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Na^+ modulates the K^+ permeability and the membrane potential of alkalophilic *Bacillus*

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In the absence of Na^+ in the medium, the membrane potential of obligately alkalophilic *Bacillus* cells was found to be decreased by the addition of K^+ to the medium, whereas K^+ addition in the presence of Na^+ had no effect. Rb^+ showed essentially the same effect as K^+. The decreased membrane potential was quickly restored by lowering the K^+ concentration in the medium or by adding Na^+ or Li^+ to the medium. Thus, in the absence of Na^+, the membrane potential of alkalophilic *Bacillus* seems to be affected by the concentration difference of K^+ between inside and outside of the cell, and Na^+ or Li^+ in the medium suppresses the K^+ effect. An exchange between extracellular Rb^+ and intracellular K^+ was observed in the absence of Na^+. However, the exchange was greatly suppressed by the addition of Na^+ or Li^+ to the medium, indicating that Na^+ in the medium modulates the K^+ permeability of the alkalophilic *Bacillus* cell membrane. The K^+-induced decrease in the membrane potential of alkalophilic *Bacillus* in the absence of Na^+ is accounted for by the increased K^+-permeability of the cell membrane.

Introduction

Many of neutrophilic bacteria utilize the electrochemical potential gradient of protons across the membrane, $\Delta\tilde{\mu}_{H^+}$, as the energy source for their functions located on the membrane such as ATP synthesis, active transport and flagellar rotation [1–3]. The $\Delta\tilde{\mu}_{H^+}$ is composed of the membrane potential and the pH gradient across the membrane. Since the intracellular pH of these bacteria is measured to be near neutral [4,5], the membrane potential is the principal constituent of the $\Delta\tilde{\mu}_{H^+}$ in the cell at neutral pH. In the case of aerobic bacteria such as *Escherichia coli* and *Bacillus subtilis*, H^+ is pumped out through the respiratory chain, and this is the major source of generation of the membrane potential. When respiration is blocked, the membrane potential is supported by the reverse reaction of ATP synthase [6,7]. Thus, the maintenance of the membrane potential seems to be the primary demand on the cell physiology of these bacteria.

Obligately alkalophilic *Bacillus* species show optimal growth at pH 10–11. Since the intracellular pH of these bacteria is lower than 9, the $\Delta\tilde{\mu}_{H^+}$ is calculated to be quite small under the growth condition [8,9]. To overcome such low $\Delta\tilde{\mu}_{H^+}$ conditions, these bacteria have developed a novel system for energy coupling to the electrochemical potential gradient of Na^+, $\Delta\tilde{\mu}_{Na^+}$. Actually, amino acid transport systems and flagellar motors of

Abbreviations. $TPMP^+$, triphenylmethylphosphonium ion, TPP^+, tetraphenylphosphonium ion.

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these bacteria are driven by the $\Delta\tilde{\mu}_{Na^+}$ [10–12]. The presence of 25 mM Na^+ in the medium is enough for the growth of these bacteria and the intracellular Na^+ concentration is estimated to be about 30 mM under the condition [12,13] This means that the membrane potential of the cell is the principal constituent of the $\Delta\tilde{\mu}_{Na^+}$. Therefore, as in the case of neutrophilic bacteria, the maintenance of the membrane potential must be quite important for the cell physiology of these alkalophilic bacteria.

It has been shown that the membrane potential of the respiring neutrophilic bacteria around neutral pH is not affected by the ionic composition of the medium [5,14]. In the previous paper [12], we reported that the membrane potential of the respiring cells of alkalophilic *Bacillus* was not affected by the presence or absence of Na^+ in the medium. Since the respiratory chain of these bacteria pumps out H^+ and produces the membrane potential [10], that result was considered to be quite reasonable. During experiments to clarify the role of membrane potential of these alkalophilic *Bacillus*, however, we found that the membrane potential in the presence of K^+ was affected by the presence or absence of Na^+ in the medium.

In this report, we describe the evidence that the K^+ permeability of alkalophilic *Bacillus* membrane is modulated by Na^+ in the medium and that the Na^+-dependence of the membrane potential of these bacteria is attributed to the secondary effect of this K^+ permeability.

