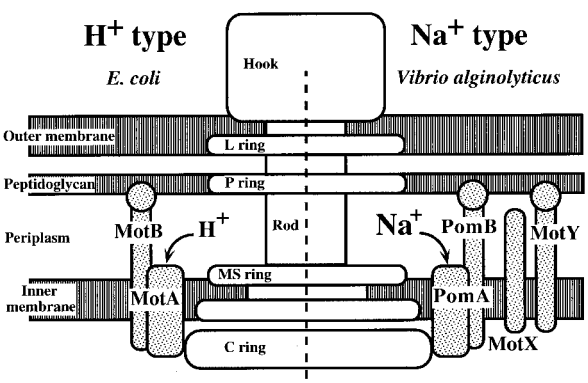

Figure 1. Bacterial flagellar motors of the sodium-driven and proton-driven type.

Amiloride specifically inhibits Na^+-driven flagellar motors by competing with Na^+ in the medium, suggesting that it interacts with the sodium binding sites located at the outer side of the motors (Sugiyama *et al*., 1988). Among amiloride analogs, phenamil is the most potent inhibitor for the Na^+-motor. In contrast to amiloride, inhibition by phenamil is non-competitive with respect to Na^+ in the medium (Atsumi *et al*., 1990), suggesting that a major interaction site of phenamil on the motor is not identical to the sodium binding site on the motor. Interestingly, motor rotation rate fluctuates greatly in the presence of phenamil, but not amiloride, a phenomenon that can be explained in terms of a relatively slow dissociation rate of phenamil from the force-generating units as compared with amiloride (Muramoto *et al*., 1996). Phenamil might interact more strongly with its site and bind more stably than amiloride to the Na^+ channel of the motor. In addition, because of 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^r), whose motors rotate more stably than wild-type in the presence of phenamil (Kojima *et al*., 1997). This steadier rotation of the Mpa^r motors can be explained by the rapid dissociation of phenamil from the high-affinity binding site in the Na^+ channel of Mpa^r motor as compared with the wild-type. Therefore, these Mpa^r mutations presumably affect genes encoding components of the force-generating units, such as *pomA*, *pomB*, *motX* and *motY*. Here, we have mapped the Mpa^r 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^r mutation of strain NMB201 is in *pomA* and/or *pomB*

On the basis of evidence presented in the previous study of Mpa^r strains (Kojima *et al*., 1997), we expected that the Mpa^r 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^r strains NMB201 and NMB205 by using the *pom* mutants with paralyzed polar

flagella derived from them. Such a Pom$^-$ Mpa^r 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$^-$ strain VIO586 which harbored pHK2 (wild-type control), suggesting that the Mpa^r phenotype of NMB161 was suppressed by the wild-type *pomA* and/or *pomB* gene. This suggests that the Mpa^r 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^r 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 *Sac*I 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^r mutation of NMB205. Then, from this *Sac*I 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$^-$) or NMB104 (PomB$^-$) cells containing pSK502 or pSK602, respectively (Figure 3(b)). Motility of the wild-type control strains, VIO586/pSK1(*pomA*$^+$) and NMB104/pHK4(*pomB*$^+$), 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^r mutation in NMB205 was in the *pomA* gene.

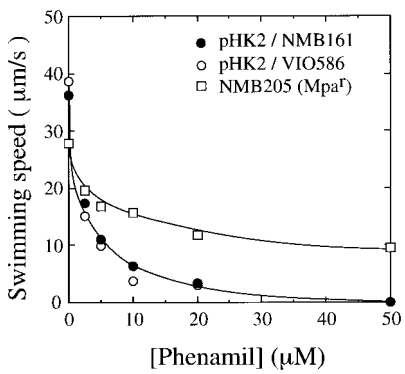

Figure 2. Inhibition of motility of NMB161/pHK2 cells by phenamil. Cells were harvested at late logarithmic phase and suspended in TMN medium (pH 7.5, 50 mM NaCl). The cell suspension was diluted about 100-fold into TMN medium containing various concentrations of phenamil. Swimming speed was measured as described in Materials and Methods.

