JOURNAL OF BACTERIOLOGY, Mar. 1990, p. 1634–1639
0021-9193/90/031634-06$02.00/0

Vol. 172, No. 3

Specific Inhibition of the Na^+-Driven Flagellar Motors of Alkalophilic *Bacillus* Strains by the Amiloride Analog Phenamil

TATSUO ATSUMI,[1] SHIGERU SUGIYAMA,[1] EDWARD J. CRAGOE, JR.,[2] AND YASUO IMAE[1]*

Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan,[1] and 2211 Oak Terrace Drive, Lansdale, Pennsylvania 19446[2]

Received 28 August 1989/Accepted 5 December 1989

Amiloride, a specific inhibitor for the Na^+-driven flagellar motors of alkalophilic *Bacillus* strains, was found to cause growth inhibition; therefore, the use of amiloride for the isolation of motility mutants was difficult. On the other hand, phenamil, an amiloride analog, inhibited motor rotation without affecting cell growth. A concentration of 50 μM phenamil completely inhibited the motility of strain RA-1 but showed no effect on the membrane potential, the intracellular pH, or Na^+-coupled amino acid transport, which was consistent with the fact that there was no effect on cell growth. Kinetic analysis of the inhibition of motility by phenamil indicated that the inhibition was noncompetitive with Na^+ in the medium. A motility mutant was isolated as a swarmer on a swarm agar plate containing 50 μM phenamil. The motility of the mutant showed an increased resistance to phenamil but normal sensitivity to amiloride. These results suggest that phenamil and amiloride interact at different sites on the motor. By examining various bacterial species, phenamil was found to be a specific and potent inhibitor for the Na^+-driven flagellar motors not only in various strains of alkalophilic *Bacillus* spp. but also in a marine *Vibrio* sp.

Flagellated bacteria swim by rotating their helical flagella as screw propellers. At the base of each flagellum, a flagellar motor is embedded in the cytoplasmic membrane and drives the flagellar rotation. The search for the energy source of the flagellar motors in various bacteria revealed that there are two types of flagellar motors; one is the H^+-driven type found in neutrophiles (21–23). This type is powered by the electrochemical potential gradient of protons across the membrane, the so-called proton motive force. The second type is the Na^+-driven type found in alkalophilic *Bacillus* spp. and a marine *Vibrio* sp. (7, 10–12) and is powered by the electrochemical potential gradient of sodium across the membrane, the so-called Na^+ motive force.

To clarify the mechanism of energy coupling in the flagellar motors, an important step is the identification of the force-generating unit, which is assumed to have a channel activity for the coupling ion in the motor. In the case of the H^+-driven flagellar motors of *Escherichia coli* and *Salmonella typhimurium*, genetic approaches revealed that the products of the *motA* and *motB* genes are required for the motor rotation (21, 26). Furthermore, Berg and colleagues (5, 6) reported that a gradual increase in the product of either the *motA* gene or the *motB* gene causes a stepwise increase in the rotational speed of the *E. coli* motor up to about 10 steps, suggesting the presence of about 10 individual force-generating units in each motor. Consistent with this, Khan and Dapice (15) used freeze-fracture electron microscopy to demonstrate that a ring structure of about 10 particles apparently comprising *motA* and *motB* gene products surrounds each motor of *E. coli*. Therefore, the *motA* and *motB* gene products are the likely candidates for the force-generating unit with H^+ channel activity. However, Chun and Parkinson (8) recently reported that the *motB* gene product appears to traverse the cytoplasmic membrane only once. Wilson and Macnab (32) reported that the increase of the *motA* gene product in the membrane does not greatly affect cell growth. These results appear to eliminate the possibility that these gene products themselves have the H^+ channel activity. In any case, more data are required to understand how the H^+ channel activity in the force-generating unit is constructed in the H^+-driven motor.

In the instance of the Na^+-driven flagellar motors, the force-generating unit of the motor is the essential site for the specific interaction with Na^+. We therefore assumed that the drugs which interfere with the interaction of Na^+ with the motor could cause the inhibition of motor rotation. A search for such drugs was successful, and Sugiyama et al. (27) found that amiloride, a potent inhibitor for the Na^+ channel in animal cells (2, 4, 18), specifically inhibits the rotation of the Na^+-driven flagellar motors of alkalophilic *Bacillus* spp. by competing with Na^+ in the medium. The results suggest that the site of action of amiloride on the motor is the Na^+ interaction site located at the outer side of the force-generating unit. Thus, amiloride is expected to be a useful tool for elucidating the energy conversion mechanism at the force-generating unit of the motor.

