Bioenergetic Properties of Alkalophilic Bacillus sp. Strain C-59 on an Alkaline Medium Containing K₂CO₃

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Alkalophilic Bacillus sp. strain C-59 could grow well on an alkaline medium containing K₂CO₃, as well as Na₂CO₃, but did not grow on K⁺-depleted medium. Right-side-out membrane vesicles, energized in the absence of Na⁺, however, could not take up [¹⁴C]methylamine actively, while vesicles equilibrated with 10 mM NaCl actively took up [¹⁴C]methylamine. The uptake of [¹⁴C]serine was also stimulated by the addition of Na⁺, and the imposition of a sodium gradient caused transient uptake. These results indicated that an Na⁺/H⁺ antiporter was involved in pH homeostasis and generation of an electrochemical sodium gradient in strain C-59 even though a growth requirement for Na⁺ was not evident. The efflux of Na⁺ from Na⁺-loaded vesicles was more rapid at pH 9.5 than at pH 7 in the presence of an electron donor. On the other hand, vesicles at pH 7 showed a more rapid efflux than at pH 9.5 when the antiporter was energized by a valinomycin-mediated K⁺ diffusion potential (inside negative).

Most alkalophilic bacteria isolated to date in alkaline media containing 1% Na₂CO₃ require Na⁺ for growth, and amino acid uptake by these cells was stimulated by the addition of Na⁺ (9, 14, 15, 17). We have reported that Na⁺ was required for α-aminosubtitytic acid uptake by the cells of some alkalophiles that were isolated in alkaline media containing K₂CO₃ instead of Na₂CO₃ (16).

It is clear that alkalophiles must maintain a cytoplasmic pH that is more acidic than the external pH. For example, cells of Bacillus firmus RAB exhibited an immediate rise in cytoplasmic pH in the absence of Na⁺ and lost viability very rapidly at alkaline pHs (13). Then, at least some alkalophiles have special functions for pH homeostasis which are related to Na⁺ and H⁺ movement. From extensive studies on the bioenergetics of alkalophiles, Kruilwich and co-workers (2, 5, 7, 20, 21, 24) indicated that an electrogenic Na⁺/H⁺ antiporter was required for pH homeostasis in obligate alkalophiles. An Na⁺/H⁺ antiporter is defined as a carrier that translocates Na⁺ and H⁺ in opposite directions across a membrane. This antiporter was very active and acidified the cytoplasm in the alkaline range of pH (18, 20, 21). In addition to the regulation of internal pH, the antiporter converted a proton motive force to an electrochemical gradient of Na⁺ which could energize solute transport. Thus, Na⁺ was reentered into the cells as a coupling ion for solute uptake. This Na⁺ circulation through the antiporter and the symporter is a most important physiological function in alkalophiles. These facts clearly underlie the expectation that alkalophiles will require Na⁺ for growth and solute transport.

Recently, we have found that alkalophilic Bacillus sp. strain C-59, which was isolated in Na₂CO₃-containing medium (11), can grow well in K₂CO₃ medium and cannot grow in K⁺-depleted alkaline medium, i.e., this strain exhibited no requirement for added Na⁺ for growth. In the current study, we have examined the effect of Na⁺ on the bioenergetic properties of Bacillus sp. strain C-59 at alkaline pH.

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MATERIALS AND METHODS

Organisms and growth conditions. Alkalophilic Bacillus sp. strains C-59 and 2b-2 were used throughout this experiment. Taxonomical studies on these strains have been reported (11, 22). Bacillus sp. strain C-59 was grown at 37°C with shaking at pH 10 in an alkaline medium (starch; 1%; yeast extract, 0.5%; polypeptone, 0.5%; K₂HPO₄, 0.1%; MgSO₄•7H₂O, 0.02%; Na₂CO₃ or K₂CO₃, 1%). Growth was measured by optical density at 660 nm with a Hitachi spectrophotometer (type 124).

Δψ and ΔpH measurements. The value for membrane potential (Δψ) was determined by a filtration assay (30) for whole cells. Bacteria were analyzed in the presence of [¹³C]tetraphenylphosphonium, which was added to a vigorously shaking cell suspension (0.3 mg of protein per ml) at a concentration of 20 μM. Counts retained by the filters in the presence of 20 μM gamicidin were subtracted. Δψ was calculated from the Nernst equation (13).