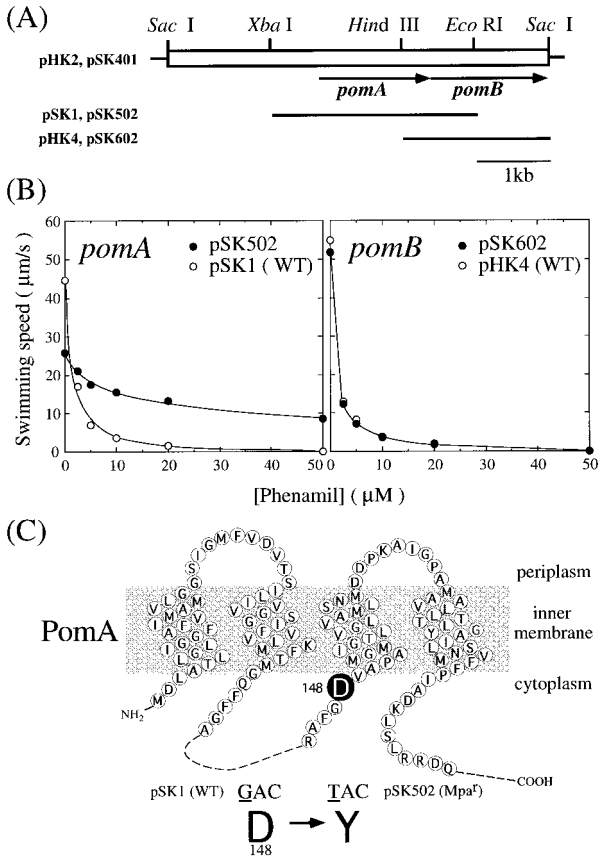

Figure 3. Single amino acid substitution in the cytoplasmic face of PomA causes phenamil resistance. (a) Restriction map of plasmids containing genes of a wild-type strain (*pomA* and *pomB*; pHK2, *pomA*; pSK1, *pomB*; pHK4) or Mpa^r strain NMB205 (*pomA* and *pomB*; pSK401, *pomA*; pSK502, *pomB*; pSK602). Genes from NMB205 were isolated by shot-gun cloning, using the VIO586 (PomA$^-$) strain as a recipient, and then subcloned. (b) Cells containing *pomA* derived from the Mpa^r strain NMB205 show phenamil-resistant motility. Swimming speed of cells containing either the *pomA* gene (left panel) or the *pomB* gene (right panel) derived from the Mpa^r strain NMB205 (●), or from a wild-type strain (○), was measured in TMN medium (NaCl 50 mM) containing various concentrations of phenamil, as described in Materials and Methods. For the host strain, VIO586 (PomA$^-$) and NMB104 (PomB$^-$) were used, in the left and right panels, respectively. (c) The predicted transmembrane regions of PomA. The Mpa^r mutation of PomA D148Y is located near the cytoplasmic end of a membrane segment of PomA. The topology of PomA was predicted from the hydropathy profile of PomA, and the topology of the MotA protein of *E. coli* (Zhou *et al*., 1995).

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), suggesting that this residue is not specific to Na^+-motors. When pSK502 was introduced into the Mpa^s (wild-type) strain, the transformants showed an Mpa^r 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^r 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^s 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.

