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A Neutral pH-insensitive Mutant of Alkalophilic *Bacillus* sp. ASSC-2

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Intracellular pH of an alkalophilic *Bacillus* sp. ASSC-2 was acidified in the K^+-limited media and could not grow at neutral pH range unless K^+ was added to the media (Oshima & Onoda, 1990). The neutral pH-insensitive mutant, *Bacillus* ASSC-2NI, was isolated by the treatment with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. The mutant could grow in the K^+-limited media even at neutral pH range in the absence of exogenously added K^+, and the intracellular pH was not acidified. The mutant has gained high K^+-transport activity and, consequently, the respiratory activities of the mutant were higher than the parent strain in the absence of added K^+ at neutral pH range. Further, the mutant has retained Na^+/H^+-antiporter activity at pH 7. The properties of the mutant indicated that if K^+-transport system was actively operating at neutral pH the intracellular pH was protected from acidification by the active Na^+/H^+-antiport system at neutral pH.

Introduction

One of the interesting problem of alkalophiles is what prevents the growth of alkalophiles at neutral pH. As for the problem in obligate alkalophiles, it is suspected to be due to loss of the membrane integrity by lowered proton motive force at neutral pH, but not the excessive acidificaiton of the intracellular pH, since the Na^+/H^+-antiporter, an important acidification system for intracellular pH of alkalophiles, is not functional at neutral pH (Garcia *et al.*, 1983; Kitada *et al.*, 1982 and Krulwich, 1983). In contrast to the obligate alkalophiles, facultative alkalophiles are capable of growth at neutral pH, though the rate is considerably less than that at alkaline pH. Accordingly, there must be any difference in the repression of growth at neutral pH between the obligate and the facultative alkalophiles. However, there are little information being directly applicable to studying this difference. We have previously reported that the facultative alkalophilic *Bacillus* ASSC-2 had considerably low K^+-transport activity in spite of rather high activity of Na^+/H^+-antiporter, and could not grow at neutral pH in the K^+-limited media because of acidification of intracellular pH (Oshima & Onoda, 1990). It thus seems to be possible that the growth repression of *Bacillus* ASSC-2 at neutral pH range might result from a limit of the capacity of the K^+-transport activity. If so, mutant strains selected for the ability to grow in the K^+-limited media at neutral

pH, should have high K^+-transport activity, and should not acidify the intracellular pH in spite of the presence of active Na^+/H^+-antiporter. We report here the isolation and properties of a neutral pH-insensitive mutant strain of alkalophilic *Bacillus* ASSC-2.

Materials and Methods

Strain and growth conditions

Alkalophilic *Bacillus* sp. ASSC-2 isolated from alkaline hot spring (Oshima *et al.*, 1987a) was used in this study. This strain was usually grown in NB-medium or P-medium as described previously (Oshima *et al.*, 1987b).

Isolation of neutral pH-insensitive mutant

The *Bacillus* ASSC-2 cells grown in NB-medium were harvested and washed twice with T_1-buffer containing 20 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 150 mM NaCl and 10 mM KCl. The cells were exposed to 50 μg/ml of *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine (NTG) for 30 min at 37°C. The cells were washed twice with T_1-buffer and spread onto the agar plate of NB-medium (pH 9.0) containing 100 μg/ml of kanamycin. One of fourteen colonies was selected as a kanamycin resistant mutant (*Bacillus* ASSC-2KR) and used as a parent strain for the isolation of neutral pH-insentitive mutant. The cells of *Bacillus* ASSC-2KR grown with shaking for 4 hrs at 37°C in NB-medium (pH 9.0) were harvested and washed twice with T_1-buffer and re-treated with 50 μg/ml of NTG for 30 min at 37°C. The cells were washed twice with T_1-buffer (pH 7.0) and spread onto the agar plate of P-medium (pH 7.0). One of four colonies appearing on the agar plate was used as a neutral pH-insensitive mutant (*Bacillus* ASSC-2NI).