Materials and Methods

Bacterial strains and growth condition. Obligately alkalophilic *Bacillus* strains used were 202-1 [15], YN-1 [16] and *Bacillus firmus* RAB [17] Cells were grown at 35°C with shaking in AB-4 medium consisting of 10 g polypeptone, 1.5 g yeast extract, 10 g glucose, 1.5 g KH_2PO_4, 0.1 g $MgCl_2$ and 10 g Na_2CO_3 in a liter of water [18]

At a late-logarithmic phase of growth, cells were harvested by centrifugation at $6000 \times g$ for 5 min at room temperature, and washed twice with TG medium consisting of 25 mM Tris-HCl buffer (pH 9 5) and 5 mM glucose. In some experiments, KG medium consisting of 80 mM K_2CO_3 buffer (pH 10) and 5 mM glucose was used instead of TG medium. Glucose was used as the energy source in all the experiments, since its transport is independent of the presence or absence of Na^+ in the medium [19,20]. When necessary, various concentrations of NaCl or KCl were added to the medium.

Measurement of the membrane potential The membrane potential was quantitated by the use of a membrane permeable radioactive cation, [^{3}H]triphenylmethylphosphonium ion ([^{3}H]TPMP$^+$) as described previously [12,21,22]. Briefly, the cells ($(1–2) \cdot 10^8$ cells per ml) were incubated with 10 μM of [^{3}H]TPMP$^+$ (0 2 mCi/mmol, New England Nuclear, Boston, MA), and aerated with shaking at 35°C. A sample (50 μl) was filtered through a membrane filter (cellulose acetate, SM 111, Sartorius-Membrane Filter GmbH, Göttingen, F.R.G.). After washing the filter with 5 ml of 0 1 M LiCl, the radioactivity trapped on the filter was measured by a scintillation spectrophotometer As a control for zero membrane potential, the cells treated with 10 μM gramicidin D were used [^{3}H]TPMP$^+$ was a generous gift of Dr R.M Macnab of Yale University. Except otherwise noted, [^{3}H]TPMP$^+$ was added to the cells at the beginning of the experiment.

To follow the detailed time course of the changes in the membrane potential of the cells, tetraphenylphosphonium ion (TPP$^+$)-selective electrode [23] was used. In this case, the cell concentration was $(5–10) \cdot 10^8$ cells per ml, and the cells at 35°C were aerated by bubbling the air through a thin needle. TPP$^+$ was added to 10 μM. The electrode potential was linear from 1 to 100 μM of TPP$^+$, and the slope was 59 mV per decade concentration at 35°C. To estimate the zero membrane potential, the cells were treated with 4 mM of a detergent, *N*-gluco-*N*-methylcaprylamide [18]

Measurement of intracellular K^+ and Rb^+ concentration Intracellular K^+ and Rb^+ concentrations were measured by the atomic absorption method as described previously [12] Briefly, a sample (about 1 10^8 cells) was filtered through a membrane filter, and the cells trapped on the filter were treated with concentrated nitric acid. The K^+ or Rb^+ content in the sample was measured by a Hitachi atomic absorption spectrometer model 180-50

Measurement of ATP content. ATP content of the cells was measured by using firefly lantern extract as described previously [12].

Measurement of O_2 consumption. The rate of O_2 consumption by the cells was measured by using an O_2-electrode (Rank Brothers, Co., U.K.). The cells in TG medium (pH 9.5) supplemented with or without 100 mM NaCl was put in the apparatus, and the rate of O_2 consumption was measured at 30 °C. Zero O_2 point was estimated by the addition of a small crystal of sodium hydrosulfite to the cell suspension.

Results

Effect of Na^+ and K^+ on the membrane potential of the cells

It has been shown that the membrane potential of alkalophilic *Bacillus* was not affected by the presence or absence of Na^+ in the medium [12]. We found, however, that this was the case only when the K^+ concentration in the medium was low.

