Relationship between Na^+-dependent cytoplasmic pH homeostasis and Na^+-dependent flagellar rotation and amino acid transport in alkalophilic *Bacillus*

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Received 27 December 1984

The cytoplasmic pH homeostasis of alkalophilic *Bacillus* strains required the presence of Na^+ in the medium, and Li^+ was found to be equivalently substitutable for Na^+. Flagellar rotation and amino acid transport of these bacteria also required Na^+ but Li^+ was not substitutable for Na^+. Na^+ concentration of about 1 mM was enough for the cytoplasmic pH homeostasis, while more than 10 mM Na^+ was required for the full activities of flagellar rotation and amino acid transport. The addition of 150 mM ethanolamine to the cells at pH 9.6 disrupted the pH homeostasis and increased the cytoplasmic pH close to the external pH. Under this condition, however, flagellar rotation and amino acid transport were not so much affected. Thus, it is clear that flagellar rotation and amino acid transport themselves require the presence of Na^+ in the medium, independent of the Na^+-dependent cytoplasmic pH homeostasis.

Na^+/H^+ antiporter *pH homeostasis* *Flagellar motor* *Amino acid transport* *Alkalophilic* Bacillus

1. INTRODUCTION

In the case of bacteria living in moderate conditions, the protonmotive force is an important energy source for various cellular functions such as amino acid transport and flagellar rotation [1–3]. Various strains of alkalophilic *Bacillus* can grow well under severe alkaline conditions such as pH 11. As expected from the evidence that their cytoplasmic pH is maintained below 9, the protonmotive force of the cells under alkaline conditions is measured to be quite small [4–6]. Many of the cellular functions in these alkalophilic *Bacillus* required the presence of Na^+ in the medium, suggesting that the sodium-motive force, instead of the protonmotive force, is utilized for these cellular functions [4–10].

Evidence has been accumulated that the Na^+/H^+ antiporter probably has an important role in the cytoplasmic pH homeostasis of alkalophilic *Bacillus* [11]. Hence, we need to consider a possibility that the Na^+-dependent nature of various cellular functions of these alkalophilic bacteria is a secondary effect of the Na^+-dependent cytoplasmic pH homeostasis. We tested the possibility, and here, we present evidence that this is not the case; the cellular functions of the alkalophilic *Bacillus* themselves require the presence of Na^+, independent of the Na^+-dependent cytoplasmic pH homeostasis.

2. MATERIALS AND METHODS

The obligate alkalophilic *Bacillus* strains used in this work were *Bacillus firmus* RAB [6] and alkalophilic *Bacillus* sp. YN-1 [9] and 202-1 [12]. The cells were grown with shaking at 37°C in AB4 medium consisting of 10 g polypeptone, 1.5 g yeast extract, 10 g glucose, 1.5 g KH_2PO_4, 7.5 g Na_2CO_3 per l (pH 9.5). Cells at the late-log phase of growth were harvested by centrifugation, washed 3 times with CG medium consisting of 50 mM 2-*N*-cyclohexylaminoethane sulfonic acid–KOH buffer (pH 9.6) and 5 mM glucose, and

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00145793/85/$3.30

resuspended in the same medium. Glucose was used as an energy source, since glucose transport is reported to be independent of Na^+ [6,7].

Cells in CG medium (pH 9.6) were mixed with various concentrations of NaCl or LiCl and incubated for 30 s at 35°C with shaking. Then, intracellular pH, membrane potential and α-aminoisobutylate (AIB) transport were measured. Cytoplasmic pH was measured by the distribution of [^{14}C]Methylamine as in [6]. [^{14}C]Methylamine (0.92 mCi/mmol) was added to the cells to a final concentration of 1.1 μM. After 2 min incubation, samples (0.5 ml) were filtered through a Whatman GF/C glass filter which was presoaked in 10 μM non-radioactive methylamine. Filters were transferred into scintillation vials without washing and drying. As a zero ΔpH control, 10 μM gramicidin was added to the cells. Membrane potential of the cells was determined by using [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$) as in [10]. The transport of AIB was measured by using [^{14}C]AIB (5 mCi/mmol) as in [7]. The final concentration of AIB was 20 μM. All the radioactive materials were obtained from New England Nuclear (Boston, MA).