During the studies with amiloride for the isolation of mutants with an altered sensitivity of motility to amiloride, we found that this agent caused an inhibition in the growth of alkalophilic *Bacillus* spp. Here we report that an amiloride analog, phenamil, specifically inhibits the Na^+-driven flagellar motors without any growth inhibition and that the drug is quite useful for the isolation of motor-specific mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The alkalophilic *Bacillus* strain used for most experiments was a streptomycin-resistant mutant of *Bacillus firmus* RAB (19), which was isolated as a spontaneous mutant on an agar plate containing 200 μg of streptomycin per ml and was named RA-1. Alkalophilic *Bacillus* sp. strains 202-1, YN-1, and 8-1 and *Bacillus alcalophilus* ATCC 27647 have been described previously (13). The other alkalophilic *Bacillus* strains used in this study were *Bacillus* sp. strains YN-2000 (29), C59-2 (16), M-29 (1), and N-6 (17). Neutrophilic strains used in this study were *Bacillus subtilis* RM125 (31), *Bacillus sphaericus*

* Corresponding author.

9602 (14), and *E. coli* RP487 (25). A marine *Vibrio* sp. that was used in this study was *Vibrio alginolyticus* 138-2 (30).

For the growth of alkalophiles, AB-4 medium (13) was used, unless otherwise noted. Neutrophiles were grown in tryptone broth (1% tryptone, 0.5% NaCl) supplemented with 10 mM DL-lactate. A marine *Vibrio* sp. was grown in a complex medium as described previously (30), although 50 mM NaCl and 250 mM KCl were added instead of 500 mM NaCl. Cells were grown at 35°C with shaking, and cell growth was monitored by using a Klett-Summerson colorimeter with a no. 66 filter.

For some experiments, RA-1 cells were grown in AB-5 medium (pH 9.5) consisting of 5 g of polypeptone, 0.75 g of yeast extract, 5 g of glucose, and 0.75 g of KH_2PO_4 per liter of water supplemented with 0.01% $MgCl_2$–0.5 mM NaCl–23 mM K_2CO_3. The Na^+ concentration in AB-5 medium was measured to be 5.0 mM by the atomic absorption method.

Measurement of swimming speed. The swimming speed of cells was measured by the photographic method as described previously (13). In some experiments, the speed was measured by direct tracing of the moving cells on the video monitor.

Cells at the late logarithmic phase of growth were harvested by centrifugation and suspended as a dense cell suspension in a motility medium. In the case of alkalophiles, TG medium consisting of 25 mM Tris hydrochloride buffer (pH 9.5) and 5 mM glucose supplemented with 5 mM NaCl was used as the motility medium. For neutrophiles, TG medium (pH 7.5) supplemented with 50 mM KCl was used, and for the marine *Vibrio* sp., TG medium (pH 7.5) supplemented with 50 mM NaCl and 350 mM KCl was used.

For the measurement of swimming speed, the concentrated cells were diluted 200-fold with the motility medium supplemented with various concentrations of NaCl and drugs. When the concentration of NaCl was varied, the total salt concentration was adjusted by the addition of a suitable amount of choline chloride. In some experiments, the cells in the growth medium were used directly for the measurement of swimming speed. In every instance, the swimming speed was measured at 25°C.

Measurement of membrane potential, intracellular pH, and α-aminoisobutyrate transport. The membrane potential, the intracellular pH, and the transport of α-aminoisobutyrate (AIB) were measured by the filtration method as described previously (13).

RA-1 cells were grown in AB-5 medium (pH 9.5). For the measurements of the membrane potential and the intracellular pH, the cells were harvested and suspended in the same medium. After the cells were incubated for 4 min at 35°C with various concentrations of phenamil, the membrane potential of the cells (3×10^8 cells or 0.11 mg of protein per ml) was measured 5 min after the addition of [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$; 5 μM, 50 mCi/mmol). Similarly, the intracellular pH was measured by adding [^{14}C]methylamine (20 μM, 1 mCi/mmol) instead of [^{3}H]TPMP$^+$ to the cells (2×10^9 cells per ml). Cells treated with 5 μM gramicidin D were used as a control.

For the measurement of AIB transport, cells were suspended in TG medium (pH 9.5) containing 5 mM NaCl and 45 mM choline chloride and were incubated with phenamil for 6 min. The AIB uptake was measured by incubating the cells (8×10^8 cells per ml) with [^{14}C]AIB (100 μM, 0.2 mCi/mmol) for 5 min.