As shown in Fig. 1A, the membrane potential of 202-1 cells in the absence of NaCl, namely in TG medium (25 mM Tris-HCl buffer (pH 9.5) supplemented with 5 mM glucose), was about −170 mV. When KCl was added to the medium to a final concentration of 200 mM, the membrane potential of the cells was gradually decreased and reached about −60 mV after 30 min. The addition of 20 mM NaCl resulted in a quick and almost complete restoration of the membrane potential of the cells. Similar results were obtained at pH 7.5 (Table I) and by the cells of RAB and YN-1 (data not shown). Thus, only in the absence of Na^+ in the medium, the membrane potential of alkalophilic *Bacillus* was decreased by K^+ in the medium.

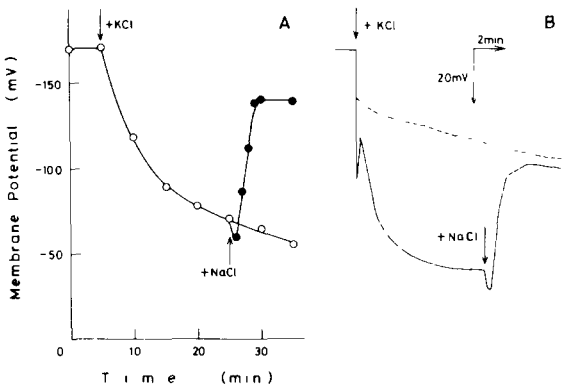

Fig 1. Effect of KCl and NaCl on the membrane potential of alkalophilic *Bacillus* (A) 202-1 cells in TG medium (pH 9.5) containing [^{3}H]TPMP$^+$ were incubated at 35 °C with aeration At the first arrow, 200 mM KCl was added (○), and at the second arrow, 20 mM NaCl was added (●) The membrane potential of the cells was measured as described in the Materials and Methods. (B) RAB cells in TG medium (pH 9 5) were incubated with 10 μM TPP$^+$, and the changes in the TPP$^+$-electrode potential were measured at 35 °C KCl and NaCl added were 200 mM and 20 mM, respectively Dotted line shows the change observed in the cells preincubated with 20 mM NaCl

The time course of the change in the membrane potential of the cells was analyzed in detail by using the TPP$^+$-electrode method. As shown in Fig. 1B, the addition of 200 mM KCl to RAB cells in TG medium (pH 9.5) caused a gradual decrease in the electrode potential, corresponding to a gradual decrease in the membrane potential of the cells. The decreased membrane potential was quickly restored by the addition of 20 mM NaCl. Consistent with this, the addition of 200 mM KCl in the presence of 20 mM NaCl caused only a slight decrease in the membrane potential. A 20 mV drop in the electrode potential observed just after the addition of 200 mM KCl was considered to be an artifact caused by the large increase in the salt concentration of the medium, since the drop was observed even in the presence of 20 mM NaCl.

TABLE I

EFFECT OF MEDIUM pH ON THE DECREASE OR THE RESTORATION OF THE MEMBRANE POTENTIAL OF 202-1 CELLS

Cells in KG medium containing 25 mM Tris (pH 9.5 or 7 5) were incubated at 35 °C for 10 min To an aliquot, 50 mM NaCl was added The membrane potential of the cells was measured after incubation for 5 min

Medium pH	Addition	Membrane potential (mV)
9 5	none	−80
	NaCl	−175
7 5	none	−60
	NaCl	−121

It is noteworthy that the addition of NaCl for the restoration of the decreased membrane potential actually induced a transient but further decrease in the membrane potential prior to the restoration (Fig. 1B). This transient decrease was not caused by the addition of 20 mM KCl instead of NaCl (data not shown), indicating that the increase in the salt concentration was not the cause of this transient decrease.

Conditions to decrease the membrane potential

To investigate the ion specificity for the decrease in the membrane potential in the absence of Na^+, RAB cells in TG medium (pH 9.5) were treated with 200 mM of various salts In addition to KCl, RbCl was found to cause a similar decrease in the membrane potential of the cells, whereas LiCl, as well as NaCl, did not (Fig. 2)

The membrane potential of the cells was gradually decreased with increasing concentrations of KCl in the medium (Fig. 2), indicating that the concentration of K^+ in the medium is an important factor for the decrease in the membrane potential.

