J. Mol. Biol. (1997) **265**, 310–318

JMB

Vibrio alginolyticus Mutants Resistant to Phenamil, a Specific Inhibitor of the Sodium-driven Flagellar Motor

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The polar flagella of *Vibrio alginolyticus* are driven by sodium motive force and those motors are specifically and strongly inhibited by phenamil, an amiloride analog that is thought to interact with a sodium channel of the flagellar motor. To study the sodium ion coupling site, we isolated motility mutants resistant to phenamil and named the phenotype Mpa^r for motility resistant to phenamil. The motility of the wild-type (Mpa^s) was inhibited by 50 μM phenamil, whereas Mpa^r strains were still motile in the presence of 200 μM phenamil. The K_i value for phenamil in the Mpa^r strain was estimated to be five times larger than that in the Mpa^s strain. However, the sensitivities to amiloride or benzamil, another amiloride analog, were not distinctly changed in the Mpa^r strain. The rotation rate of the wild-type Na^+-driven motor fluctuates greatly in the presence of phenamil, which can be explained in terms of a relatively slow dissociation rate of phenamil from the motor. We therefore studied the stability of the rotation of the Mpa^r and Mpa^s motors by phenamil. The speed fluctuations of the Mpa^r motors were distinctly reduced relative to the Mpa^s motors. The steadier rotation of the Mpa^r motors can be explained by an increase in the phenamil dissociation rate from a sodium channel of the motor, which suggests that a phenamil-specific binding site of the motor is mutated in the Mpa^r strain.

Keywords: flagella; amiloride; *Vibrio*; motor; sodium-motive force

Introduction

Bacterial flagellar motors are molecular machines powered by the electrochemical potential gradient of specific ions across the membrane (Blair, 1995; Imae & Atsumi, 1989). The flagellar motors can be classified by coupling ion: some are H^+-driven and some are Na^+-driven. The motor contains several independently functioning force-generating units, which should interact with coupling ion at the external surface of the cytoplasmic membrane and function as ion channels. It has been estimated that the H^+-driven motor of *Escherichia coli* and the Na^+-driven motor of an alkalophilic *Bacillus* have eight and five to nine force-generating units, respectively (Block & Berg, 1984; Blair & Berg, 1988; Muramoto *et al*., 1994). For studies of the flagellar motor, the Na^+-driven type presents certain advantages: the sodium motive force can be easily changed by the Na^+ concentration of the medium and the motor rotation can be specifically and competitively inhibited by amiloride (Sugiyama *et al*., 1988), which are potent inhibitors of Na^+ channel, Na^+/H^+ exchangers, and Na^+/Ca^{2+} exchangers (Cragoe *et al*., 1992). An amiloride analog, phenamil, whose inhibition is non-competitive with Na^+, is more potent than amiloride without affecting cell growth of alkalophiles (Atsumi *et al*., 1990; Sugiyama *et al*., 1988). The inhibitors are thought to interact with the force-generating units of the motors (Sugiyama *et al*., 1988; Atsumi *et al*., 1990; Muramoto *et al*., 1994, 1995).

Some marine *Vibrio* species have both types of motors in one cell. A single polar flagellum (Pof) rotates by a Na^+-driven motor and multiple lateral

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Abbreviations used: Pof, polar flagellum; Laf, lateral flagellum; Mpa^r, motility resistant to phenamil; LDM, laser-dark field microscopy.

0022–2836/97/030310–09 $25.00/0/mb960732

flagella (Laf) rotate by H^+-driven motors (Atsumi *et al*., 1992a; Kawagishi *et al*., 1995). The polar flagella work better for swimming in a low-viscosity environment than the lateral flagella (Atsumi *et al*., 1996). The restriction of the function of the polar flagellum appears to trigger the expression of lateral flagella (Kawagishi *et al*., 1996; McCarter *et al*., 1988). The polar flagella are covered with a sheath that is contiguous with the outer membrane (Follett & Gordon, 1963; Allen & Baumann, 1971) and the single filament is easily observed under the dark-field microscope.

The high-speed rotation of flagella has been analyzed by laser-dark field microscopy (LDM; Kudo *et al*., 1990; Kami-ike *et al*., 1991). Using this system, it was shown that the rotation of polar flagella was very fast in *V. alginolyticus*; the speeds of 600 r.p.s. were observed in the presence of 50 mM NaCl at room temperature, and surprisingly, the speeds increase up to 1700 r.p.s. in the presence of 300 mM NaCl at 35°C (Magariyama *et al*., 1994, 1995; Muramoto *et al*., 1995). It was also shown that the rotation of this motor was stable at high speed (Muramoto *et al*., 1995). The standard deviation of the rotation period was only 7% to 16% of the average rotation period in polar flagella of *V. alginolyticus*. When the rotation rate was reduced by decreasing external concentration of NaCl or adding amiloride or carbonylcyanide *m*-chlorophenylhydrazone (CCCP), the motor rotated smoothly. On the other hand, we found that the polar flagella showed remarkably larger speed fluctuations when the rotation rate was reduced by phenamil (Muramoto *et al*., 1996). The speed fluctuations induced by phenamil can be explained by the slow dissociation rate of the inhibitor from the force-generating unit.

To identify the force-generating units and investigate the sodium ion coupling site of the motor, it would be useful to identify the target of amiloride and its analogs in the flagellar motor. In this work, we synthesized phenamil, which had not been commercially available, isolated *V. alginolyticus* motility mutants resistant to phenamil and characterized the mutants with respect to the effect of phenamil on the rotation of the motor.