ΔpH was measured by the distribution of a weak base, [¹⁴C]methylamine, in a flow dialysis apparatus (26). Whole cells (0.9 mg of protein) were suspended with 200 μl of 0.05 M Na₂CO₃ or K₂CO₃ buffer (pH 10) in the upper chamber. Buffer was pumped through the lower chamber (spiral channel) at a rate of 1 ml/min. Fractions (1 ml) were collected and assayed for radioactivity by liquid scintillation spectrometry. The upper and lower chamber were separated by dialysis tubing. The upper chamber was stirred with a magnetic stirring bar under a stream of oxygen. [¹⁴C]Methylamine was added to a final concentration of 55 μM. [¹⁴C]Methylamine uptake by membrane vesicles was also measured by a flow dialysis method. The vesicles were energized by the addition of Tris-ascorbate (20 mM) and TMDP (tetramethyl-p-phenylenediamine, 0.2 mM). Vesicle concentrations of 1.1 mg of protein per ml were used. The ΔpH was calculated by the formula of Waddell and Butler (29). The internal volumes of Bacillus sp. strain C-59 cells and vesicles were found to be 6.0 and 1.1 μl/mg of protein, respectively.

²²Na⁺ efflux experiments. Na⁺ efflux experiments were performed by following the time-dependent decrease in
intravesicular $^{22}\text{Na}^+$ content of $^{22}\text{Na}^+$-loaded membrane vesicles as described by Bassilis et al. (1). Right-side-out membrane vesicles in 50 mM $K_2\text{CO}_3$ buffer (pH 9.5) were suspended in the same buffer containing 10 mM $\text{MgSO}_4$ and 10 mM NaCl at a final concentration of 20 mg/ml. Then, a small portion of carrier-free $^{22}\text{Na}^+$ was added to the vesicle suspension (3,500 cpm/mmol), and the suspension was incubated on ice for 2 to 3 h. When $^{22}\text{Na}^+$ efflux was conducted by $K^+$ diffusion potential, valinomycin was added to give a final concentration of 20 $\mu$M. $\text{Na}^+$ efflux determinations were initiated by diluting 2.5 $\mu$L of membrane vesicle solution into 400 $\mu$L of 50 mM $K_2\text{CO}_3$ or $K_2\text{PO}_4$ buffer containing 10 mM $\text{MgSO}_4$ and electron donors (Tris-ascorbate, 20 mM, plus TMPD, 0.15 mM) and quickly agitated with a Vortex mixer at room temperature. When a $K^+$ diffusion potential was produced, concentrated vesicles that had been equilibrated with $^{22}\text{Na}^+$ in the presence of valinomycin were diluted 800-fold into 100 mM choline chloride buffered with 10 mM Tris chloride (pH 7 and 9.5) or 100 mM $K_2\text{CO}_3$ as a control. At given times, the $\text{Na}^+$ efflux reaction was terminated by filtering the diluted sample. The filter (0.45 $\mu$m pore size; Millipore Corp.) was washed once with 2 ml of equilibrium solution. Nonspecific binding of $^{22}\text{Na}$ on the filters was estimated from the amount of radioactivity retained on the filter following filtration of diluted sample that had stood for 2 h at room temperature and was subtracted from the $\text{Na}^+$ efflux data.

Transport of [14C]serine by cells or membrane vesicles. Preparation of right-side-out membrane vesicles for transport and antiporter studies was performed by the osmotic shock procedure (12). Protein was determined by the method of Lowry et al. (23) with bovine serum albumin as a standard. The uptake of $[14\text{C}]$serine was determined by a filtration assay described previously (15). The final concentrations of protein and serine were 0.2 mg/ml and 300 $\mu$M for cells, or 1 mg/ml and 15 $\mu$M for membrane vesicles, respectively.

Materials. $^{22}\text{NaCl}$ (carrier free), $[14\text{C}]$serine (173 mCi/mmol), $[14\text{C}]$methyamine (46 mCi/mmol), and $[^3\text{H}]$tetraphenylphosphonium (35.5 Ci/mmol) were all obtained from New England Nuclear Corp. Valinomycin, gramicidin, and CCCP (carbonyl cyanide chlorophenylhydrazine) were from Sigma Chemical Co. Ascorbic acid and TMPD were purchased from Tokyo Kasei Co., Ltd. All other chemicals were obtained commercially at the highest purity available.