Isolation of motility mutants. Mutagenesis was carried out by using ethyl methanesulfonate (24). RA-1 cells were suspended in 25 mM Tris hydrochloride buffer (pH 7.5) containing 150 mM NaCl, and the cells (2 ml, 9×10^8 cells per ml) were mixed with 7.6 ml of 1 M Tris hydrochloride buffer (pH 7.4) containing 0.4 ml of ethyl methanesulfonate and incubated for 5 min at 35°C. After the cells were diluted 30-fold with AB-5 medium, they were incubated overnight at 35°C with shaking.

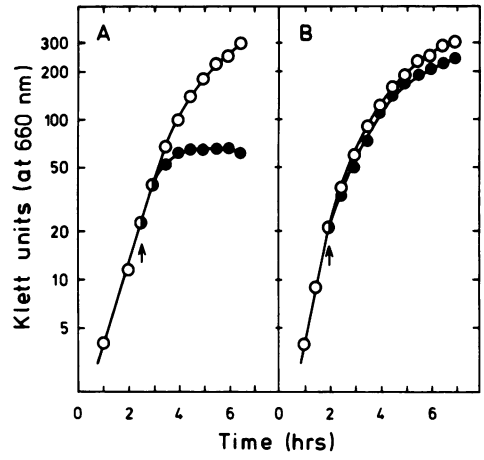

FIG. 1. Effect of amiloride and phenamil on the growth of an alkalophilic *Bacillus* strain, RA-1. Cells were grown at 35°C in AB-5 medium (pH 9.5), and at the times indicated by the arrows, 400 μM amiloride (A) or 50 μM phenamil (B) was added. Symbols: ○, no addition; ●, growth with the drug.

For the selection of the mutants with phenamil-resistant motility, AB-5 swarm agar (AB-5 medium [pH 9.5] containing 0.45% agar) supplemented with 50 μM phenamil was used. A drop of overnight culture was spotted onto the center of the swarm agar plate, and the plates were incubated at 35°C for 12 h. The mutants, which produced a distinct swarm on the plate, were isolated and purified. Then, the mutants were tested for streptomycin resistance and also for sensitivity to the anti-RA-1 flagellar antibody, by which motility of RA-1 was specifically inhibited. One such mutant was named RA-30.

Chemicals and anti-flagellar antibody. Amiloride and phenamil were prepared as described previously (9). Radioactive materials were the products of DuPont, NEN Research Products (Boston, Mass.), and [^{3}H]TPMP$^+$ was a gift from R. M. Macnab (Yale University, New Haven, Conn.). Anti-RA-1 flagellar antibody was prepared by injecting a rabbit with purified RA-1 flagella.

RESULTS

Effect of amiloride and phenamil on cell growth. We have previously reported (27) that amiloride specifically inhibits the Na^+-driven flagellar motors of alkalophilic *Bacillus* spp. by competing with Na^+ at the Na^+ interaction site of the motor. Therefore, amiloride was expected to be useful for isolating the motor-specific mutants. It was found, however, that amiloride caused a delayed inhibition in the growth of alkalophilic *Bacillus* spp. As shown in Fig. 1A, the growth of RA-1 cells at 5 mM Na^+ was inhibited by 400 μM amiloride, although the inhibition started about 30 min after the addition of amiloride. Under this condition, motility was immediately and completely inhibited (27). The increase in Na^+ concentration up to 150 mM restored motility but not cell growth (data not shown). Similar results were obtained with other alkalophilic *Bacillus* strains, YN-1 and 202-1. These results indicate that the growth inhibition by amiloride is a

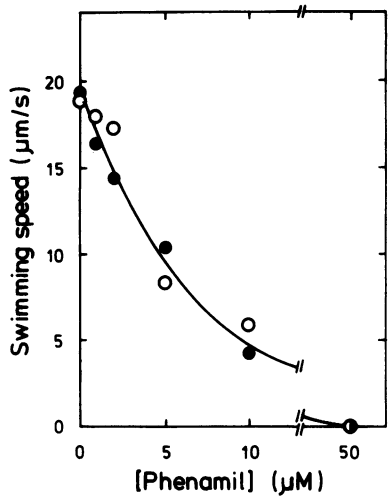

FIG. 2. Inhibition by phenamil of motility of RA-1 under conditions of growth. At the time indicated by the arrow in Fig. 1B, various concentrations of phenamil were added to the cells, and cultivation was continued at 35°C. The swimming speeds of the cells were measured after incubation for 5 min (○) or 1 h (●).

secondary effect of amiloride action on some unknown processes in cell growth. It is noteworthy that the cells grown in amiloride showed some elongation and some irregularity in shape, suggesting that the growth inhibition by amiloride occurs at least somewhere in the sequence of cell wall synthesis.