Results

Isolation of motility mutants resistant to phenamil

We tried to isolate mutants whose flagellar motors could function in the presence of amiloride or its more potent analogs, phenamil and benzamil. Experiments using amiloride or benzamil, which are commercially available, were not successful because their effects seem to be weak in the growth medium of *V. alginolyticus* whose ionic strength and Na^+ concentration are high (Atsumi *et al*., 1992b). Phenamil, which is known to be the most potent inhibitor of Na^+-driven motors was then synthesized by Cragoe's methods (Cragoe *et al*., 1967). It has been reported that the cell growth of alkalophiles and *V. parahaemolyticus* is not affected by phenamil, even at concentrations that fully block rotation of the flagellar motors (Atsumi *et al*., 1990, 1992b; Kawagishi *et al*., 1996). *V. alginolyticus* cells grew normally in VC medium containing a high concentration (500 μM) of phenamil, while motility of the wild-type cells was completely inhibited (data not shown). This indicates that phenamil works as a specific inhibitor of the Na^+-motors of *V. alginolyticus*, without affecting cell growth.

V. alginolyticus VIO5 (Pof^+ Laf^- Rif^r) cells were treated with ethylmethane sulfonate (EMS) and streaked onto the surface of 0.25% agar VD plates supplemented with 500 μM phenamil, and the plates were incubated. Cells that spread out from the point of inoculation on the plate were isolated, and two isolates were named NMB201 and NMB205. We confirmed the resistant phenotype in a 0.25% agar VG plate containing 200 μM phenamil (Figure 1). Because these mutants and the parent showed very tumble-biased swimming in the presence of inhibitors, it was quite difficult to measure their swimming speed. We therefore isolated smooth swimming (*che*) mutants, NMB136 (from VIO5), NMB157 (from NMB201), and NMB160 (from NMB205). These *che* mutants swam at the same swimming speed as the parent strains, and showed the same inhibition profiles against the phenamil concentrations (data not shown). Thus, the motor function seems not to be affected in the *che* mutants. NMB136 (phenamil-sensitive) and NMB157 (phenamil-resistant) were then characterized more closely. We named the phenotype Mpa^r for *m*otility *r*esistant to *p*hen*a*mil. Accordingly, the wild-type phenotype was designated as Mpa^s.

Effect of specific inhibitors on the mutants

We first examined whether the motility of Mpa^r cells (NMB157) and Mpa^s cells (NMB136) showed

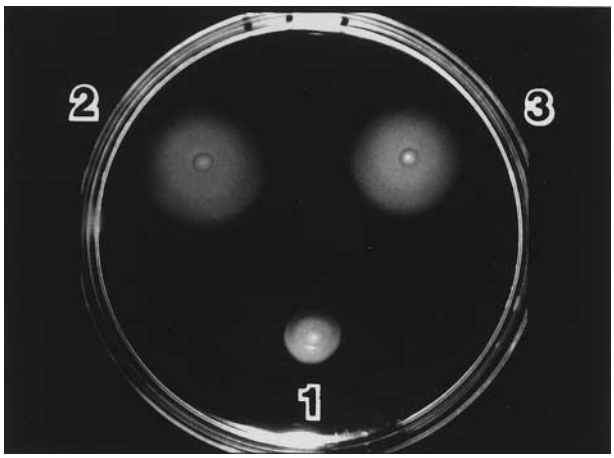

Figure 1. Swarming abilities of the mutants in the presence of phenamil. A VG semi-solid plate containing 200 μM phenamil was incubated at 30°C for 20 hours. 1, the Mpa^s parent strain (VIO5); 2 and 3, the Mpa^r mutants (NMB201 and NMB205).

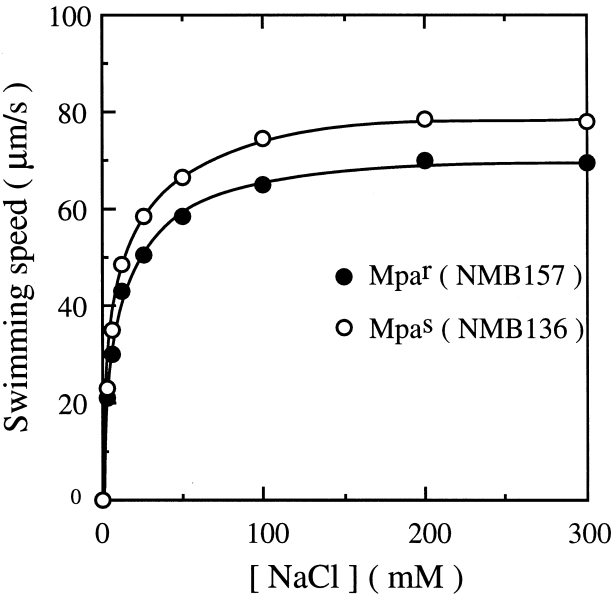

Figure 2. Relationship between Na^+ concentration of media and swimming speed of Mpa^r cells. Cells from the Mpa^r strain (●) or the Mpa^s strain (○) were harvested at late logarithmic phase and suspended in TNM medium (pH 7.5). The cell suspension was diluted about 100-fold into the TNM medium containing various concentrations of NaCl. Swimming speed was measured within one minute as described in Materials and Methods.

similar dependence on the external Na^+ concentration. Their motility profiles were indistinguishable, although Mpa^r cells swam a little slower than Mpa^s cells (Figure 2). This suggests that the affinity for Na^+ is not affected by the mutation and the Na^+ binding region is essentially unchanged in Mpa^r cells.