Flagellar rotation at 35°C was measured as the swimming speed of the cells as in [9]. The concentrated cells in AB4 medium were diluted 2000-fold or more with CG medium (pH 9.6) containing various concentrations of NaCl and LiCl. The amount of Na^+ contaminated from AB4 medium under the condition was less than 0.1 mM.

3. RESULTS

3.1. *Na^+-dependent cytoplasmic pH homeostasis*

Cytoplasmic pH of *B. firmus* RAB in a growth medium measured by the distribution of [^{14}C]methylamine was about 8.2 and stayed constant between the extracellular pH of 8.5 and 11.5. As in [6], Na^+ was required for the pH homeostasis of this bacterium. We observed that the cytoplasmic pH of the cells washed with CG medium (pH 9.6) without Na^+ was close to the extracellular pH and that the addition of Na^+ resulted in a clear restoration of the cytoplasmic pH to 8.5 within 2 min. Fig.1 shows the relationship between the Na^+ concentration in the medium and the cytoplasmic pH. The concentration of Na^+ required for the pH homeostasis was about

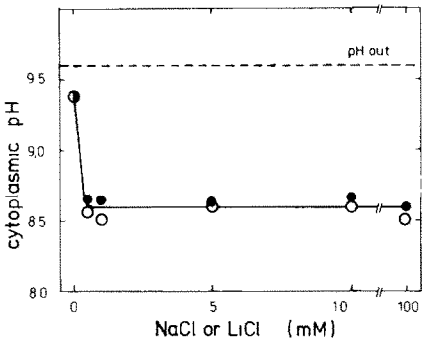

Fig.1. Effect of Na^+ and Li^+ on the cytoplasmic pH of *B. firmus* RAB. Cells in CG medium (pH 9.6) were mixed with various concentrations of NaCl (○) and LiCl (●) as indicated. After 0.5 min incubation at 35°C, the cytoplasmic pH was measured using [^{14}C]methylamine. Results are from 3 experiments.

0.5 mM or higher, and Na^+ concentrations lower than 0.1 mM had almost no effect. In addition to Na^+, we found that Li^+ was also effective for the pH homeostasis of this bacterium (fig.1). The concentration of Li^+ required for the pH homeostasis was quite similar to that of Na^+, indicating that Li^+ is almost equivalently substitutable for Na^+ on the pH homeostasis. Essentially similar results were obtained with other obligatory alkalophiles, alkalophilic *Bacillus* sp. YN-1 and 202-1 (not shown).

The membrane potential of *B. firmus* RAB cells, another important bioenergetic parameter of the cells, was not affected by the omission of Na^+ from the medium (table 1). The membrane potential of the cells at pH 9.6 was about -200 mV, and the addition of Na^+ or Li^+ showed no increase in the membrane potential. Alkalophilic *Bacillus* strains YN-1 and 202-1 showed similar results.

Table 1

Effect of Na^+ and Li^+ on the membrane potential of *B. firmus* RAB in CG medium (pH 9.6)

Addition	Membrane potential (mV)
+ none	−200
+ 100 mM NaCl	−195
+ 100 mM LiCl	−195

Thus, in the presence of either Na^+ or Li^+, both of the important bioenergetic parameters, cytoplasmic pH and membrane potential, of these alkalophiles are normal as in the growth medium.

3.2. *Na^+-dependent flagellar rotation and amino acid transport*

It has been reported that the flagellar rotation of alkalophilic *Bacillus* required the presence of Na^+ in the medium [6,9,10]. As shown in fig.2a, flagellar rotation of *B. firmus* RAB measured as the swimming speed of the cells required about 10 mM Na^+ in the medium for the maximum speed. In contrast to the case of cytoplasmic pH homeostasis described in the previous section, flagellar rotation was not restored by the addition of Li^+. The addition of 1 mM Na^+ induced slow but definite translational swimming in most cells, while the addition of 100 mM Li^+ did not induce any swimming. Of course, the addition of Na^+ in the presence of 100 mM LiCl induced normal swimming, indicating that Li^+ at this concentration had no inhibitory effect on the flagellar rotation. Flagellar rotation of other strains, YN-1 and 202-1, was also induced by Na^+ but not by Li^+.