Conditions to restore the membrane potential

The decreased membrane potential of K^+-treated 202-1 cells was restored by the addition of Na^+ or Li^+. With increasing concentrations of NaCl or LiCl, the final value of the membrane potential was incerased, and about 5 mM of either NaCl or LiCl was enough to restore the membrane potential almost completely (Fig. 3). Compared to Na^+, Li^+ showed a similar but slightly weaker effect on the restoration. The decreased membrane potential caused by the addition of Rb^+ was also restored by Na^+ or Li^+ (data not shown).

The decreased membrane potential of the K^+-treated cells was also restored quickly by decreasing K^+ concentrations in the medium. Fig 4 shows that with decreasing the final KCl concentrations, the membrane potential of RAB cells was gradually increased and reached about -160 mV at 4 mM KCl. This indicates that the membrane potential is almost completely restored by the dilution of KCl concentration from 200 mM to 4 mM. Essentially the same results were obtained in 202-1 and YN-1. Thus, these results strongly support the idea that the K^+ concentration in the medium is the only effective factor for altering the membrane potential of the alkalophilic *Bacillus* in the absence of Na^+.

Effect of Na^+ on the K^+ permeability of cell membrane

The K^+-induced decrease in the membrane

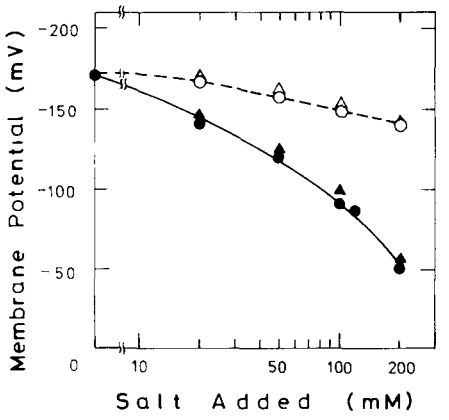

Fig 2. Relationship between the membrane potential of RAB cells and various concentrations of salts in the medium The cells in TG medium (pH 9 5) containing [^{3}H]TPMP$^+$ were incubated with various salts for 20 min at 35°C KCl (●), RbCl (▲), NaCl (○), LiCl (△)

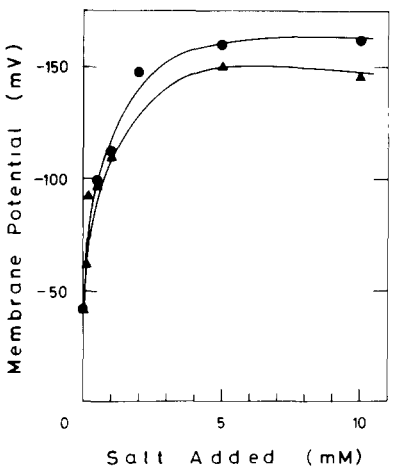

Fig 3 Restoration of the membrane potential of the K^+-treated 202-1 cells with various concentrations of NaCl or LiCl The cells were preincubated for 20 min at 35°C in KG medium (pH 9 5), and then, various concentrations of NaCl (●) or LiCl (▲) were added. The membrane potential of the cells was measured after incubation for 5 min

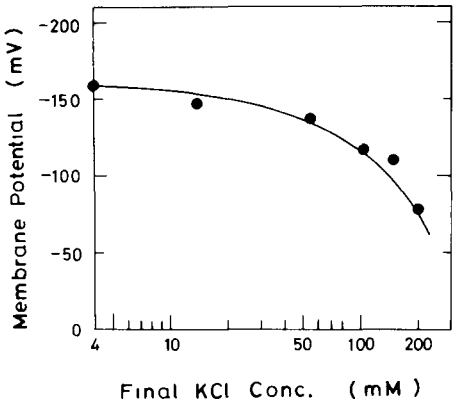

Fig 4 Restoration of the membrane potential of the K^+-treated RAB cells by the dilution of KCl concentration The cells (about 4 10^9 cells per ml) in TG medium (pH 9 5) were preincubated with 200 mM KCl for 20 min. The cells were then diluted 50-fold in TG medium (pH 9 5) containing [^{3}H]TPMP$^+$ and various concentrations of KCl The membrane potential of the cells was measured after incubation for 10 min