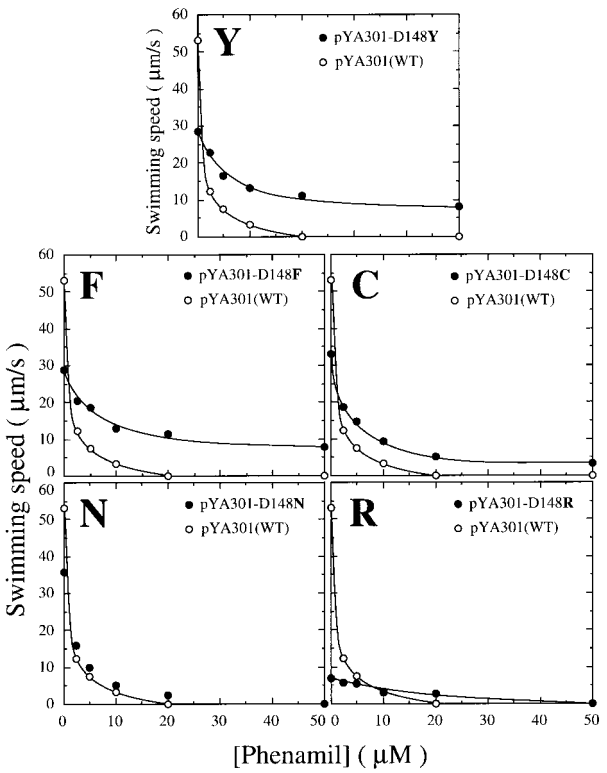

Figure 4. Phenamil resistance of PomA Asp148 mutants. Swimming speeds were measured in TMN medium containing various concentrations of phenamil as indicated in Materials and Methods, for the strain NMB188 ($PomA^-$ Che^-) expressing the mutant PomA proteins D148F, D148C, D148N, D148R, and D148Y. Filled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

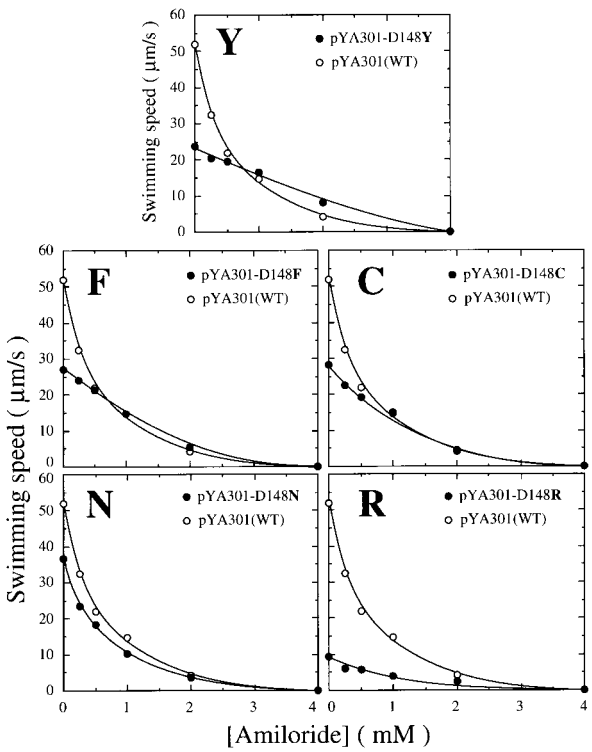

Figure 5. Sensitivity to amiloride of PomA Asp148 mutants. The experimental conditions and symbols are as indicated for Figure 4, except the TMN medium contained the indicated concentrations of amiloride.

Another Mpa^r mutation is in the *pomB* gene

As described above, we assumed that the Mpa^r 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^s phenotype (data not shown). The Mpa^r 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 *Eco*RI 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^r phenotype. We also introduced the P16S mutation into wild-type *pomB* by site-directed mutagenesis, and confirmed Mpa^r 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^s strain VIO5, the transformed cells showed an Mpa^r phenotype.