RESULTS

Growth characteristics. Bacillus sp. strain C-59 was originally isolated as a xylanase-producing bacterium (11) on Na$_2$CO$_3$-containing medium. Investigation of the effect of Na$^+$ or K$^+$ on the growth of Bacillus sp. strain C-59 showed that it could also grow well on K$_2$CO$_3$ medium. Indeed, the specific growth rate in K$_2$CO$_3$ medium was slightly higher than in Na$_2$CO$_3$ (0.57 and 0.43 h$^{-1}$, respectively). This strain could not grow on K$^+$-depleted medium (data not shown).

As the external pH was kept at more than 9.5 during the first 6 h, Bacillus sp. strain C-59 can grow in very alkaline conditions without added Na$^+$. $\Delta$P analysis. To analyze the bioenergetic properties of Bacillus sp. strain C-59, $\Delta$PH and $\Delta$P were measured, which together constituted the $\Delta$ (25). The flow dialysis pattern of methylamine uptake by cells of Bacillus sp. strains C-59 and 2b-2 showed that cells of C-59 actively took up methylamine in the presence of K$_2$CO$_3$ as well as Na$_2$CO$_3$. This was consistent with the generation of $\Delta$PH, inside acid, without added Na$^+$. On the other hand, the cells of 2b-2 failed to take up methylamine in the absence of Na$^+$. Methylamine uptake by the cells of C-59 was inhibited by the inclusion of gramicidin (20 $\mu$m) in both Na$_2$CO$_3$ and K$_2$CO$_3$ media. The magnitude and orientation of the $\Delta$PH and $\Delta$P of cells in C-59 and 2b-2 are shown in Table I. In the absence of added Na$^+$, the cells of C-59 generated a substantial $\Delta$PH, acid in, in alkaline conditions, while the internal pHs of the cells of 2b-2 and Bacillus firmus RAB, which require added Na$^+$ for growth and amino acid transport, were 10.0, i.e., $\Delta$PH = 0. Cells incubated with either Na$^+$ or K$^+$ exhibited appreciable $\Delta$P values, and there was essentially no difference between Na$^+$ and K$^+$ medium. These results indicate that primary proton pumping allowed the formation of $\Delta$PH, and a secondary Na$^+$/H$^+$ antiporter plays a vital role in pH homeostasis in the alkaline region. The question then arises how Bacillus sp. strain C-59 could keep the cytoplasmic pH lower than in the external milieu without added Na$^+$. Does C-59 possess a special function for the regulation of internal pH other than an Na$^+/H^+$ antiporter, or does this antiporter have a special high affinity for Na$^+$ like that of Bacillus alcalophilus (20)? To examine this question in more detail, methylamine uptake by membrane vesicles was measured by a flow dialysis assay. Membrane vesicles energized in the absence of Na$^+$ could not take up methylamine actively (Fig. 1). By contrast, the vesicles equilibrated with 10 mM NaCl actively took up methylamine. This was consistent with generation of $\Delta$P, inside acid. This indicates that an Na$^+$/$H^+$ antiporter is involved in pH homeostasis in C-59 which requires relatively lower concentration of Na$^+$. In the presence of 50 mM K$_2$CO$_3$, some indication of intravesicular acidification was also observed (see Discussion).

Transport system. The investigation was then extended to [14C]serine uptake by cells or membrane vesicles from C-59 and 2b-2. Serine uptake by cells of C-59 was stimulated by the addition of Na$^+$ to a maximal level at around 10 mM Na$^+$ (Fig. 2A). Further addition of Na$^+$ up to 100 mM produced no increased stimulation. Conversely, cells of 2b-2 were stimulated for serine uptake at higher levels than for C-59; 2b-2 also demonstrated a requirement for higher concentrations of Na$^+$, attaining maximal uptake at Na$^+$ concentrations of 100 mM or greater. Figure 2B shows the effect of Na$_2$CO$_3$ and K$_2$CO$_3$ concentration on serine uptake by cells of C-59. The profile of serine uptake in the presence of Na$_2$CO$_3$ was similar to that in the presence of NaCl. Serine uptake was also stimulated by the addition of K$_2$CO$_3$. In this case, serine uptake was higher than for Na$_2$CO$_3$ at concentrations higher than 20 mM, reaching a maximum at approximately 50 mM.

