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Amiloride at pH 7.0 inhibits the Na^+-driven flagellar motors of *Vibrio alginolyticus* but allows cell growth

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Amiloride, a specific inhibitor for the Na^+-driven flagellar motors of alkalophilic *Bacillus*, is known to inhibit secondarily the growth of alkalophiles. The motility of a marine *Vibrio*, *V. alginolyticus*, was almost completely inhibited by 2 mM amiloride either at pH 7.0 or 8.5. We found that this concentration of amiloride inhibited the cell growth completely at pH 8.5 but only slightly at pH 7.0. Kinetic analysis of the inhibition of motility by amiloride at pH 7.0 showed that the inhibition was competitive with Na^+ in the medium. Thus, amiloride at pH 7.0 is really a specific and useful tool for the analysis of the Na^+-driven flagellar motors of *Vibrio*.

Na^+-driven flagellar motor; *Vibrio*, Amiloride

1. INTRODUCTION

Flagellate bacteria swim in the medium by rotating their helical flagella as screw propellers using a rotary motor embedded in the cytoplasmic membrane at the base of each flagellum. These motors are powered by the electrochemical potential gradient of ions across the membrane (see [1,2] for review); neutrophilic bacteria such as *Bacillus subtilis* and *Escherichia coli* have H^+-driven motors, and alkalophilic *Bacillus* and marine *Vibrio* have Na^+-driven motors. Thus, the force-generating unit in the motor is assumed to have a channel-like structure for coupling ion flux and rotation.

To clarify the mechanism of energy coupling in the flagellar motors, characterization of the force-generating unit is most important, and for this purpose, some inhibitors, which can specifically interact with the unit, would be quite useful. If cells grow well in the presence of such inhibitors, mutants with altered force-generating units can easily be isolated [3]. In the case of the Na^+-driven flagellar motors of alkalophilic *Bacillus*, we have shown that amiloride, a potent inhibitor of Na^+ channels of various organisms [4], inhibits the motors by competing with Na^+ at the Na^+-interacting site located on the external side of the force-generating unit [2,5]. However, amiloride was later found to inhibit the growth of alkalophilic *Bacillus* secondarily [3], indicating that the use of amiloride for the analysis of the Na^+-driven flagellar motors has certain limitations. An analog, phenamil, inhibits the motors without affecting cell growth [3], but its interaction site has not yet been clarified.

In this paper, we describe that amiloride at pH 7.0 is a useful drug for investigating the force-generating unit of the Na^+-driven flagellar motors of *V. alginolyticus*.

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2. MATERIALS AND METHODS

V. alginolyticus NapI is a respiration-coupled Na^+ pump-deficient mutant [6]. Cells were grown at 35°C with shaking in a complex medium [6] supplemented with 50 mM NaCl and 250 mM KCl. The pH of the medium was adjusted to 7.0 or 8.5 by KOH.

For motility measurements, cells in late log phase were harvested, washed once with motility medium consisting of 50 mM HEPES-KOH, 5 mM glucose, 250 mM KCl and 50 mM NaCl, and resuspended in a small volume of the same medium. The pH of the medium was adjusted to 7.0 or 8.5 by KOH. Swimming speed of the cells was measured as described previously [7,8] by diluting the cells 200-fold with motility medium supplemented with 10 mM serine, which is an attractant for the cells and suppresses the directional change of swimming for at least 1 min. When the Na^+ concentration in the medium was varied, KCl was added to keep the salt concentration constant.

Membrane potential of the cells was measured by the filtration method using [^{3}H]triphenylmethylphosphonium ion (0.2 mCi/mmol; DuPont-New England Nuclear) as described previously [8].

Amiloride (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethylsulfoxide and stored at −20°C.