To avoid this secondary effect of amiloride, we have tested several amiloride analogs and found that phenamil, which is also a potent inhibitor of the Na^+ channels in animal cells (3, 18) and the Na^+-driven flagellar motors of alkalophilic *Bacillus* spp. (27), showed almost no growth inhibition. As shown in Fig. 1B, the growth of RA-1 cells at 5 mM Na^+ was almost normal in the presence of 50 μM phenamil. Under this condition, motility was immediately and completely inhibited, but cell shape and colony-forming ability were normal. Figure 2 shows the relationship between phenamil concentration and motility inhibition. Phenamil, at a concentration of 5 or 10 μM, caused considerable inhibition of motility, and the inhibition continued after a doubling of the cell concentration. Motility was not restored even after continued exposure to phenamil for 6 h (data not shown). These results indicate that the cell growth observed in the presence of phenamil is not due to a rapid decrease in the effective concentration of phenamil in the growth medium during cultivation.

Effect of phenamil on cellular physiology. Since cell growth was not affected by phenamil, this agent is assumed to have an inhibitory effect on motility without affecting other aspects of cellular physiology. Consistent with this, the membrane potential, which is a major component of the driving force of the Na^+-coupled systems in alkalophiles (20), was not affected by phenamil in concentrations up to 50 μM. Similarly, the intracellular pH, which is maintained at about 8 in alkalophiles by the function of the Na^+/H^+ antiporter (20, 28), was not affected by the same concentration of phenamil. The latter result is consistent with the finding that phenamil is a weak inhibitor of the Na^+/H^+ antiporter in animal cells (18). Furthermore, as shown in Fig. 3, Na^+-coupled AIB transport was not affected by phenamil. These results indicate that the inhibition of motility of alkalophilic *Bacillus* spp. by phenamil is not due to the decrease in the

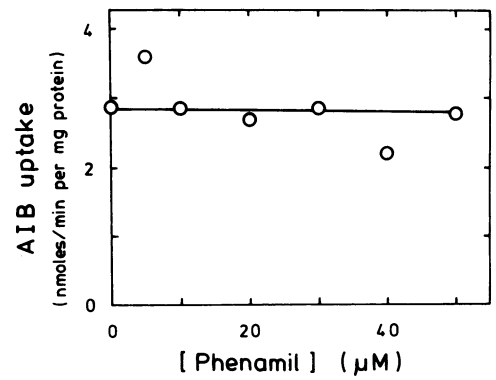

FIG. 3. Effect of phenamil on Na^+-coupled AIB transport in RA-1.

Na^+ motive force or to an abnormality of the intracellular conditions. Thus, it is concluded that phenamil is a truly specific and potent inhibitor of the Na^+-driven flagellar motors of alkalophilic *Bacillus* spp.

Mechanism of motility inhibition by phenamil. The motility of alkalophiles is inhibited by the competition of amiloride with Na^+ (27). We investigated whether the inhibition of motility by phenamil is caused by the same mechanism.

Figure 4 shows the reversibility of the inhibition. The addition of 5 μM phenamil to RA-1 cells caused an immediate reduction in the swimming speed of the cells to 50% of that of controls, and it remained at about this level for several minutes. When the phenamil concentration was decreased to 1 μM by diluting the cells fivefold, the swimming speed of the cells was immediately increased to the value observed in the presence of 1 μM phenamil. These results indicate that the inhibitory action of phenamil on the Na^+-driven flagellar motors is caused by a rapid and reversible interaction between phenamil and the binding site of the motor.

For amiloride, the increase in Na^+ but not other cations in the medium resulted in a clear restoration of motility (27). However, the increase in concentration of any cations,

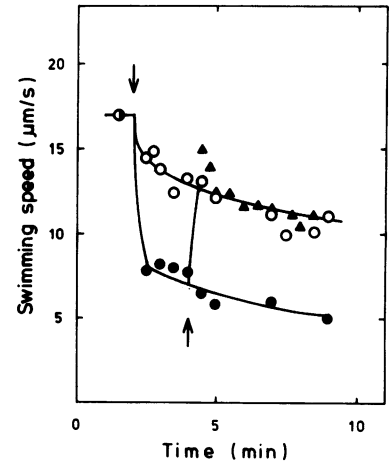

FIG. 4. Reversibility of the inhibition of motility by phenamil. RA-1 cells in TG medium (pH 9.5) supplemented with 5 mM NaCl were treated with 1 μM (○) or 5 μM (●) phenamil, as indicated by the first arrow. At the second arrow, the cells treated with 5 μM phenamil were diluted fivefold with the same medium without phenamil (▲).