Next, we examined the swimming speed of Mpa^r cells in various concentrations of phenamil, amiloride or benzamil (Figure 3). The motility of Mpa^s cells was completely inhibited by 50 μM phenamil. In contrast, Mpa^r cells still swam at 12 μm/second and were stopped only by 500 μM phenamil. When amiloride was used, differences between Mpa^r and Mpa^s cells were less pronounced. Mpa^r cells swam 20 or 30% faster than Mpa^s cells in the range of 0.5 mM to 2.0 mM amiloride. Difference in swimming speed were observed when Na^+ concentration was varied in the presence of 1 mM amiloride. At lower Na^+ concentrations, Mpa^r cells swam faster than Mpa^s cells (data not shown). This difference indicates that the mutation affects the amiloride sensitivity of the motor. However, we could not detect any difference between the swarming abilities of Mpa^r and Mpa^s strains in a semi-solid plate containing 1 mM amiloride. The inhibition profile by benzamil, whose potency is intermediate between phenamil and amiloride (Sugiyama *et al*., 1988; Imae., 1991), showed no difference between the Mpa^r and Mpa^s strain. These results suggest that the mutation affects mainly the phenamil interaction site in the Na^+-driven motor.

To quantify the effect of the mutation on phenamil binding, we estimated the inhibition constant (K_i) in both strains. We measured their swimming speeds in the presence of 6.25, 25, and 100 mM NaCl under various concentrations of phenamil. Figure 4 is a Dixon plot of the phenamil concentration and the Na^+ influx estimated from the swimming speed by a kinetic analysis described previously (Sugiyama *et al*., 1988). This shows that the inhibition profile of phenamil in a Mpa^r strain changes into a rather competitive manner with Na^+. The K_i values of the Mpa^s and Mpa^r strains were 0.14 μM and 0.74 μM, respectively.

Fluctuation of the rotation rate in the phenamil-resistant mutant

It has been shown that the rotation rate becomes very unstable in the presence of phenamil but not in the presence of amiloride or CCCP, even when the inhibitors are used at concentrations that reduce the rotation rate by the same amount (Muramoto *et al*., 1996). We investigated the stability of Mpa^r flagellar rotation at 50 mM NaCl in the presence of phenamil and amiloride. The Mpa^r and Mpa^s cell bodies were fixed on the glass surface, and the free rotation of a single flagellum was measured by using laser-dark field microscopy (LDM). Figure 5 shows the periodic change in photon counts observed under each condition. When the rotation rate of a Mpa^s flagellum was decreased to about 200 r.p.s. by 1 μM phenamil, large fluctuations in rotation rate were observed (Figure 5(A)). In the case of a Mpa^r cell, when the rotation rate was decreased to about 200 r.p.s. by 5 μM phenamil, the speed fluctuations were significantly smaller than in the Mpa^s strain (Figure 5(B)). On the other hand, when the rotation rate was decreased to about 200 r.p.s. by 0.5 mM amiloride, both the Mpa^r and Mpa^s flagella rotated very smoothly and there was little change of the rotation period, peak-to-peak interval (Figure 5(C) and 5(D)). The fluctuations seen with phenamil in the Mpa^r strain were still larger than those seen with amiloride.

Rotation rates in the presence of phenamil or amiloride were calculated from the rotation periods and their distribution plotted on histograms (Figure 6). When the rotation rate was reduced by phenamil, the rotation rate of the Mpa^s flagella fluctuated between 50 and 500 r.p.s., and the slow rotation sometimes persisted for relatively long times (Figure 6(A)). The variation in the rotation period of Mpa^r flagella was smaller, and periods of slow rotation were shorter (Figure 6(B)). In the presence of amiloride, the rotation rates of both Mpa^r and Mpa^s flagella were distributed sharply around the average rotation rate (Figure 6(C) and (D)).

To evaluate this difference in various cells, we obtained the standard deviation of the rotation rate (σ_ω) and expressed the fluctuation as $(\sigma_\omega/\bar{\omega})\times$

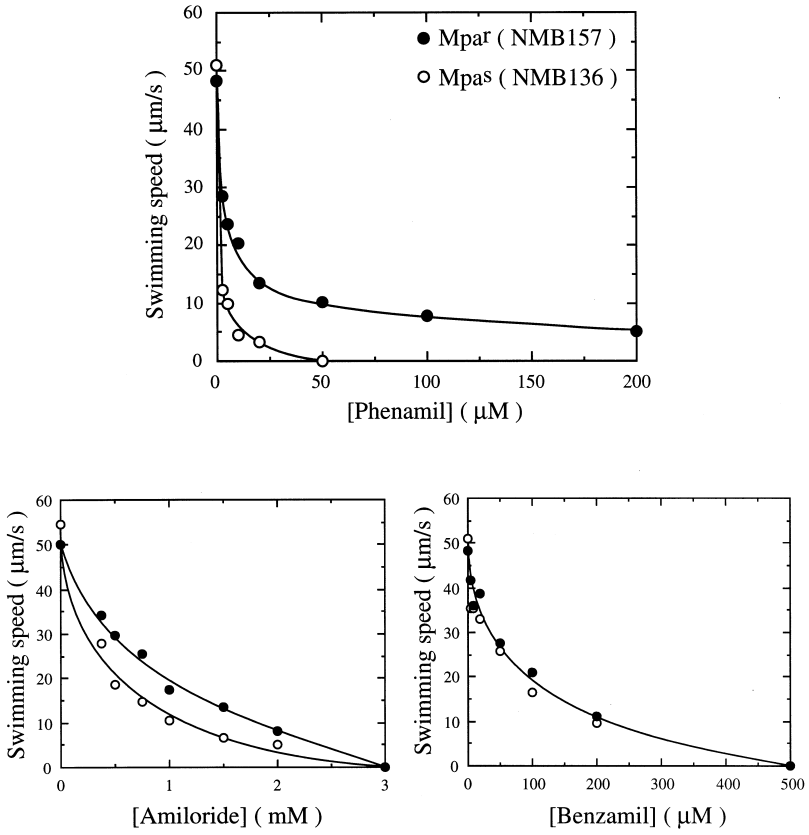

Figure 3. Motility inhibition of Mpa^r cells by specific inhibitors. Cells of the Mpa^r strain (●) or the Mpa^s strain (○) were suspended in TNM medium containing various concentrations of inhibitors, and the swimming speed was measured as described in Materials and Methods.