The different Na$^+$ requirements for serine uptake were notable in membrane vesicles. At concentrations of added Na$^+$ less than 10 mM, the vesicles from 2b-2 exhibited little serine uptake, whereas the vesicles from C-59 showed

### TABLE 1. $\Delta$P patterns of alkalophilic Bacillus species as a function of Na$^+$ and K$^+$ content of the medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Na$_2$CO$_3$ (50 mM)</th>
<th>K$_2$CO$_3$ (50 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta$PH (mV)</td>
<td>$\Delta$P (mV)</td>
</tr>
<tr>
<td></td>
<td>$\Delta$PH (mV)</td>
<td>$\Delta$P (mV)</td>
</tr>
<tr>
<td>Bacillus sp. strain 2b-2</td>
<td>+84</td>
<td>-142</td>
</tr>
<tr>
<td>Bacillus sp. strain C-59</td>
<td>+90</td>
<td>-131</td>
</tr>
<tr>
<td>B. firmus RAB*</td>
<td>+65</td>
<td>-162</td>
</tr>
</tbody>
</table>

* Data from Kitada et al. (13).
maximum accumulation (Fig. 3). Thus, Na⁺/serine symport in vesicles from C-59 showed a requirement for a lower concentration of added Na⁺. Since there was almost no serine uptake in the absence of added Na⁺, it is likely that serine was taken up by the electrochemical sodium gradient which was formed through an Na⁺/H⁺ antiporter. The result in Fig. 4 showed that this was the case. The membrane vesicles were washed thoroughly with 50 mM Tris hydrochloride (pH 8) and suspended in the same buffer containing 10 mM MgSO₄ and [¹⁴C]serine. A sodium ion gradient across the membrane was imposed by the addition of NaCl (50 mM) into the above solution. The addition of Na⁺ caused a transient uptake of serine (Fig. 4). When K⁺ was substituted for Na⁺, no stimulation of serine uptake was observed.

**Efflux of ²²Na⁺ from membrane vesicles of Bacillus sp. strain C-59.** The results in Fig. 1 and 4 suggest that the membrane from C-59 possesses an Na⁺/H⁺ antiporter and that this antiporter plays a crucial role in the maintenance of relatively acidified cytoplasm and in generating a sodium gradient. This antiporter activity was examined by measuring the efflux of ²²Na from ²²Na-loaded vesicles that were energized either by the addition of an electron donor, ascorbate-TMPD, or by the establishment of a valinomycin-mediated potassium diffusion potential. In the experiment in Fig. 5, vesicles equilibrated with 10 mM radioactive Na⁺ inside at pH 9.5 were diluted 100-fold into the buffer containing 10 mM NaCl at pH 7 or 9.5. In the absence of an electron donor, there was almost no ²²Na efflux at either pH. In the presence of the electron donor, rapid efflux of ²²Na was observed at both pHs; ²²Na efflux at pH 9.5 was more rapid than that at pH 7. The addition of CCCP (20 μM)
inhibited $^{22}\text{Na}$ efflux in the presence of the electron donor. Although the data are not shown, the presence of 10 to 50 mM nonradioactive Na$^+$ in the dilution medium had no effect on the patterns of $^{22}\text{Na}$ efflux. When the antiporter was energized by a valinomycin-mediated potassium diffusion potential (inside negative), there was also rapid $^{22}\text{Na}$ efflux at both pHs. The vesicles showed more rapid efflux at pH 7 than at pH 9.5.

**DISCUSSION**

The growth data showed that *Bacillus* sp. strain C-59 could grow in alkaline conditions without added Na$^+$. Flow dialysis analysis indicated that methylamine was taken up in 50 mM K$_2$CO$_3$ buffer, reflecting a ΔpH, acid in. Since the alkaline medium used here contained a fermentable sugar, starch, acidic metabolites might be produced to lower the internal pH. To avoid this possibility, methylamine uptake was examined by flow dialysis with right-side-out membrane vesicles. There was no appreciable uptake of methylamine in the absence of Na$^+$. Thus, it is possible that C-59 really required Na$^+$ for growth but that the alkaline medium might contain considerable amounts of Na$^+$ contamination. The results in Fig. 1 indicated that Na$^+$ was necessary to maintain lower cytoplasmic pH than external pH in the alkaline region and that an electrogenic Na$^+$/H$^+$ antiporter might be involved in this reaction.