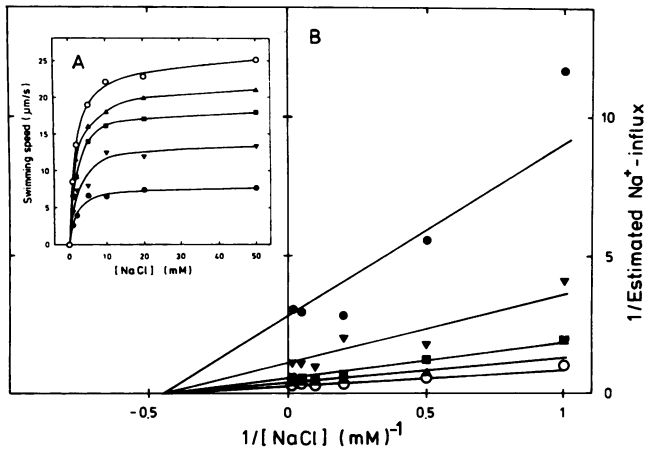

FIG. 5. Effect of Na^+ concentration on the inhibition of motility by phenamil in RA-1 cells. Cells were suspended in TG medium (pH 9.5) supplemented with various concentrations of NaCl. The total salt concentration added to TG medium was adjusted to 50 mM by varying the concentration of choline chloride. Various concentrations of phenamil were then added, and after 1 min, the swimming speed of the cells was measured. Phenamil concentrations of 0 μM (○), 1 (▲), 2 (■), 5 (▼), and 10 μM (●) were used. (A) Measured data; (B) double-reciprocal plot of the data shown in panel A after the kinetic treatment described previously (27). The inverse of the estimated Na^+ influx through the motor shown in the ordinate corresponds to the inverse of $v^2/\varepsilon \times Na^+$ motive force, where v is the swimming speed and ε is the efficiency. For this estimation, ε was assumed to be constant. The units are given as mV s^2 μm^{-2} (27).

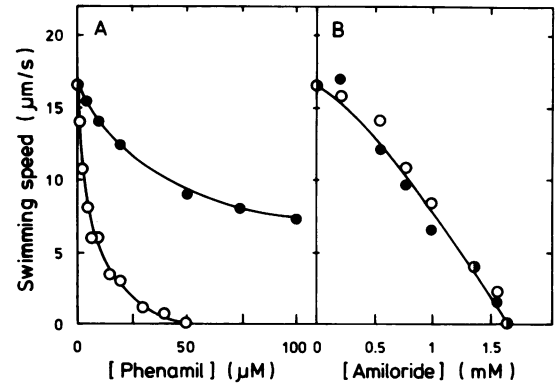

FIG. 6. Effect of phenamil and amiloride on the swimming speed of a wild-type swarmer (RA-1 [○]) and a phenamil-resistant swarmer (RA-30 [●]). Cells in TG medium (pH 9.5) containing 5 mM NaCl and 45 mM choline chloride were treated with various concentrations of phenamil (A) or amiloride (B).

including Na^+, Li^+, and K^+, did not alter the inhibitory action of phenamil (data not shown). This suggests that amiloride and phenamil inhibit motility by different mechanisms. To clarify this point, the relationship between swimming speed and Na^+ concentration in the medium at various phenamil concentrations was analyzed. As shown in Fig. 5A, the motility of RA-1 cells showed a clear Na^+ dependence, and the addition of phenamil caused reductions in motility. However, the motility inhibition by phenamil was not restored significantly by increasing the Na^+ concentration in the medium. These data were further analyzed by the previously described kinetic treatment method (27), in which the influx of Na^+ for motor rotation was estimated from the swimming speed. The kinetic data shown in Fig. 5B indicate that phenamil inhibits the rotation of the Na^+-driven flagellar motors in a noncompetitive manner with Na^+ in the medium; namely, the inhibition by phenamil on the motor rotation is not due to the alteration in the affinity of Na^+ to the motor.

From the Dixon plot of the data presented in Fig. 5A, the K_i of phenamil for the motor was calculated to be 1.5 μM. The data presented in Fig. 5B show that the K_m value of Na^+ for the motor is estimated to be 2.2 mM, irrespective of the presence or absence of phenamil.