100 (%). The data obtained from four cells are summarized in Table 1. In the presence of phenamil, the speed fluctuations of Mpa^s cells increased to 40%, and in Mpa^r cells 24%. When we used 0.5 mM amiloride as a control, both Mpa^s and Mpa^r strains showed the same fluctuation, about 16%. In the absence of phenamil or amiloride, the motors of Mpa^s and Mpa^r strains rotated at 640 and 550 r.p.s., respectively, with fluctuations of 15% (data not shown). These results indicate that the fluctuations are significantly reduced in the Mpa^r strain, relative to the wild-type.

Discussion

To study the Na^+-driven motor, we isolated motility mutants resistant to phenamil (Mpa^r). Phenamil is the most potent specific inhibitor of a Na^+-driven motor and does not severely affect cell growth or physiology. The growth and Na^+-dependent motility of the Mpa^r mutant were the same as those of the parent Mpa^s strain, which is wild-type as far as the polar flagellum is concerned. The mutant was significantly more resistant to phenamil, exhibiting an inhibition constant about five times larger than that of the Mpa^s strain. This suggests that the Mpa^r mutation affects the interaction between phenamil and the motor.

The sensitivity to benzamil was not changed in the mutant and the sensitivity to amiloride was only slightly reduced. These results suggest that the mutation affects mainly the phenamil interaction site in the flagellar motor, and to a lesser extent the amiloride interaction site. In previous work, motility mutants resistant to phenamil were isolated from an alkalophilic *Bacillus*. These mutants suggested that amiloride and phenamil have different interaction sites in the motor (Atsumi *et al*., 1990), because the sensitivity to amiloride was not affected. This difference between the two species might reflect differences in the mutations or in the Na^+ binding regions.

It has been observed that the motor rotation rate fluctuates in the presence of phenamil but not amiloride or CCCP (Muramoto *et al*., 1996) and it was suggested that this phenomenon is due to the longer duration of phenamil binding to the motor. If there are several force-generating units in one motor and each unit functions independently, then the number of functional units is proportional to the rotation rate and changes in this number cause the fluctuations of rotation rate. In the case of phenamil, since the dissociation rate constant should be small, phenamil binds stably to the motor. So unstable rotation at subnormal speed is detected by LDM. On the other hand, the interaction of amiloride with the force-generating units should be more rapid, and an average speed corresponding to the average number of active force-generating units is detected. The fluctuations of rotation rate, given by the tethering technique, in the H^+-motor of *E. coli* were explained by a model incorporating dissociation and association of the force-generating units (Kara-Ivanov *et al*., 1995).

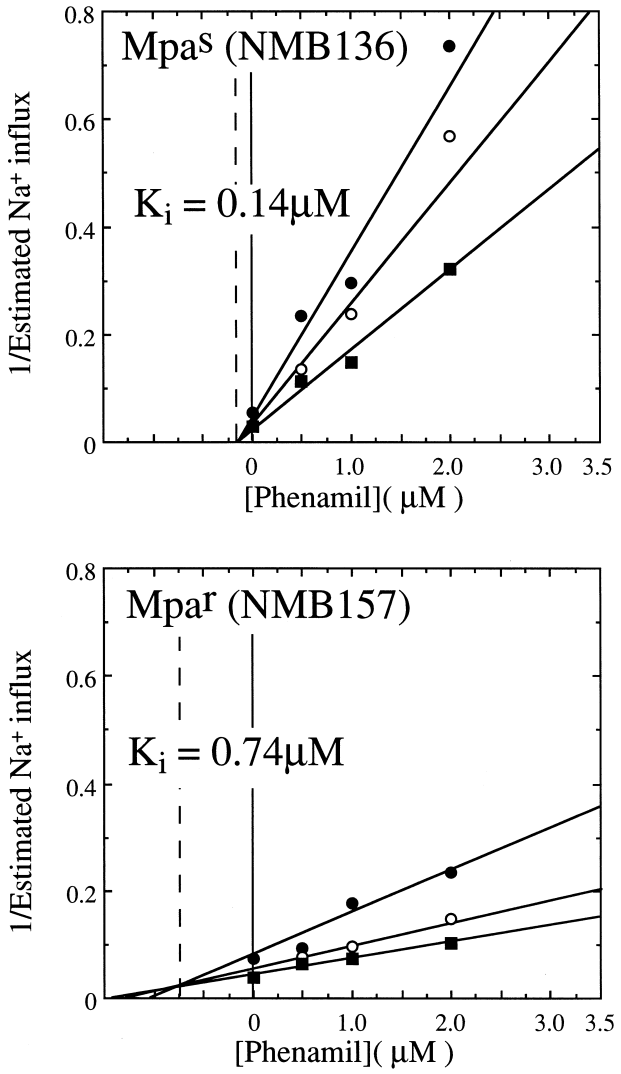

Figure 4. Effect of various concentrations of phenamil on the estimated Na^+ influx through the motors of Mpa^s (upper) and Mpa^r (lower). Cells were suspended in TNM medium containing 6.25 mM (●), 25 mM (○), or 100 mM (■) NaCl and various concentrations of phenamil were added. The swimming speed was measured as described in Materials and Methods and the Na^+ influx was estimated as described previously (Sugiyama *et al*., 1988). The sodium motive force was calculated by using the membrane potential of −110 mV and the intracellar Na^+ of 50 mM (Tokuda *et al*., 1988). A reciprocal plot (Dixon plot) of those data was performed. The units in the ordinate are given as mV s^2 μm^{-2}.