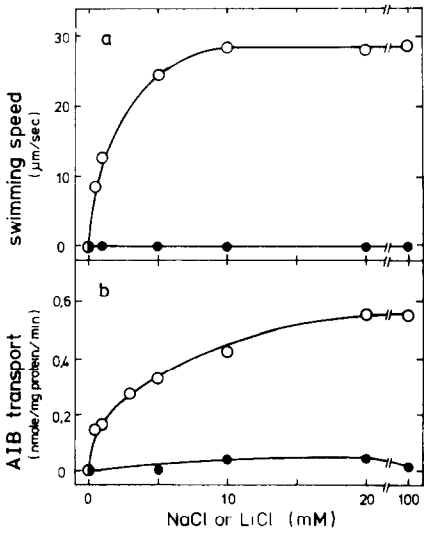

Fig.2. Effect of Na^+ and Li^+ on the swimming speed (a) and AIB transport (b) of *B. firmus* RAB. Cells in CG medium (pH 9.6) were mixed with various concentrations of NaCl (○) or LiCl (●) as indicated. After 0.5 min incubation at 35°C, swimming speed and AIB transport were measured.

The transport of α-aminoisobutylate (AIB) in alkalophilic *Bacillus* also required the presence of Na^+ in the medium, and much evidence suggests that AIB is symported with Na^+ [13,14]. Fig.2b shows that the AIB uptake in *B. firmus* RAB was stimulated clearly by Na^+ but very poorly by Li^+. Thus, as in the case of flagellar rotation, the AIB transport system absolutely required Na^+.

3.3. *Disruption of cytoplasmic pH homeostasis by ethanolamine*

It has been shown that membrane permeable weak acids and weak bases cause the disruption of cytoplasmic pH homeostasis [15,16]. The addition of 150 mM ethanolamine to *B. firmus* RAB cells at pH 9.6 caused a clear increase in their cytoplasmic pH close to the external pH even in the presence of Na^+ (table 2). Under this condition, however, more than half of the activities of flagellar rotation and AIB transport remained. Consistently, the membrane potential of the cells was not affected so much. Thus, it is clear that the increase in the cytoplasmic pH up to about 9.6 had no severe effect at least on these cellular functions.

4. DISCUSSION

The cytoplasmic pH homeostasis of alkalophilic *Bacillus* is considered to be a consequence of the

Table 2

Effect of ethanolamine on the cytoplasmic pH, membrane potential, flagellar rotation, and AIB transport in *B. firmus* RAB

	Ethanolamine added (mM)		
	0	100	150
Cytoplasmic pH	8.2	8.8	9.4
Membrane potential (mV)	−188	−162	−146
Swimming speed (μm/ml)	29	27	26
AIB transport (nmol/mg protein per min)	0.31	0.26	0.18

Cells in CG medium (pH 9.6) containing 75 mM NaCl were diluted 10–20-fold with CG medium, with or without ethanolamine as indicated. Before dilution, pH and Na^+ concentration of the ethanolamine-containing medium were adjusted to 9.6 by NaOH and to 75 mM by NaCl, respectively

function of Na^+/H^+ antiporter, which exchanges intracellular Na^+ for H^+ in the medium and acidifies the cytoplasm of these bacteria living in alkaline conditions. Since Li^+ was also active for the pH homeostasis, the Na^+/H^+ antiporter of these alkalophiles seems to utilize Li^+ instead of Na^+, like the Na^+/H^+ antiporter of higher animals [11].

In contrast to the cytoplasmic pH homeostasis, flagellar rotation and amino acid transport of these alkalophilic *Bacillus* absolutely required the presence of Na^+ in the medium and were not supported by Li^+. Furthermore, the disruption of the cytoplasmic pH homeostasis by ethanolamine did not cause a severe inhibition of these cellular functions. Thus, flagellar rotation and amino acid transport themselves require Na^+, independent of the Na^+-dependent cytoplasmic pH homeostasis. These results are clearly against the claim of Chernyak et al. [17]; they suggested that the Na^+-dependent nature of flagellar rotation and amino acid transport in alkalophilic *Bacillus* might be a secondary effect of the Na^+-dependent cytoplasmic pH homeostasis. Rather, the results give further support to the idea that these cellular functions are driven by the sodium-motive force in these alkalophiles.

ACKNOWLEDGEMENTS

We thank Professors T.A. Krulwich, K. Horikoshi and Y. Nosoh for provision of bacterial strains and Professor F. Oosawa of our Institute for critical reading of the manuscript. This work was supported by Grants-in-Aid for Special Project Research on Bioenergetics (no.59108006) and on Bioelectrical Response (no.59123004) from the Japanese Ministry of Education, Science and Culture.

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