3. RESULTS

The polar flagellar motor of *V. alginolyticus* is known to be Na^+-driven [6,8–10]. As shown in Fig. 1, the motility of *V. alginolyticus* NapI cells, which were grown in complex medium and had only polar flagella, was almost completely inhibited by 2 mM amiloride in motil-

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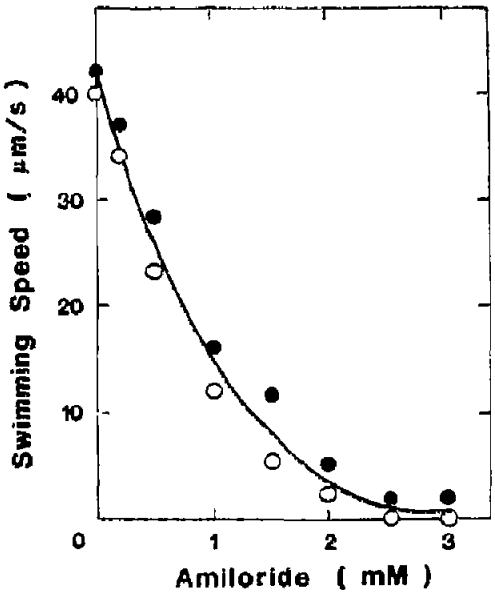

Fig. 1. Effect of amiloride on the swimming speed of *V. alginolyticus* NapI cells. Cells in motility medium containing 50 mM NaCl were mixed with various concentrations of amiloride, and the swimming speed was measured within 1 min. The pH of the medium was 7.0 (○) or 8.5 (●).

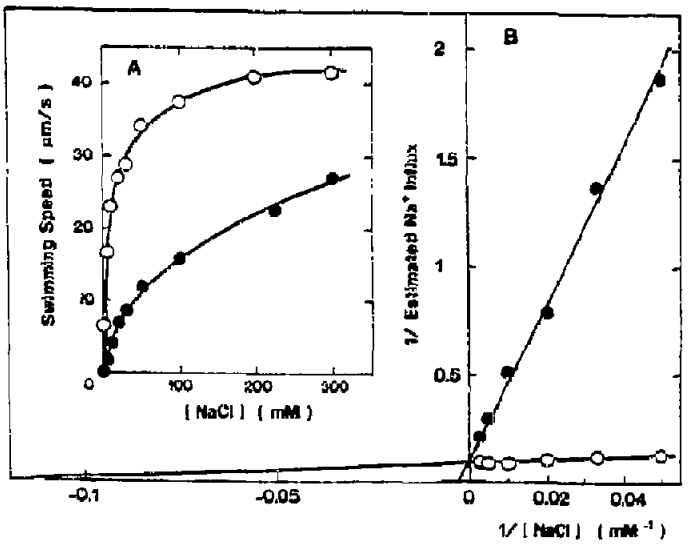

Fig. 3. Effect of Na^+ concentration on the inhibition of motility by amiloride in NapI cells. Cells in motility medium (pH 7.0) containing various concentrations of Na^+ were treated with (●) or without (○) 1 mM amiloride. (A) Measured data. (B) A double-reciprocal plot of the data shown in panel (A) after the kinetic treatment as described previously [5]. In the treatment, the Na^+ influx through the motor was assumed to be proportional to $v^2/(\varepsilon \times$ sodium-motive force), where v is the swimming speed and ε is the efficiency. The sodium-motive force was calculated by using the membrane potential of −110 mV and the intracellular Na^+ of 50 mM [6], and ε was assumed to be 1. The units in the ordinate are given as $mV \cdot s^2 \cdot \mu m^{-2}$ [5].

ity medium containing 50 mM Na^+. This inhibitory effect was seen at both pH 7.0 and pH 8.5.

In order to clarify whether amiloride specifically inhibits motility or affects physiology more generally, we measured the effect of amiloride on cell growth in complex medium containing 50 mM Na^+. As shown in Fig. 2A, at pH 7.0 growth was only slightly inhibited by 2 mM amiloride. Under these conditions, motility was immediately and almost completely inhibited, and was not restored by the further growth of the cells (data not shown). Even at 3 mM amiloride, the growth rate was reduced only by half. At pH 8.5, however, 2 mM amiloride completely inhibited growth (Fig. 2B). The motility of cells grown for 4 h at pH 7.0 in the presence of 2 or 3 mM amiloride was quickly restored by lowering the concentration of amiloride. These results clearly indicate that at pH 7.0, amiloride can function as a specific inhibitor for the Na^+-driven flagellar motors of *V. alginolyticus*. Consistent with this, the membrane potential of the cells at pH 7.0 was −110 mV, and was not affected by the addition of 2 or 3 mM amiloride.