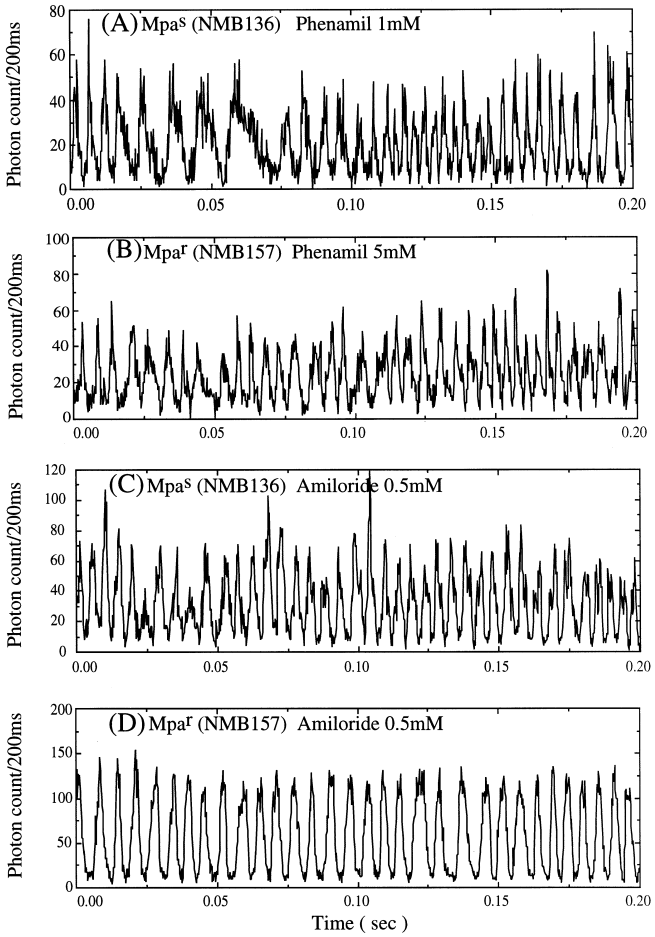

Figure 5. Mpa^s and Mpa^r flagellar rotation detected by LDM. Peak intervals of the intensity changes of the scattered light from the rotating flagella correspond to the rotation period. Cells harvested at late logarithmic phase were stuck on the cover glass and the rotation of single flagella were measured by LDM for about six seconds in the presence of phenamil or amiloride which decrease the rotation rate to about 200 r.p.s. (A) The Mpa^s flagella with 1 μM phenamil; (B) the Mpa^r flagella with 5 μM phenamil; (C) the Mpa^s flagella with 0.5 mM amiloride; (D) the Mpa^r flagella with 0.5 mM amiloride.

The model is essentially the same as our model though the time resolution is extremely different.

The phenamil resistance of the mutant isolated in this work can be explained by an increase in the dissociation rate of phenamil, a decrease in the association rate, or both. If the dissociation rate increases, the binding time of phenamil will be shorter, reducing the amplitude of the speed fluctuations. If the association rate decreases, then the binding time should not change, and fluctuation would not be affected. The LDM analysis indicates that the Mpa^r strain shows significantly smaller fluctuations than the Mpa^s strain. Accordingly, we suggest that phenamil may dissociate from the motor faster in the mutant and this causes the resistance to phenamil.

The interaction of phenamil and amiloride with the motor is speculated as follows. Amiloride is suggested to associate with the same site in the motor as do sodium ions, and quickly associate and dissociate with the motor, causing the competitive inhibition with Na^+. On the other hand, phenamil is assumed to interact at another high affinity site and can bind more stably to the motor than amiloride, causing the non-competitive inhibition with Na^+. Moreover, phenamil can also associate with the amiloride binding site because of structural similarity. In the Mpa^r motor, we think that the high affinity site for phenamil is mutated,

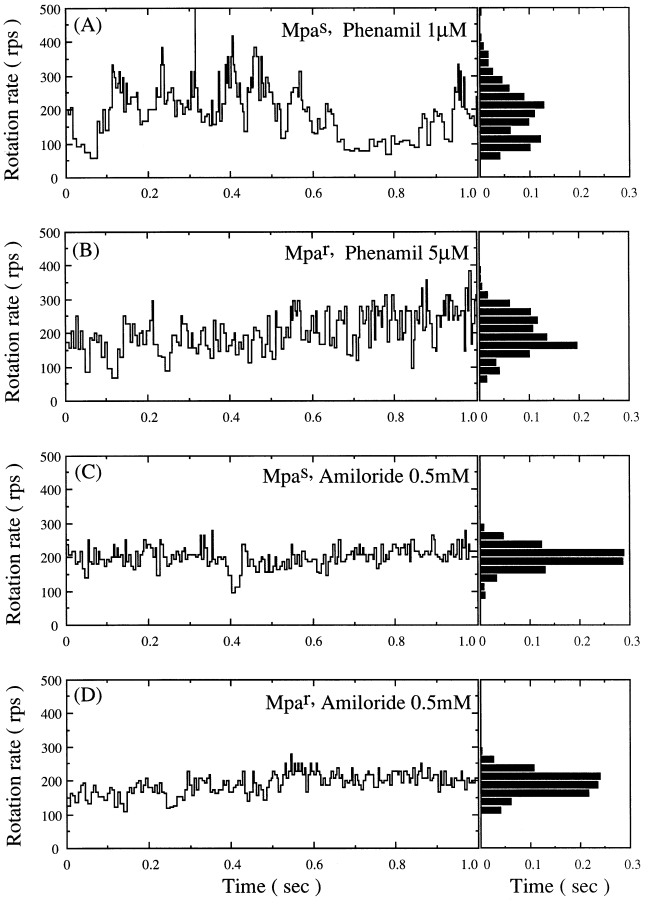

Figure 6. The rotation rate and its distribution of the Mpa^s and Mpa^r flagella in the presence of phenamil and amiloride. The rotation rate calculated from the data of Figure 5 for one second as described in Materials and Methods was shown in the left graphs. Histogram of rotation rate for one second was shown in the right graphs. (A) The Mpa^s flagella with 1 μM phenamil; (B) the Mpa^r flagella with 5 μM phenamil; (C) the Mpa^s flagella with 0.5 mM amiloride; (D) the Mpa^r flagella with 0.5 mM amiloride. Average rotation rate (ω) was 182 r.p.s. (A), 194 r.p.s. (B), 198 r.p.s. (C),188 r.p.s. (D). The values of the fluctuation were 42% (A), 29% (B), 15% (C), 17% (D).