Measurement of the O_2 consumption

The cells grown in the NB-medium were harvested and washed three times with T_1-buffer and resuspended in the T_2-buffer containing 20 mM Tris-HCl pH 9.0 and 10 mM $MgCl_2$. The cell suspension (100 μl, 0.17 mg dry weight) was injected into 3 ml of suitable buffer in the thermo-regulated reaction vessel and O_2 consumption of the cells was measured polarographically with a Clark oxygen electrode (YSI-type 53 oxygen electrode) equipped with a recorder (Hitachi, Ltd 056). The O_2 electrode was calibrated by the method of Robinson and Cooper (1970).

Measurement of NADH oxidase activities

NADH oxidase activities were assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH at 30°C. The reaction mixture contained 20 mM Tris-HCl, 134 mM NADH (Tris salt) and appropriate amount of membrane fraction prepared as described previously (Oshima *et al.*, 1987) in a final volume of 2 ml. The

reaction was started by addition of NADH.

Fluorescence assay

Intracellular pH (acid inside) of whole cells were monitored by the quenching of fluorescence of 9-aminoacridine. The cells in the logarithmic phase were harvested and washed with T_1-buffer or T_1'-buffer (the same as T_1-buffer but omission of 10 mM KCl). The assay was started by the addition of 100 μl of cell suspension (about 2.5 mg dry weight) to 2 ml of the suitable buffer system containing 4 μM 9-aminoacridine. The fluorescence was measured using Hitachi model 850 fluorescence spectrophotometer with excitation at 400 nm and emission at 455 nm.

Measurement of K^+-transport activities

Cells grown in NB-medium were harvested and washed three times with T_3-buffer containing 0.5 M Na_2SO_4 instead of 150 mM NaCl of T_1'-buffer (pH 9) and resuspended in T_1'-buffer (pH 7.0). After 2.5 min of incubation KCl was added to be 0.2 mM at the indicated time, the cells were collected by centrifugation (Michels & Bakker, 1987). Then, K^+ concentrations in the cells were measured by the atomic absorption method as described by Matsukura and Imae (1987) with a Hitachi atomic absoption spectrometer model 170–40.

Measurement of Na^+/H^+-antiporter activities in whole cells

Na^+/H^+-antiporter activities were measured in whole cells by Li^+ extrusion from Li^+ loaded cells. The cells grown in NB-medium were harvested and washed three times with T_4-buffer (pH 9) containing 20 mM Tris-HCl, 5 mM $MgCl_2$, and 150 mM LiCl. Then, cells were collected by centrifugation and resuspended in T_1'-buffer (pH 7). At time intervals, the cells were collected by centrifugation (Michels & Bakker, 1987) and internal concentration of Li^+ was measured by atomic abosorption method (Matsukura & Imae, 1987).

Protein determinations

Protein was determinated by the method of Lowry *et al.* (1951) or by using Bradford protein assay (Bio-Rad). Bovine albumin was used as a standard.

Chemicals

Peptone and Ehrlich meat extract were obtained from Kyokuto Pharmaceutical Industrial Co. Polypeptone was purchased from Nihon Pharmaceutical Co. Ltd. 9-aminoacridine was a product of Nacalai Tesque. Tetraphenylphosphonium bromide was supplied by Sigma Chemical Co. All other chemicals were reagent grade and obtained from commercial sources.

Results

Effects of pH on the growth of the mutant and parent strains

As shown in Fig. 1, the pH-dependent growth rate of *Bacillus* ASSC-2KR in NB-medium was essentially similar to that of wild type strain (Oshima *et al.*, 1987b). On the other hand, the mutant strain *Bacillus* ASSC-2NI gain the ability to grow in the same medium even around pH 6.0. Further, the mutant can also grow in K^+-limited medium (P-medium) at neutral pH range (Fig. 2) but the parent strain could not grow below pH 7.5. As previously reported, the wild type strain *Bacillus* ASSC-2 could not grow in P-medium unless K^+ was added to the medium (Oshima & Onoda, 1987b). Effect of K^+ concentrations on growth of the mutant and parent strains were examined in P-medium at pH 7.0. The mutant grew well at very low concentration of K^+ or even in the absence of K^+, but the parent strain required more than 1 mM of K^+ for its growth (Fig. 3).