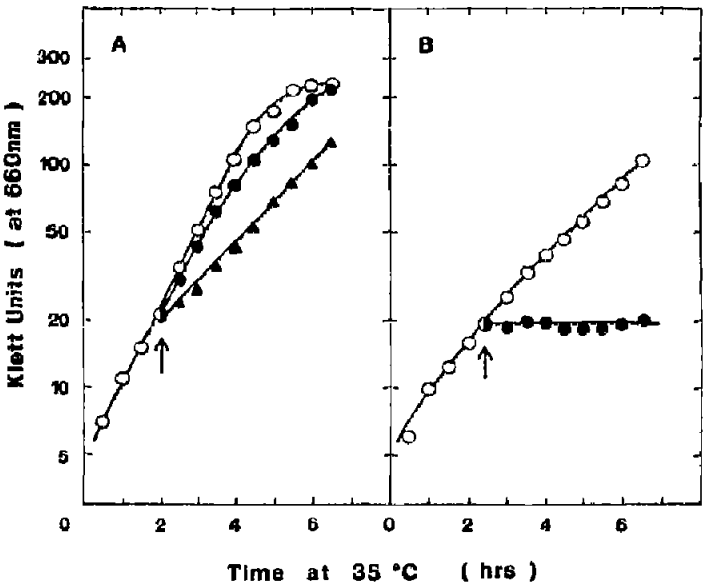

Fig. 2. Effect of pH on the growth inhibition of NapI cells by amiloride. Cells were grown at 35°C in growth medium containing 50 mM NaCl. (A) pH 7.0. (B) pH 8.5. At the time point indicated (arrow), amiloride was added to the medium: (○) 0 mM; (●) 2 mM; (▲) 3 mM.

In the case of the Na^+-driven flagellar motors of alkalophilic *Bacillus*, amiloride inhibits the flagellar motors by competing with Na^+ in the medium [2,5]. In order to see whether this is also the case for the flagellar motors of *V. alginolyticus*, its swimming speed at various Na^+ concentrations in motility medium at pH 7.0 was measured with or without 1 mM amiloride. As shown in Fig. 3A, the inhibitory effect of amiloride was more significant at lower Na^+ concentrations, suggesting that it acts by competing with Na^+ in the medium.

To test this, the data were analyzed by a kinetic treatment described previously [5], in which the Na^+ influx for motor rotation was estimated from the swimming speed. Fig. 3B shows the double-reciprocal plot of the estimated Na^+ influx through the motor and the Na^+

concentration in the medium. In the absence of amiloride, the relationship was linear and the Michaelis constant for Na^+ was calculated to be 9 mM. In the presence of 1 mM amiloride, the relationship was also linear, and the K_m for Na^+ was increased to 330 mM without any effect on the V_{max}. These results indicate that amiloride at pH 7.0 inhibits motility by competing with Na^+ in the medium.

As well as Na^+, Li^+ can partly support the motility of *V. alginolyticus* [11,12]. At 50 mM Li^+, swimming speed was reduced from about 5 μm/s to almost zero by 1 mM amiloride, but at 300 mM Li^+, it was reduced only by half (from 11 μm/s to 5 μm/s). These results indicate that Li^+ functions in the same manner as Na^+ for flagellar rotation, though less efficiently.

4. DISCUSSION

By the use of phenamil, we had previously isolated various motility mutants of alkalophilic *Bacillus* with altered sensitivity to phenamil [3]. However, the target of phenamil action has not yet been identified, and furthermore, phenamil is not commercially available. We showed here that a commercially available drug, amiloride, allows the growth of *V. alginolyticus* at pH 7.0 but inhibits its Na^+-driven flagellar motors by competing with Na^+ at the Na^+-interacting site of the force-generating unit. Thus, amiloride at pH 7.0 is a useful inhibitor for the analysis of the force-generating units of the Na^+-driven flagellar motors of *V. alginolyticus*, especially for the isolation of mutants with altered force-generating units.

The pH dependence of growth inhibition by amiloride has also been seen in *Streptococcus faecalis*; 2 mM amiloride inhibited cell growth at pH 7.5 but not at pH 5.5 [13]. In contrast, we showed that the inhibitory activity of amiloride on the motility of *V. alginolyticus* was not pH dependent. Thus, the pH dependence of growth inhibition by amiloride in these bacteria is not a simple reflection of the pH dependence of amiloride activity itself. Since amiloride is a permeable weak base [4], a pH-dependent permeation rate of amiloride through the cytoplasmic membrane may be a factor. Further analysis is required to clarify the point.

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