so phenamil additionally interacts with the amiloride binding site, causing phenamil to dissociate more rapidly than in the Mpa^s motor and the intermediate inhibition profile between amiloride and phenamil. The mutation might also cause subtle structural changes around the common interaction site, because inhibition by amiloride was also slightly affected by the mutation. Benzamil, which is larger than phenamil, can slightly interact with the high affinity site for phenamil and the mutation may not greatly affect their inhibition profile.

The interaction between amiloride analogs and their targets has been studied in various Na^+-transporters and with anti-amiloride antibodies (Cragoe *et al*., 1992; Lin *et al*., 1994). In the fibroblast Na^+/H^+ exchanger (NHE1), which has ten transmembrane segments, a mutant resistant to amiloride was isolated (Counillon *et al*., 1993). This mutant has point mutations that convert Phe165 and Phe168 into Tyr. These residues are located in the middle of the fourth putative transmembrane segment. However, in the case of the epithelial Na^+ channel (ENAC), which is supposed to have six transmembrane segments, a recent report suggested that the amiloride-binding site resides on an extracellular loop of the α subunit and not the putative second transmembrane domain which forms a channel pore (Li *et al*., 1995). The amiloride-binding site may therefore be in very different locations in different proteins, although an additional possibility that the mutations in Na^+/H^+ exchanger mapped in membrane segments might cause a change in conformation that affects an extracellular binding site for amiloride cannot be eliminated. It is also known that phenamil is a more potent inhibitor of the Na^+ channel than the Na^+/H^+ exchanger (Cragoe *et al*., 1992). Since the Na^+-driven motor is also inhibited potently by phenamil, it might have more features in common with the Na^+ channel. However, the additional high affinity binding site of phenamil in the scheme described above has not yet been identified in the Na^+ channel.

Recently, the *motX* and *motY* genes, which seem to encode components of the force-generating units, were isolated from *Vibrio parahaemolyticus* (McCarter, 1994a,b). MotX is inferred to be a component of the Na^+ channel of the motor because overproduction of MotX was lethal in *E. coli* in proportion to the external Na^+ concentration, but this effect was suppressed by the addition of amiloride. The C-terminal domain of MotY is homologous to many peptidoglycan-interacting proteins. So, MotX and MotY, each of which has a putative single transmembrane region, form a channel component and a stator component, respectively, as the H^+ motor components of MotA and MotB (Blair, 1995). The *pom* (polar flagella motility) genes, whose mutants have paralysed polar flagella, identical to *motX* and *motY*, were detected in *V. alginolyticus* (Okunishi *et al*., 1996) and novel *pom* genes have been isolated (Asai, Y., Okunishi, I., Kojima, S., Kawagishi, I. & Homma, M., unpublished). We expected that the Mpa^r mutation would map to one of the genes coding for the motor proteins, or to other components of the force-generating units. Actually, the Mpa^r mutation was mapped in a *pom* gene other than *motX* and *motY* genes (Kojima, S., Asai, Y., Kawagishi, I. & Homma, M., unpublished). We hope to determine the target of phenamil and thereby obtain information about the mechanism by which Na^+ is used to drive the motor.

Materials and Methods

Bacterial strains, growth conditions and media

V. alginolyticus VIO5 (Okunishi *et al*., 1996) was used as the parent strain to isolate mutants. It is a Pof$^+$ Laf$^-$ Rif^r

Table 1. Summary of the results of effect of phenamil and amiloride on the stability of rotation rate of Mpa^r and Mpa^s strains obtained from four independent cells

		1	2	3	4	Average
Mpa^s (NMB136)	Rotation rate (r.p.s.)	197	207	131	182	179
1 μM Phenamil	Standard deviation (r.p.s.)	79	79	51	77	72
	Fluctuation (%)	40	38	39	42	40
Mpa^r (NMB157)	Rotation rate (r.p.s.)	170	221	160	194	186
5 μM Phenamil	Standard deviation (r.p.s.)	47	42	34	57	45
	Fluctuation (%)	28	19	21	29	24
Mpa^s (NMB136)	Rotation rate (r.p.s.)	198	138	162	158	164
0.5 mM Amiloride	Standard deviation (r.p.s.)	30	23	20	19	23
	Fluctuation (%)	15	17	12	12	14
Mpa^r (NMB157)	Rotation rate (r.p.s.)	188	270	258	258	243
0.5 mM Amiloride	Standard deviation (r.p.s.)	32	30	29	33	31
	Fluctuation (%)	17	11	11	13	13

strain derived from strain VIK4 (Okunishi *et al*., 1996), which is a rifampicin-resistant derivative of the wild-type strain 138-2 (Tokuda *et al*., 1988).

Cells were grown at 30°C with shaking in VG medium containing 1% (w/v) polypeptone, 0.5% (w/v) glycerol, 0.4% (w/v) K_2HPO_4, and 0.5 M NaCl. To select motility mutants, 0.25% (w/v) agar VD plates (0.25% agar in VD medium containing 0.5% polypeptone, 0.2% glucose, 0.5% yeast extract, 0.4% K_2HPO_4, 50 mM NaCl, and 250 mM KCl) were used.