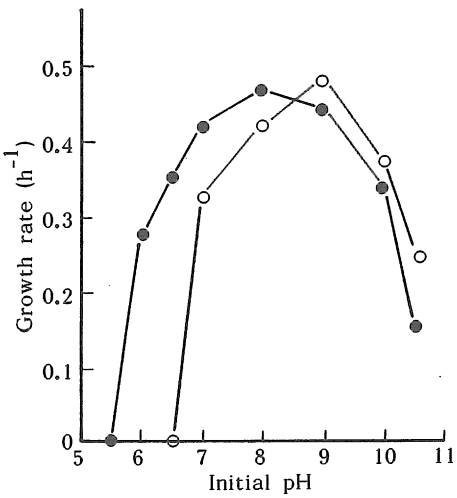

Fig. 1. Effect of pH on growth rate of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI in NB-medium. Both strains preincubated at 37°C over night were inoculated into NB-medium at final OD_{600} of 0.05, and incubated aerobically at 37°C. The pH of NB-medium was adjusted to the desired pH by the addition of NaOH or HCl. Symbols: ○, *Bacillus* ASSC-2KR; ●, *Bacillus* ASSC-2NI.

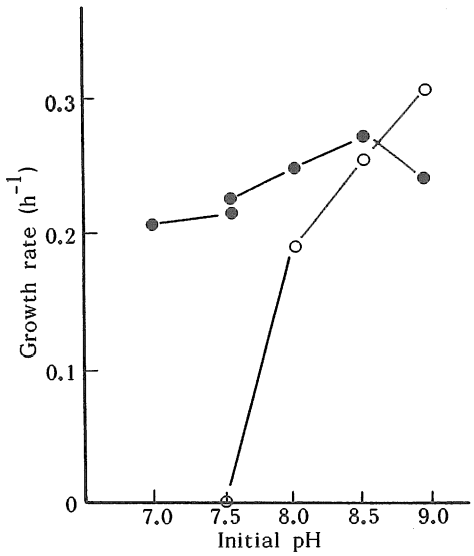

Fig. 2. Effect of pH on growth rate of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI in P-medium. Both strains grown over night at 37°C in NB-medium were harvested and washed twice with P-medium (pH 7.0). Then the washed cells were inoculated into P-medium at final OD_{600} of 0.05. The pH of P-medium was retained with 50 mM MOPS-NaOH (pH 7.0 and 7.5) of 50 mM Tris-HCl (pH 7.5 to 9.0). Symbols: ○, *Bacillus* ASSC-2KR; ●, *Bacillus* ASSC-2NI.

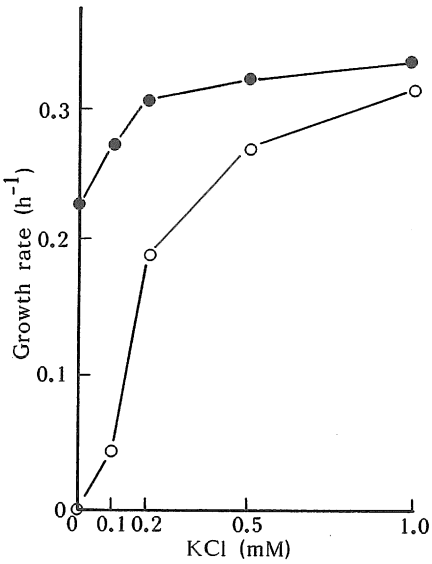

Fig. 3. Effect of KCl concentration on growth rate of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI. The growth was measured in P-medium (pH 7) supplemented with various concentration of KCl. Other conditions were the same as in Fig. 2. Symbols: ○, *Bacillus* ASSC-2KR; ●, *Bacillus* ASSC-2NI.