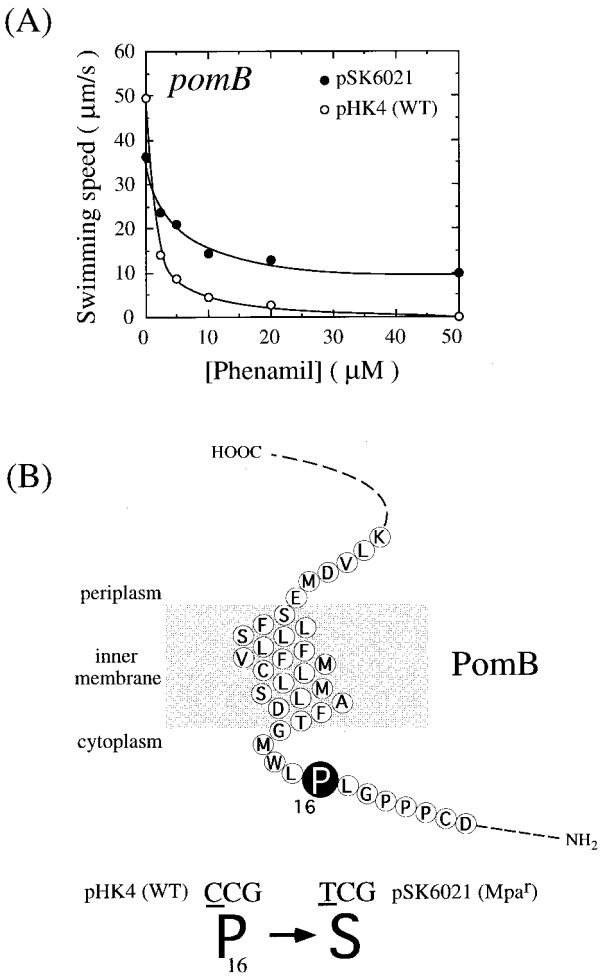

Figure 6. The mutation of another Mpa^r strain, NMB201, maps to the *pomB* gene. (a) Cells containing *pomB* derived from the Mpa^r strain NMB201 showed phenamil-resistant motility. The *pomB* gene of this strain, cloned by using PCR, was introduced into the *pomB* mutant (NMB104). Swimming speeds of these cells were measured as described in Materials and Methods. (b) The predicted transmembrane region of PomB. The Mpa^r 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).

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 *lacI*^{q} 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 be then 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 IC_{50} 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 IC_{50} 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

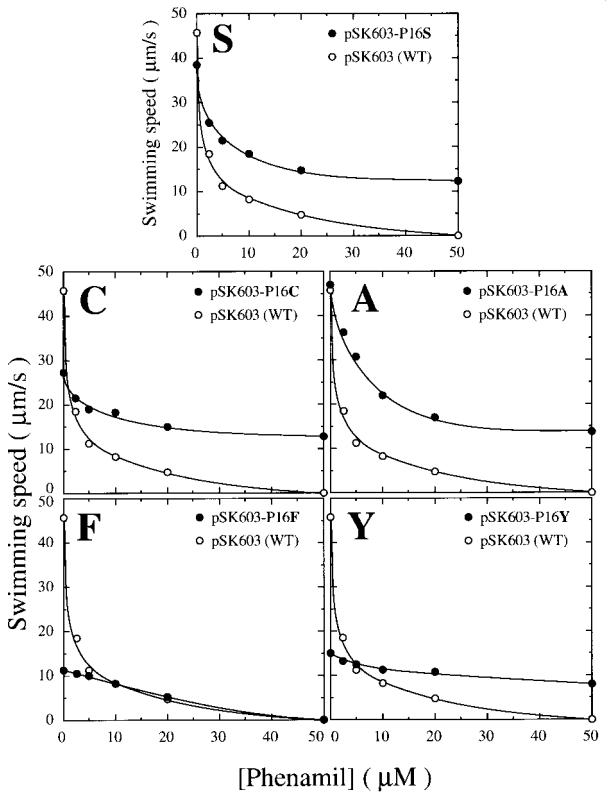

Figure 7. Phenamil resistance of PomB Pro16 mutants. Plasmids coding the PomB mutants P16S, P16A, P16C, P16F and P16Y were co-introduced into the strain NMB104 along with pMMB206, which contains the *lacI*^{q} gene. Mutant *pomB* genes were induced by 1 mM IPTG, as described in Materials and Methods, and swimming speeds were measured in TMN medium containing various concentrations of phenamil, as indicated in Materials and Methods. Filled (●) and open (○) symbols show the speed of mutants and wild-type, respectively.

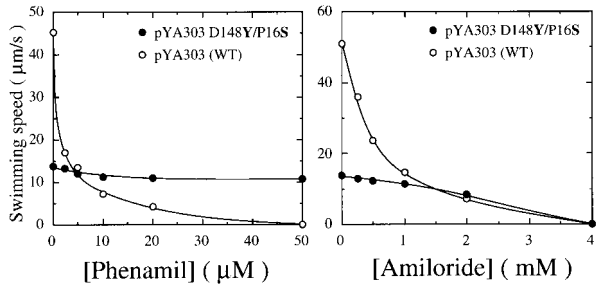

Figure 8. Motility inhibition of the PomA D148Y/PomB P16S double mutant by phenamil and amiloride. The plasmid containing both PomA D148Y and PomB P16S mutations (pYA303-D148Y/P16S) was introduced into the strain NMB188 (PomA$^-$ Che$^-$). Swimming speeds were measured as indicated in Materials and Methods.