Vibrio buffer containing 10 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 350 mM NaCl was used to wash the cells and TNM medium containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose, 50 mM NaCl, 250 mM KCl was used to measure the swimming speed. When the concentration of Na^+ in the motility medium was varied, KCl was added to keep the salt concentration constant.

Chemicals and anti-flagellar antibody

Amiloride and benzamil was purchased from Sigma Chemical Co. and Research Biochemical Incorporated, respectively. Phenamil, which was not commercially available, was synthesized by the method of Cragoe *et al*. (1967).

Anti-polar flagellar antibody was prepared by injecting a rabbit with purified polar flagella of *V. alginolyticus* strain YM4 (Pof^+ Laf^-) whose parent is the same as VIO5 (Kawagishi *et al*., 1995).

Isolation of motility mutants

Mutagenesis was carried out by using ethyl methanesulfonate (EMS). Cells of VIO5 were harvested by centrifugation from 1.0 ml culture at late logarithmic phase, washed once with *Vibrio* buffer, and resuspended in 0.5 ml of *Vibrio* buffer. Then, 15 μl of EMS was added to the suspension and incubated with mild shaking for one hour at 30°C. After washing the cells once with *Vibrio* buffer, they were resuspended in 0.5 ml of *Vibrio* buffer. EMS-treated cells were streaked onto the surface of a 0.25% agar VD plate supplemented with 500 μM phenamil, and the plate was incubated at 30°C for 12 hours. Mutants that spread out from the point of inoculation were isolated. Phenamil-resistant candidates were tested for rifampicin resistance and also for reactivity to the anti-polar flagellar antibody.

Smooth swimming mutants were isolated from the phenamil-resistant mutants to easily measure their swimming speeds. The *che* mutants were selected as described previously (Homma *et al*., 1996).

Measurement of swimming speed

Cells were harvested at late logarithmic phase and resuspended in TNM medium at half the original culture volume. Then a small amount of the cell suspension was diluted about 100-fold into TNM medium, and motility of the cells was observed under a dark-field microscope and recorded on video tape. Swimming speed was determined as described previously (Atsumi *et al*., 1996). The average swimming speed was obtained by measuring more than 20 swimming tracks.

Measurement and analysis of single flagellar rotation

High speed flagellar rotation was measured by laser-dark field microscopy (LDM) as described previously (Kudo *et al*., 1990; Muramoto *et al*., 1995). Cells were harvested at late logarithmic phase, washed once with TNM medium, and resuspended in the same medium. The cell suspension was poured into the space between slide and cover glass on which thin spacers were placed. After ten minutes, cells not stuck to the slide were washed by the flow of TNM medium. Rotation of the polar flagellum of a stuck cell was measured by using LDM for about six seconds. When the effect of inhibitor was measured, the stuck cells were washed with the flow of TNM medium supplemented with a suitable amount of inhibitor.

The photon counting gate time was set at 200 μs. The rotation rate was calculated as a reciprocal of the period of peak intervals, and average rotation rate ($\bar{\omega}$) and its standard deviation (σ_ω) for one second were calculated by the following equations (Muramoto *et al*., 1996).

$$\bar{\omega} = \frac{\sum_{k=1}^{N} \omega(k)\tau(k)}{\sum_{k=1}^{N} \tau(k)} = \frac{N}{t_0} \tag{1}$$

$$\sigma_\omega = \sqrt{\frac{\sum_{k=1}^{N} (\omega(k) - \bar{\omega})^2 \tau(k)}{\sum_{k=1}^{N} \tau(k)}}. \tag{2}$$

Here, $\omega(k)$ and $\tau(k)$ are the rotation rate and period for kth revolution, t_0 is the duration of measurement, N is the total number of revolutions.

Acknowledgements

We thank Dr Robert M. Macnab for invaluable discussions and Dr Kohtaro Tomizawa for assistance with the phenamil synthesis. We especially thank Dr David F. Blair for critical reading of the manuscript. This work was supported in part by grants-in-aid for scientific researches (to T.A., I.K. and M.H.) from the Ministry of Education, Science and Culture of Japan and by R & D program from NEDO of Japan (to I.K. and M.H.).

References

Allen, R. D. & Baumann, P. (1971). Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. *J. Bacteriol.* **107**, 295–302.

Atsumi, T., Sugiyama, S., Cragoe, E. J., Jr & Imae, Y. (1990). Specific inhibition of the Na^+-driven flagellar motors of alkalophilic *Bacillus* strains by the amiloride analog phenamil. *J. Bacteriol.* **172**, 1634–1639.

Atsumi, T., McCarter, L. & Imae, Y. (1992a). Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. *Nature,* **355**, 182–184.

Atsumi, T., Maekawa, Y., Tokuda, H. & Imae, Y. (1992b). Amiloride at pH 7.0 inhibits the Na^+-driven flagellar motors of *Vibrio alginolyticus* but allows the cell growth. *FEBS Letters,* **314**, 114–116.

Atsumi, T., Maekawa, Y., Yamada, T., Kawagishi, I., Imae, Y. & Homma, M. (1996). Effect of viscosity on swimming by the lateral and polar flagella of *Vibrio alginolyticus*. *J. Bacteriol.* **178**, 5024–5026.

Blair, D. F. (1995). How bacteria sense and swim. *Annu. Rev. Microbiol.* **49**, 489–522.

Blair, D. F. & Berg, H. C. (1988). Restoration of torque in defective flagellar motors. *Science,* **242**, 1678–1681.

Block, S. M. & Berg, H. C. (1984). Successive incorporation of force-generating units in the bacterial rotary motor. *Nature,* **309**, 470–472.