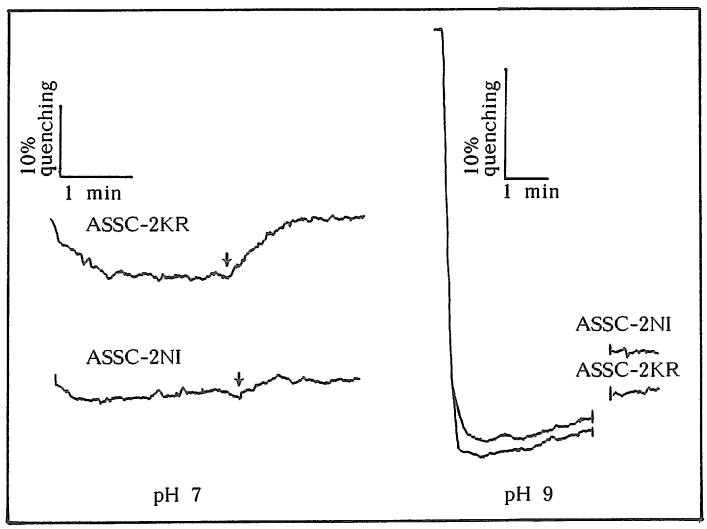

Fig. 4. Internal pH of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI in P-medium at pH 7 and 9. Both strains grown in NB-medium (pH 9) were harvested and washed twice with P-medium (pH 9). Fluorescence of 9-aminoacridine were monitored after the cells were suspended in P-medium (pH 7 or 9). The pH of P-medium was retained with 50 mM Tris-HCl. At the time indicated by the arrows KCl was added to a concentration of 10 mM.

Intracellular pH of the mutant and parent strains at neutral pH

In *Bacillus* ASSC-2, inability of the growth at pH 7 in P-medium due to a excessive acidification of the intracellular pH (Oshima & Onoda, 1990). Thus, the ability of pH-maintenance (internal alkaline) of the mutant and parent strains in P-medium at pH 7 were examined qualitatively by the flurescene change of 9-aminoacridine. When the parent strain grown at pH 9.0 were suspended into P-medium (pH 7.0) containing 9-aminoacridine, fluorescence quenching was observed unless K^+ was added to the suspension, but in the case of the mutant strain quenching did not occur (Fig. 4). These results indicate that the intracellular pH of the mutant strain was not acidified below pH 7.0 under the conditions tested. When the experiments were carried out at pH 9.0, fluorescence levels of 9-aminoacridine in the mutant and parent strains were maintained constantly (intracellular acidic) (Fig. 4).

Intracellular pH regulatory systems of the mutant and parent strains at neutral pH

The intracellular pH regulatory system of *Bacillus* ASSC-2 at neutral pH range consists of primary respiratory pump and K^+-transport systems (Oshima & Onoda, 1990). From the results described above, it was expected that the intracellular pH in the mutant strain was well regulated by the highly active respiratory system and/or K^+-transport system. Thus, the respiratory and K^+-transport activities of the mutant and parent strains were compared in various pHs. As shown in Fig. 5, the endogenous substrate-dependent respiratory activities of the mutant strain in K^+-limited solution were higher than that of the parent strain in all pHs tested, and the rate of stimulation at neutral pH range were higher than at alkaline pH range. The respiratory activities of the parent strain were stimulated when K^+ was added, and such stimulation by K^+ in

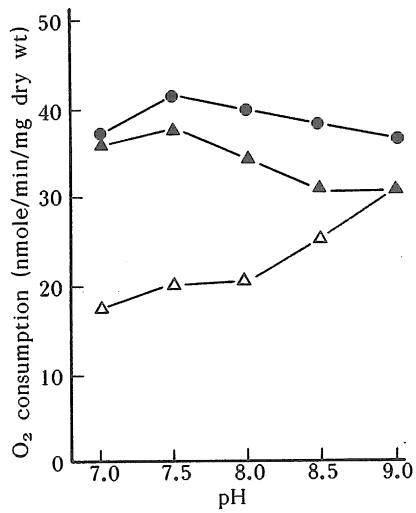

Fig. 5. Effect of pH on the endogenous respiratory activities of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI. The endogenous respiratory activities of *Bacillus* ASSC-2KR (△, ▲) and *Bacillus* ASSC-2NI (●) were measured in the presence (▲) and absence (△, ●) of 50 mM KCl in the 20 mM Tris-HCl (pH 7.0 to 9.0) buffer containing 5 mM $MgCl_2$ as described in **Materials and Methods**.

the parent strain was also observed when membrane permeable cation (TPP) was added to the solution (Table 1). Further, the NADH oxidase activity in the membrane fractions of both strains were measured in the presence and absence of K^+. However, K^+ had not any effect on the activity of the NADH oxidase in both the strains (Table 2).