Brey et al. (3) had proposed that the Na$^+$/H$^+$ antiporter was not needed for pH homeostasis in *Escherichia coli* because there was no evidence that Na$^+$ was required for its growth. They concluded that an K$^+$/H$^+$ antiporter played a vital role for pH homeostasis in *E. coli* because K$^+$ was absolutely required for growth and was continually accumulated to high concentration in the cells. In the case of C-59, however, K$^+$ did not appreciably stimulate methylamine uptake by membrane vesicles (Fig. 1). It was unlikely that a K$^+$/H$^+$ antiporter regulated the internal pH in the alkaline region.

It has been reported that *Bacillus alcalophilus* did not exhibit any requirement for added Na$^+$ for growth but possessed the Na$^+$/H$^+$ antiporter, having a higher affinity for Na$^+$, which served the vital physiological function of maintaining lower cytoplasmic pH (20).

Observations of respiration-dependent and valinomycin-induced $^{22}\text{Na}$ efflux supported the idea that $^{22}\text{Na}$ efflux from membrane vesicles from C-59 would represent the activity of the electrogenic Na$^+$/H$^+$ antiporter. Na$^+$-dependent H$^+$ influx could not be determined, but this $^{22}\text{Na}$ efflux was not due to a respiration-dependent sodium pump (27, 28) because the efflux was inhibited by the addition of CCCP (20 μM).

At an internal pH of 9.5, respiration-dependent $^{22}\text{Na}$ efflux was more rapid at an external pH of 9.5 than at a pH of 7.0. Thus, high external H$^+$ concentration (pH 7) had no stimulatory effect on $^{22}\text{Na}$ efflux. This result was in good agreement with the fact that the antiporter must normally function at an alkaline pH. On the contrary, $^{22}\text{Na}$ efflux was more rapid at pH 7 when the antiporter used a valinomycin-mediated K$^+$ diffusion potential. This means that the external H$^+$ concentration stimulated $^{22}\text{Na}$ efflux from the vesicles. Similar results have been obtained in the experiments on ATP synthesis in starved cells of *Bacillus firmus* RAB (8), but the antiporter in that strain is not active at neutral pH (4).

It is so far not clear why the external pH had different effects on the $^{22}\text{Na}$ efflux rate between respiration-dependent and valinomycin-induced energization. Since the actual membrane potential could not be determined, it is unclear whether the value of the diffusion potential at pH 9.5 and at pH 7 is the same. The hypothesis by Haines (10) about a localized pathway, however, is suggestive of one model, i.e., some of the protons pumped out by respiration are made available to the proton-utilizing antiporter in some localized way, as observed in ATP synthesis by *B. firmus* RAB (6, 8, 19).

Finally, we have to refer to the effects of K$_2$CO$_3$ on methylamine and serine uptake. As shown in Fig. 1 and 2B, methylamine and serine were taken up actively in the presence of K$_2$CO$_3$. The potassium carbonate used was the highest grade, but 50 mM K$_2$CO$_3$ buffer (pH 10) contained 12 ppm (0.5 mM) of Na$^+$ from the atomic absorption analysis.

**FIG. 5.** $^{22}\text{Na}^+$ efflux from $^{22}\text{Na}$-loaded membrane vesicles of *Bacillus* sp. strain C-59 at pH 7.0 (A) and 9.0 (B). Efflux of $^{22}\text{Na}^+$ was energized by either respiration (A) or K$^+$ diffusion potential (B). Control experiments (A) were carried out in the presence of either CCCP (10 μM) (A) or K$_2$CO$_3$ (50 mM) (B).
Therefore, methylamine uptake by cells might be due to the contaminating Na\(^+\) in K\(_2\)CO\(_3\) buffer, because membrane vesicles required considerable amounts of Na\(^+\) for its uptake. It is unlikely, however, that the stimulation of serine uptake was due to the contaminating Na\(^+\), because serine uptake was higher in K\(_2\)CO\(_3\) over 50 mM concentration (Fig. 2B). It will be of interest to study the influence of K\(_2\)CO\(_3\) and CO\(_3^{2-}\) on physiological properties in alkalophiles, especially those able to grow on K\(_2\)CO\(_3\) medium.

**LITERATURE CITED**