A mutant with phenamil-resistant motility. On a swarm plate containing 50 μM phenamil, RA-1 cells showed almost no swarming, and therefore we could isolate mutants with phenamil-resistant motility as swarmers on the plate. As shown in Fig. 6A, the motility of RA-30, a swarmer isolated from RA-1, showed a clear resistance to phenamil. At 50 μM phenamil, the motility of wild-type cells was completely inhibited, whereas that of the mutant cells was reduced only by half. In contrast, the motilities of RA-1 and RA-30 showed almost the same sensitivity to amiloride (Fig. 6B). These results strongly suggest that amiloride and phenamil interact at different sites on the motor. In consideration of these facts and the results presented above, it is concluded that, unlike amiloride, the site of action of phenamil on the motor is not simply the Na^+ interaction site on the motor.

Phenamil specifically inhibits the Na^+-driven flagellar motors. When using amiloride, an increase in the ionic strength of the medium significantly reduced the inhibitory action on motility (27). However, this was not seen with phenamil (data not shown). Therefore, phenamil can be applied to various bacterial species with different salt concentration requirements.

The effects of phenamil on the motility of various bacterial species are summarized in Table 1. Phenamil caused a potent inhibition of motility in bacterial species such as obligately alkalophilic *Bacillus* strains RAB, 202-1, 8-1, and YN-1; a facultatively alkalophilic *Bacillus* strain; YN-2000; and a marine *Vibrio* sp., *V. alginolyticus*. All of these species are known to have Na^+-driven flagellar motors (7, 13, 29, 30). In contrast, the motility of bacterial species with H^+-driven flagellar motors, such as *E. coli* and neutrophilic *Bacillus* spp. (21), was not affected by phenamil. It is noteworthy that the growth of these bacteria was also not affected by this concentration of phenamil (data not shown).

As shown in Table 1, it is noteworthy that some alkalophilic *Bacillus* strains showed weak or no Na^+-dependent motility and that the motility of these strains was found to be rather resistant to phenamil. We observed that the optimal pH for their motility was in the alkaline region (data not shown). However, even at the optimal pH, they showed considerably slower swimming speeds compared with the swimming speeds of other alkalophiles with tightly Na^+-dependent motility (Table 1).

DISCUSSION

In a previous report (27), we showed that amiloride inhibits rotation of the Na^+-driven flagellar motors by competing with Na^+ in the medium, and therefore, it was expected to be useful for the isolation of motor-specific mutants. However, we found that amiloride also caused the growth inhibition of alkalophilic *Bacillus* spp. (Fig. 1A). This result does not affect the interpretation of the mode of action of amiloride on the motors, since the motility inhibition by amiloride begins almost instantaneously, but growth inhibition by amiloride is observed only about 30 min after the

TABLE 1. Effect of phenamil on the motility of various bacterial species

Strain	Swimming speed (μm/s)[a]		
	$-Na^+$	$+Na^+$	$+Na^+$ + phenamil
Motility tightly Na^+ dependent			
Alkalophilic *Bacillus* strains:			
RAB (RA-1)[b]	0	22	0
202-1	0	41	0
8-1	0	19	0
YN-1	0	28	6
YN-2000	0	21	6
Marine *Vibrio* sp., *V. alginolyticus*	0	69	1
Motility weakly or not Na^+ dependent			
Alkalophilic *Bacillus* strains:			
M-29	8	11	11
ATCC 27647	7	11	7
C59-2	3	4	2
N-6	7	7	4
Neutrophiles			
E. coli	28	29	33
B. subtilis	22	21	22
B. sphaericus	35	37	36

[a] Motility was measured in TG medium. The pH of the medium was 9.5 for alkalophiles and 7.5 for neutrophiles and the *Vibrio* sp. For alkalophiles and neutrophiles, the medium was supplemented with 50 mM KCl ($-Na^+$) or 50 mM NaCl ($+Na^+$). For the *Vibrio* sp., the medium was supplemented with 400 mM KCl ($-Na^+$) or 400 mM NaCl ($+Na^+$). Phenamil was added to a final concentration of 100 μM.

[b] Motility at pH 7.5 was also tightly Na^+ dependent and was completely inhibited by 100 μM phenamil.

addition of amiloride. It was noted that cells grown in the presence of amiloride showed some morphological abnormalities, suggesting that one of the sites for this secondary effect of amiloride occurs in the sequence of cell wall synthesis. In any event, it is clear that the use of amiloride is not appropriate for the isolation of motor-specific mutants.

To avoid this secondary effect of amiloride, we have examined several amiloride analogs and found that phenamil, a more potent inhibitor for the Na^+-driven flagellar motors of alkalophilic *Bacillus* spp. (27), did not cause any growth inhibition (Fig. 1B). Then, we showed that it was possible to isolate motor-specific mutants by using phenamil.