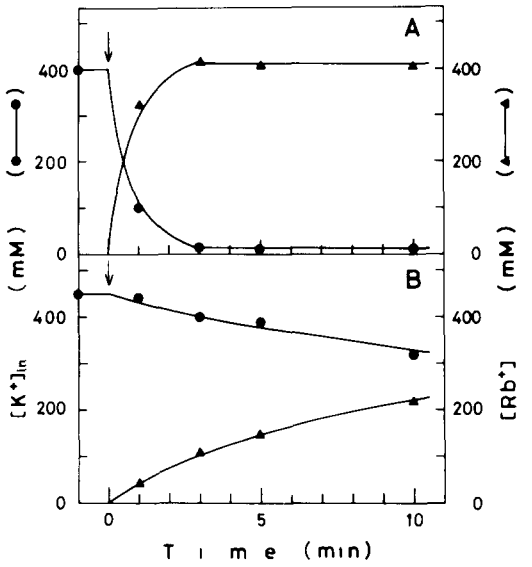

Fig 5 Exchange between intracellular K^+ and extracellular Rb^+ in RAB cells. The cells in TG medium (pH 9 5) with or without 200 mM NaCl were incubated at 35 °C, and 25 mM RbCl was added at the time zero K^+ (●) and Rb^+ (▲) contents in the cells were measured by the atomic absorption method (A) no NaCl, (B) 200 mM NaCl

potential in the absence of Na^+ might be explained if the K^+ permeability of the cell membrane would be considerably high in the absence of Na^+. To test this possibility, the K^+ permeability of the cells in the presence or absence of Na^+ in the medium was measured as the rate of exchange between intracellular K^+ and extracellular Rb^+.

Fig. 5 shows that the addition of 25 mM RbCl to RAB cells in the absence of Na^+ caused a quick and large decrease in the intracellular K^+ concentration. The decrease in the intracellular K^+ was found to be accompanied with a compensatory increase in the intracellular Rb^+ concentration. In contrast, in the presence of 200 mM NaCl in the medium, the addition of 25 mM RbCl caused only a small and slow decrease in the intracellular K^+ and also a small increase in the intracellular Rb^+. Similar results were obtained in 202-1 and YN-1. These results clearly indicate that in the absence of Na^+ in the medium, a K^+–Rb^+ exchange system appears in the alkalophilic *Bacillus* membrane. Since K^+ exchanged almost equivalently with Rb^+, the exchange seems to be electroneutral.

With increasing Na^+ concentrations in the medium, the rate of K^+–Rb^+ exchange was gradually decreased (Fig. 6). Thus the K^+–Rb^+ exchange, namely the K^+ permeability of alkalophilic *Bacillus* membrane, is affected by the Na^+ concentration in the medium. These results strongly support the idea that the high K^+-permeability in the absence of Na^+ is responsible for the K^+-induced decrease in the membrane potential of the cells.

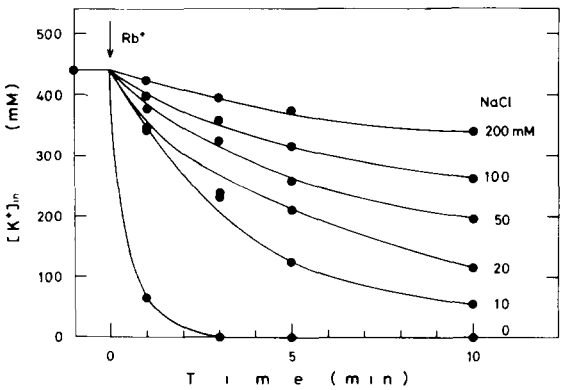

Fig 6 Effect of various concentrations of NaCl on the K^+–Rb^+ exchange in RAB cells. After preincubating the cells in TG medium (pH 9.5) with the indicated concentrations of NaCl, 25 mM RbCl was added at the time zero