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 IC_{50} 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^r 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^r mutants), and speculated that mutations occurred in a high-affinity phenamil-binding site (Kojima *et al.*, 1997). Here, we showed that two Mpa^r 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^r 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^r mutations in PomA and PomB also conferred some amiloride resistance. In addition, the competition of amiloride with Na^+ in the medium was significantly decreased in these Mpa^r mutants (data not shown). This may indicate that the amiloride-binding site is affected by the Mpa^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^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.

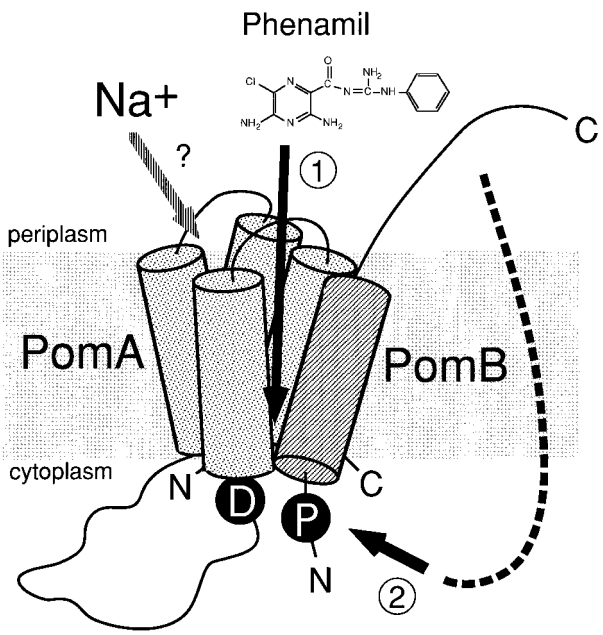

Figure 9. Working model for the interaction of phenamil with the PomA/PomB Na^+ channel complex. If phenamil interacts directly with PomA Asp148 or PomB Pro16, it has to go through the channel pore (①) or permeate the lipid bilayer (②) to reach the cytoplasmic side of the transmembrane segments.

In the case of the H^+-motor, it was suggested that MotA and MotB form a complex that functions as a H^+ 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^+ 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 μM phenamil the D148Y/P16S double mutant exhibited an Mpa^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^+ 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^r mutations in PomA and PomB conferred some amiloride resistance and an internal Na^+ 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^+/H^+ exchanger. Mutations that cause a large decrease in K_i for amiloride were found to occur in the second transmembrane segment of the α 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 extracellar domain of αrENaC, 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 Mpa^s 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) K_2HPO_4, 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 % K_2HPO_4, 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 $MgCl_2$, 5 mM glucose, 50 mM NaCl, 250 mM KCl. For the induction of the *pom* genes in plasmids, cells were grown in VC medium containing 1 mM IPTG (isopropyl-1-thio-β-D-garactoside). *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