Counillon, L., Franchi, A. & Pouyssegur, J. (1993). A point mutation of the Na^+/H^+ exchanger gene (*NHE1*) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis. *Proc. Natl Acad. Sci. USA,* **90**, 4508–4512.

Cragoe, E. J., Jr, Woltersdorf, O. W., Jr., Bicking, J. B., Kwong, S. F. & Jones, J. H. (1967). Pyrazine diuretics. II. N-amidino-3-amino-5-substituted 6-halopyrazinecarboxamides. *J. Med. Chem.* **10**, 66–75.

Cragoe, E. J., Jr, Kleyman, T. R. & Simchowitz, L. (1992). *Amiloride and its Analogs: Unique Cation Transport Inhibitors*. VCH Publishers, Inc., New York.

Follett, E. A. C. & Gordon, J. (1963). An electron microscope study of *Vibrio* flagella. *J. Gen. Microbiol.* **32**, 235–239.

Homma, M., Oota, H., Kojima, S., Kawagishi, I. & Imae, Y. (1996). Chemotactic responses to an attractant and a repellent in the flagellar systems of *Vibrio alginolyticus*. *Microbiology,* **146**, 2777–2783.

Imae, Y. (1991). Use of Na^+ as an alternative to H^+ in energy transduction. In *New Era of Bioenergetics* (Mukohata, Y., ed.), pp. 197–221. Academic Press, Inc., Tokyo.

Imae, Y. & Atsumi, T. (1989). Na^+-driven bacterial flagellar motors. *J. Bioenerg. Biomemb.* **21**, 705–716.

Kami-ike, N., Kudo, S., & Hotani, H. (1991). Rapid changes in flagellar rotation induced by external electric pulses. *Biophys. J.* **60**, 1350–1355.

Kara-Ivanov, M., Eisenbach, M. & Caplan, S. R. (1995). Fluctuations in rotation rate of the flagellar motor of *Escherichia coli*. *Biophys. J.* **69**, 250–263.

Kawagishi, I., Maekawa, Y., Atsumi, T., Homma, M. & Imae, Y. (1995). Isolation of the polar and lateral flagellum-defective mutants in *Vibrio alginolyticus* and identification of their flagellar driving energy sources. *J. Bacteriol.* **177**, 5158–5160.

Kawagishi, I., Imagawa, M., Imae, Y., McCarter, L. & Homma, M. (1996). The sodium-driven polar flagellar motor of marine *Vibrio* as the mechanosensor that regulates lateral flagellar expression. *Mol. Microbiol.* **20**, 693–699.

Kudo, S., Magariyama, Y. & Aizawa, S.-I. (1990). Abrupt changes in flagellar rotation observed by laser dark-field microscopy. *Nature,* **346**, 677–680.

Li, X. J., Xu, R. H., Guggino, W. B. & Snyder, S. H. (1995). Alternatively spliced forms of the α subunit of the epithelial sodium channel: distinct sites for amiloride binding and channel pore. *Mol. Pharmacol.* **47**, 1133–1140

Lin, C., Kieber-Emmons, T., Villalobos, A. P., Foster, M. H., Wahlgren, C. & Kleyman, T. R. (1994). Topology of an amiloride-binding protein. *J. Biol. Chem.* **269**, 2805–2813.

Magariyama, Y., Sugiyama, S., Muramoto, K., Maekawa, Y., Kawagishi, I., Imae, Y. & Kudo, S. (1994). Very fast flagellar rotation. *Nature,* **371**, 752.

Magariyama, Y., Sugiyama, S., Muramoto, K., Kawagishi, I., Imae, Y. & Kudo, S. (1995). Simultaneous measurement of bacterial flagellar rotation rate and swimming speed. *Biophys. J.* **69**, 2154–2162.

McCarter, L. L. (1994a). MotY, a component of the sodium-type flagellar motor. *J. Bacteriol.* **176**, 4219–4225.

McCarter, L. L. (1994b). MotX, the channel component of the sodium-type flagellar motor. *J. Bacteriol.* **176**, 5988–5998.

McCarter, L., Hilmen, M. & Silverman, M. (1988). Flagellar dynamometer controls swarmer cell differentiation of V. parahaemolyticus. *Cell*, **54**, 343–351.

Muramoto, K., Sugiyama, S., Cragoe, E. J., Jr & Imae, Y. (1994). Successive inactivation of the force-generating units of sodium-driven bacterial flagellar motors by a photoreactive amiloride analog. *J. Biol. Chem.* **269**, 3374–3380.

Muramoto, K., Kawagishi, I., Kudo, S., Magariyama, Y., Imae, Y. & Homma, M. (1995). High speed rotation and speed stability of the sodium-driven flagellar motor in *Vibrio alginolyticus*. *J. Mol. Biol.* **251**, 50–58.

Muramoto, K., Magariyama, Y., Homma, M., Kawagishi, I., Sugiyama, S., Imae, Y. & Kudo, S. (1996). Rotational fluctuation of the sodium-driven flagellar motor of *Vibrio alginolyticus* induced by binding of inhibitors. *J. Mol. Biol.* **259**, 687–695.

Okunishi, I., Kawagishi, I. & Homma, M. (1996). Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*. *J. Bacteriol.* **178**, 2409–2415.

Sugiyama, S., Cragoe, E. J., Jr & Imae, Y. (1988). Amiloride, a specific inhibitor for the Na^+-driven flagellar motors of alkalophilic *Bacillus*. *J. Biol. Chem.* **263**, 8215–8219.

Tokuda, H., Asano, M., Shimamura, Y., Unemoto, T., Sugiyama, S. & Imae, Y. (1988). Roles of the respiratory Na^+ pump in bioenergetics of *Vibrio alginolyticus*. *J. Biochem.* **103**, 650–655.

Edited by J. Karn

(Received 5 July 1996; received in revised form 21 October 1996; accepted 28 October 1996)