Relationship between the K^+ permeability and the membrane potential

From the results shown in the previous sections, it is evident that the concentration of Na^+ required for the restoration of the membrane potential was considerably lower than that required for the suppression of K^+-permeability. To investigate the quantitative relationship between the rate of the K^+ permeation and the size of the membrane potential of RAB cells, a well-known K^+ ionophore, valinomycin, was used to make the cell membrane quantitatively permeable to K^+. At first, the cells were incubated with 200 mM NaCl to minimize the K^+ permeability, and then, various concentrations of valinomycin were added to induce the different rates of K^+ permeation into the cells. As shown in Fig. 7, the valinomycin-induced K^+ permeation, which was measured as the K^+–Rb^+ exchange rate, was detected clearly at valinomycin concentrations higher than 2 nM, whereas the K^+-induced decrease in the membrane potential was detected at valinomycin concentrations higher than 20 nM. Thus, compared to the K^+ permeability, a concentration greater of about 10-fold valinomycin was required to alter

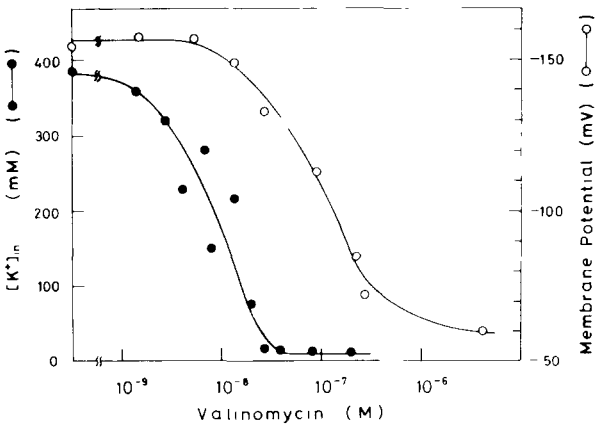

Fig 7 Relationship between valinomycin concentration and the K^+–Rb^+ exchange or the membrane potential in RAB cells. The cells in TG medium (pH 9 5) containing 200 mM NaCl were incubated at 35 °C with various concentrations of valinomycin for 5 min For the measurement of the K^+–Rb^+ exchange, the intracellular K^+ was measured 3 min after the addition of 25 mM RbCl The membrane potential was measured after incubating the cells with [^{3}H]TPMP$^+$ and 200 mM KCl for 10 min Intracellular K^+ content (●), the membrane potential (○)

the membrane potential. This indicates that the alkalophilic *Bacillus* cells have the ability to maintain the high membrane potential even when there is a considerable leakage of K^+ through the membrane.

Based on these results, we can conclude that the K^+-induced decrease in the membrane potential of the cells in the absence of Na^+ is caused by the considerable increase in the K^+ permeability of the cell membrane induced by the absence of Na^+.

ATP content and O_2 consumption rate in the absence of Na^+

The ATP content of alkalophilic *Bacillus* was not affected by the presence or absence of Na^+ in the medium [12,19]. However, the addition of KCl to the cells in the absence of Na^+ was found to cause a gradual decrease in the ATP content. By the incubation for 30 min with 200 mM KCl, the ATP content of RAB cells was decreased from 3.5 mM to 0.2 mM. Time course of the decrease in the ATP level looked parallel with that of the decrease in the membrane potential shown in Fig. 1. The addition of Na^+ to the medium caused a quick restoration of ATP level coupled with a quick restoration of the membrane potential. Thus, the decrease in the membrane potential seems to cause the decrease in the ATP content of the cell.

The rate of respiration of RAB cells in TG medium (pH 9.5), which was measured as the rate of O_2 consumption using an oxygen electrode, was not affected by the presence or absence of Na^+ in the medium or by the addition of 200 mM KCl in the absence of Na^+ These results are consitent with those reported by Kitada et al. [19], although our experimental condition is slightly different from theirs. Hence, the decrease in the membrane potential and the ATP content in the K^+-treated cells in the absence of Na^+ was not attributed to the decrease in the activity of the energy producing system, namely the respiratory chain.