From the kinetic analysis of the inhibition of motility of alkalophilic *Bacillus* spp. by phenamil, phenamil was shown to inhibit the Na^+-driven flagellar motors in a noncompetitive manner with Na^+ in the medium. This indicates that, unlike amiloride, a major interaction site of phenamil on the motor is not identical to the Na^+ interaction site of the motor. Furthermore, a mutant of an alkalophilic *Bacillus* sp. that was isolated as a phenamil-resistant swarmer showed no change in the sensitivity of its motility to amiloride. Thus, it is quite likely that the interaction site for phenamil on the motor is different from the site for amiloride, since the latter is strictly located at the Na^+ interaction site of the motor.

The inhibitory action of amiloride is strongly affected by not only the Na^+ concentration but also the ionic strength of the medium (27). However, we found that the changes in both ionic strength and NaCl concentration in the medium did not affect the inhibitory potency of phenamil on motility. This indicates that as a motility inhibitor, phenamil is more widely usable than amiloride is. By examining the inhibitory effect of phenamil on the motility of various bacterial species, it was found that phenamil has no inhibitory effect on the H^+-driven flagellar motors of various neutrophiles but inhibits the Na^+-driven flagellar motors of various species of alkalophilic *Bacillus* and also that of a marine *Vibrio* sp. These results suggest that the interaction site of phenamil on Na^+-driven flagellar motors has a common and rather uniform structure among various bacterial species.

Although many strains of alkalophilic *Bacillus* showed tightly Na^+-dependent and phenamil-sensitive motility, several strains whose growth appears to have weak or no Na^+ dependence (1, 16, 17, 19) also showed weak or no Na^+-dependent motility. Furthermore, phenamil showed a weak or almost no inhibitory effect on the motility of these strains. Thus, the flagellar motors of these bacteria seem to be different from typical Na^+-driven flagellar motors. It will be interesting if these strains, although they live in alkaline environments and show optimal motility in the alkaline pH range, do not have Na^+-driven flagellar motors.

ACKNOWLEDGMENTS

We thank R. Aono, K. Horikoshi, M. Kitada, T. A. Krulwich, Y. Nosoh, and H. Tokuda for providing us with bacterial strains.

This work was supported in part by grants-in-aid for Scientific Research on Priority Areas of "Bioenergetics" and "Sliding Motility" (to Y.I.) from the Ministry of Education, Science and Culture, Japan.

LITERATURE CITED

1. **Aono, R., and K. Horikoshi.** 1983. Chemical composition of cell walls of alkalophilic strains of *Bacillus*. J. Gen. Microbiol. **129**:1083–1087.
2. **Asher, C., E. J. Cragoe, Jr., and H. Garty.** 1987. Effects of amiloride analogues on Na^+ transport in toad bladder membrane vesicles. J. Biol. Chem. **262**:8566–8573.
3. **Barbry, P., O. Chassande, D. Duval, B. Rousseau, C. Frelin, and M. Lazdunski.** 1989. Biochemical identification of two types of phenamil binding sites associated with amiloride-sensitive Na^+ channels. Biochemistry **28**:3744–3749.
4. **Benos, D. J.** 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. Am. J. Physiol. **242**:c131–c145.
5. **Blair, D. F., and H. C. Berg.** 1988. Restoration of torque in defective flagellar motors. Science **242**:1678–1681.
6. **Block, S. M., and H. C. Berg.** 1984. Successive incorporation of force-generating units in the bacterial rotary motor. Nature (London) **309**:470–472.
7. **Chernyak, B. V., P. A. Dibrov, A. N. Glagolev, M. Y. Sherman, and V. P. Skulachev.** 1983. A novel type of energetics in a marine alkali-tolerant bacterium: $\Delta\bar{\mu}_{Na}$-driven motility and sodium cycle. FEBS Lett. **164**:38–42.
8. **Chun, S. Y., and J. S. Parkinson.** 1988. Bacterial motility: membrane topology of the *Escherichia coli* Mot B protein. Science **239**:276–278.
9. **Cragoe, E. J., Jr., O. W. Woltersdorf, J. B. Bicking, C. F. Kwong, and J. H. Jones.** 1967. Pyrazine diuretics. II. N-Amidino-3-amino-5-substituted 6-halopyrazinecarboxamides. J. Med. Chem. **10**:66–75.
10. **Hirota, N., and Y. Imae.** 1983. Na^+-driven flagellar motors of an alkalophilic *Bacillus* strain YN-1. J. Biol. Chem. **258**:10577–10581.
11. **Hirota, N., M. Kitada, and Y. Imae.** 1981. Flagellar motors of alkalophilic *Bacillus* are powered by an electrochemical potential gradient of Na^+. FEBS Lett. **132**:278–280.
12. **Imae, Y., and T. Atsumi.** 1989. Na^+-driven bacterial flagellar motors. J. Bioenerg. Biomembr. **21**:705–716.
13. **Imae, Y., H. Matsukura, and S. Kobayasi.** 1986. Sodium-driven flagellar motors of alkalophilic *Bacillus*. Methods Enzymol. **125**:582–592.
14. **Imae, Y., and J. L. Strominger.** 1976. Conditional spore cortexless mutants of *Bacillus sphaericus* 9602. J. Biol. Chem. **251**:

1493–1499.
15. **Khan, S., and M. Dapice.** 1988. Effects of *mot* gene expression on the structure of the flagellar motor. J. Mol. Biol. **202:** 575–584.
16. **Kitada, M., and K. Horikoshi.** 1987. Bioenergetic properties of alkalophilic *Bacillus* sp. strain C-59 on an alkaline medium containing K_2CO_3. J. Bacteriol. **169:**5761–5765.
17. **Kitada, M., K. Onda, and K. Horikoshi.** 1989. The sodium/proton antiport system in a newly isolated alkalophilic *Bacillus* sp. J. Bacteriol. **171:**1879–1884.
18. **Kleyman, T. R., and E. J. Cragoe, Jr.** 1988. Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. **105:**1–21.
19. **Krulwich, T. A., A. A. Guffanti, R. F. Bornstein, and J. Hoffstein.** 1982. A sodium requirement for growth, solute transport, and pH homeostasis in *Bacillus firmus* RAB. J. Biol. Chem. **257:**1885–1889.
20. **Krulwich, T. A., D. B. Hicks, D. Seto-Young, and A. A. Guffanti.** 1988. The bioenergetics of alkalophilic bacilli. Crit. Rev. Microbiol. **16:**15–36.
21. **Macnab, R. M.** 1987. Flagella, and Motility and chemotaxis, p. 70–83 and 732–759, respectively. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
22. **Manson, M. D., P. Tedesco, H. C. Berg, F. M. Harold, and C. Van der Drift.** 1977. A protonmotive force drives bacterial flagella. Proc. Natl. Acad. Sci. USA **74:**3060–3064.
23. **Matsuura, S., J. Shioi, and Y. Imae.** 1977. Motility in *Bacillus subtilis* driven by an artificial protonmotive force. FEBS Lett. **82:**187–190.
24. **Osborn, M., S. Person, S. Phillips, and F. Funk.** 1967. A determination of mutagen specificity in bacteria using nonsense mutants of bacteriophage T4. J. Mol. Biol. **26:**437–447.
25. **Parkinson, J. S., and P. T. Revello.** 1978. Sensory adaptation mutants of *E. coli*. Cell **15:**1221–1230.
26. **Silverman, M., P. Matsumura, and M. Simon.** 1976. The identification of the *mot* gene product with *Escherichia coli*-lambda hybrids. Proc. Natl. Acad. Sci. USA **73:**3126–3130.
27. **Sugiyama, S., E. J. Cragoe, Jr., and Y. Imae.** 1988. Amiloride, a specific inhibitor for the Na^+-driven flagellar motors of alkalophilic *Bacillus*. J. Biol. Chem. **263:**8215–8219.
28. **Sugiyama, S., H. Matsukura, and Y. Imae.** 1985. Relationship between Na^+-dependent flagellar rotation and amino acid transport in alkalophilic *Bacillus*. FEBS Lett. **182:**265–268.
29. **Sugiyama, S., H. Matsukura, N. Koyama, Y. Nosoh, and Y. Imae.** 1986. Requirement of Na^+ in flagellar rotation and amino-acid transport in a facultatively alkalophilic *Bacillus*. Biochim. Biophys. Acta **852:**38–45.
30. **Tokuda, H., M. Asano, Y. Shimamura, T. Unemoto, S. Sugiyama, and Y. Imae.** 1988. Roles of the respiratory Na^+ pump in bioenergetics of *Vibrio alginolyticus*. J. Biochem. **103:**650–655.
31. **Uozumi, T., T. Hoshino, K. Miwa, S. Horinouchi, T. Beppu, and K. Arima.** 1977. Restriction and modification in *Bacillus* species. Mol. Gen. Genet. **152:**65–69.
32. **Wilson, M. L., and R. M. Macnab.** 1988. Overproduction of the MotA protein of *Escherichia coli* and estimation of its wild-type level. J. Bacteriol. **170:**588–597.