Strains or plasmids	Genotype or description[a]	References or sources
V. alginolyticus strains		
VIO586	Rif^r Pof^+ Laf^- $PomA^-$	(Asai *et al*., 1997)
NMB104	Pof^+ Laf^- $PomB^-$	(Asai *et al*., 1997)
NMB201	Rif^r Pof^+ Laf^- Mpa^r	(Kojima *et al*., 1997)
NMB205	Rif^r Pof^+ Laf^- Mpa^r	(Kojima *et al*., 1997)
NMB161	Rif^r Pof^+ Laf^- Mpa^r $PomB^-$	(Asai *et al*., 1997)
NMB188	Rif^r Pof^+ Laf^- $PomA^-$ Che^-	This work
E. coli strains		
DH5α	F^- λ^- *recA1 hsdR17 endA1 supE44 thi-1 relA1 gyrA96* Δ(*argF-lacZYA*)U169 Φ80d*lacZ*ΔM15	(Grant *et al*., 1990)
XL1-Blue	*recA1 hsdR17 supE44* Δ(*lac-proAB*){F′::Tn*10 proAB lacI*^{q}ZΔM15}	Stratagene
Plasmids		
pSU21	*cat*(Cm^r) P_{lac}*lacZ*α	(Bartolomé *et al*., 1991)
pSU41	*kan*(Km^r) P_{lac}*lacZ*α (MCS same as that in pSU21)	(Bartolomé *et al*., 1991)
pYA301	0.8 kb *Bam*HI fragment (P_{lac}-*pomA*) in pSU41	This work
pYA303	1.9 kb *Bam*HI-*Sac*I fragment (P_{lac}-*pomAB*) in pSU41	This work
pHK2	2.9 kb *Sac*I fragment (*pomAB*) in pSU21	(Asai *et al*., 1997)
pHK4[b]	1.2 kb *Hin*dIII-*Sac*I fragment (P_{lac}-*pomB*) in pSU41	This work
pSK1	1.5 kb *Xba*I-*Eco*RI fragment (*pomA*) in pSU41	(Asai *et al*., 1997)
pSK1-Δ28	329 bp deleted from *Xba*I site of pSK1 (lack of the native promoter of *pomA*)	(Asai *et al*., 1997)
pSK401	2.9 kb *Sac*I fragment (*pomAB*) from NMB205 in pSU21	This work
pSK502	1.5 kb *Xba*I-*Eco*RI fragment (*pomA*) from NMB205 in pSU41	This work
pSK602[b]	1.2 kb *Hin*dIII-*Sac*I fragment (P_{lac}-*pomB*) from NMB205 in pSU41	This work
pSK5021	0.8 kb *Bam*HI-*Eco*RI fragment (P_{lac}-*pomA*) from NMB201 in pSU41	This work
pSK6021	1.1 kb *Bam*HI-*Sac*I fragment (P_{lac}-*pomB*) from NMB201 in pSU41	This work
pSK603	1.1 kb *Bam*HI-*Sac*I fragment (P_{lac}-*pomB*) in pSU41	This work
pMMB206	*cat*(Cm^r) *IncQ lacI*^{q} Δ*bla* $P_{tac\text{-}lac}$ *lacZ*α	(Morales *et al*., 1991)

[a] Cm^r, chloramphenicol-resistant; Km^r, kanamycin-resistant; MCS, multicloning site; P_{lac}, *lac* promoter; $P_{tac\text{-}lac}$, tandemly located *tac* and *lac* promoters; Rif^r, rifampicin-resistant.

[b] The 1.2 kb *Hin*dIII-*Sac*I 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 PRISMT™ Dye 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^r strain NMB205 (Kojima *et al*., 1997) were digested with *Sac*I and ligated. These DNA libraries were introduced into cells of the *pomA* mutant (VIO586) 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^r 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′-dCTCGGATCCGCCCAATGGCAGTATTTGA-3′, and generated a *Bam*HI site. The antisense-primer (designated *pomA*-E2) was 5′-dCTCGAATTCAAGTTACTCGTCAATCTCA-3′, and generated an *Eco*RI site. PCR primers complementary to each end of *pomB* were also synthesized: The sense-primer (designated *pomB*-A) was 5′-dATTGGATCCCGTGCCCTTGAGATTGAC-3′, and generated a *Bam*HI site. The antisense-primer (designated *pomB*-Z) was 5′-dTAGGATCCGAGCTCACTTTTAAGGCG-3′, and contained both a *Sac*I site and a *Bam*HI 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 fragments containing the full-length genes were digested with *Bam*HI and *Eco*RI for *pomA*, or with *Bam*HI and *Sac*I 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 *Hin*dIII-*Sac*I fragment containing the 3′-terminal 155 bp of *pomA* and full-length *pomB* which has the PomB-P16S mutation was amplified, digested with *Hin*dIII and *Sac*I, and ligated into pSU41 (named pHK4-P16S). Then the *Hin*dIII fragment from pYA301-D148Y was inserted into the *Hin*dIII 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 (*pomAB*$^+$), so it was named pYA303-D148Y/P16S.

Isolation of PomA$^-$ Che$^-$ 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 VIO586 (PomA$^-$), and EMS mutagenesis was carried out on the transformants (Asai *et al*., 1997). A *che* mutant was selected as described (Homma *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 100-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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