Discussion

The cell membrane of obligately alkalophilic *Bacillus* was found to have a unique property for K^+ permeability; the K^+ permeability is high in the absence of Na^+ in the medium but is sharply

decreased with increasing concentrations of Na^+ in the medium. In the absence of Na^+, as an expected consequence of the high K^+-permeability, the membrane potential of the cells decreased with increasing K^+ concentrations in the medium, just as in the presence of a small amount of a K^+ ionophore, valinomycin. The decreased membrane potential was restored by lowering the K^+ concentration in the medium or by adding Na^+ to the medium. Thus, as a secondary effect of the Na^+-dependent modulation of K^+-permeability of the cell membrane, the membrane potential of alkalophilic *Bacillus* is affected by Na^+ and K^+. Li^+ was almost equivalent to Na^+, and Rb^+ was almost equivalent to K^+. Three strains isolated by different laboratories showed essentially the same properties, suggesting that this peculiar property of the cell membrane is widely distributed in obligately alkalophilic *Bacillus* species.

The addition of 5 mM Na^+ was enough for the complete restoration of the decreased membrane potential, although a significant rate of K^+ permeation was detected at 10 mM Na^+. Thus, the cells seem to have the ability to maintain the membrane potential even when there is some leakage of K^+. By the use of valinomycin, we showed that this was the case. Namely, in the presence of Na^+, the valinomycin concentration required for the induction of K^+-leakage was about 10-times lower than that required for the K^+-induced decrease in the membrane potential of the cells. In the case of alkalophilic *Bacillus*, the respiratory chain pumps out H^+ and this is considered to be the main source for the generation of the membrane potential of the cells [10]. Then, the H^+-pumping activity of alkalophilic *Bacillus* seems to have enough power to overcome the K^+ permeability detected at 10 mM Na^+.

Although the K^+ permeability was high in the absence of Na^+, the addition of K^+ to the medium caused not an intantaneous but a gradual decrease in the membrane potential of the cell. This may indicate that the observed K^+ permeability is not sufficiently high to cause the instantaneous effect of the K^+ addition. Furthermore, the intracellular ATP content was found to be decreased with a time course similar to the decrease in the membrane potential. Therefore, the gradual decrease in the membrane potential may also have some relation to the ATP-coupled maintenance system of the membrane potential such as the reverse reaction of ATP synthase.

In the absence of Na^+ in the medium, it has been shown that the intracellular pH of these bacteria is increased to the medium pH, since the pH homeostasis is maintained by the Na^+/H^+ antiporter [9,10,24]. Therefore, the high intracellular pH in the absence of Na^+ might be the cause for the increased K^+ permeability. However, we showed that the Na^+-modulated K^+ permeability was also observed at pH 7.5. Also, at 10 mM Na^+, the cells showed significant leakage of K^+, but the pH homeostasis was well maintained [9]. These results clearly excluded the possibility of the involvement of the high intracellular pH in the high K^+-permeability.

As shown in Fig. 1B, the addition of Na^+ to the K^+-treated cells caused a further but transient decrease in the membrane potential. Since the addition of Na^+ would cause a quick decreaes in the intracellular pH by the function of Na^+/H^+ antiporter, the antiporter might be considered to have some role in the phenomenon. However, this possibility is unlikely, because the Na^+–H^+ exchange of the antiporter is electrogenic, with $H^+ > Na^+$ [24,25]. Rather, the influx of Na^+ itself may cause the phenomenon. In that case, it is likely that the intracellular Na^+ plays a modulatory role for the K^+ permeability.

Kashket [14] reported that the membrane potential of respiring *E. coli* cells was significantly affected by K^+ or Na^+ in the medium, when the pH of the medium was higher than 8.1 In this case, however, the addition of both K^+ and Na^+ caused an increase in the membrane potential, and also the effects of K^+ or Na^+ were attributed to the K^+/H^+- and Na^+/H^+ antiporters. Thus, the phenomenon observed in *E. coli* at alkaline pH is completely different from that reported here.

So far, we have no evidence to explain the mechanism on the Na^+-dependent modulation of K^+ permeability in the alkalophillic *Bacillus*. It may be worthwhile to search for Na^+-regulated K^+-channel proteins in the membrane. Whatever the mechanism would be, it is necessary to pay attention on the cation composition of the medium for the energetic analysis of alkalophilic *Bacillus*. When the role of Na^+ in the Na^+-coupled system

is investigated, it is especially important whether or not the observed phenomenon is related to the alteration of the K^+ permeability or the membrane potential of the cell.

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