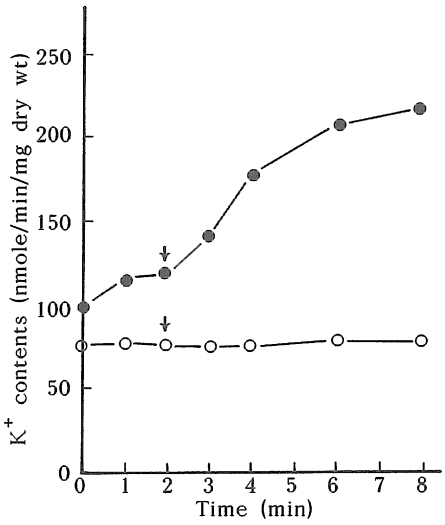

Fig. 6. K^+-uptake activities at pH 7 in *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI. K^+-depleted cells of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI were prepared as described in **Materials and Methods**. At the time indicated by the arrow, KCl was added at final concentration of 0.5 mM and intracellular concentrations of K^+ in *Bacillus* ASSC-2KR (○) and *Bacillus* ASSC-2NI (●) were measured.

Table 1. Effect of TPP on endogenous respiratory activity of *Bacillus* ASSC-2KR

O_2 consumption (nmole/min/mg dry wt)		
None	KCl (10 mM)	TPP (250 μM)
22.5	40.5	38.2

The endogenous respiratory activities were measured at pH 7. Other conditions were the same as in Fig. 5.

Table 2. Effect of KCl on NADH oxidase activities of the *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI

Addition	nmole/mg protein			
	ASSC-2KR		ASSC-2NI	
	pH 7	pH 9	pH 7	pH 9
None	744	934	960	955
KCl	755	966	1047	964

KCl was added to a final concentration of 50 mM.

Next, the K^+-transport activities of the two strains were compared at pH 7.0. In the previous paper, we have reported that the wild type strain *Bacillus* ASSC-2 could not take up K^+ at pH 7 even when extracellular concentration of K^+ was 1 mM (Oshima & Onoda, 1990). As shown in Fig. 6, the mutant strain could take up K^+

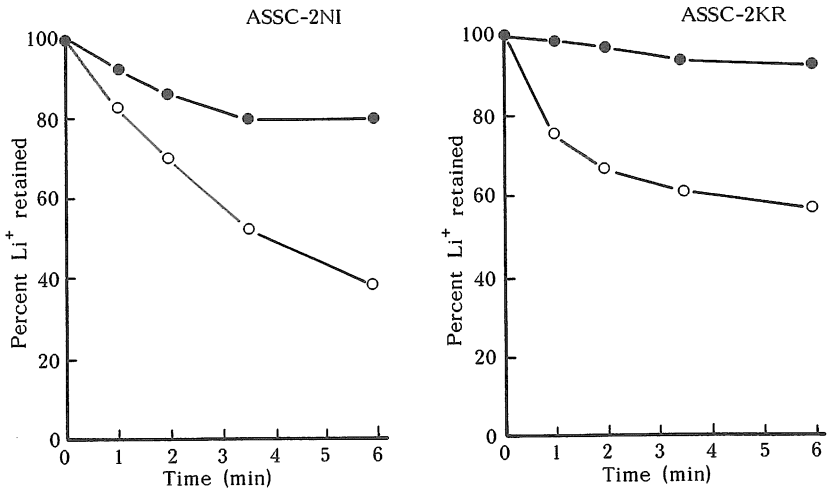

Fig. 7. Li^+-extrusion from Li^+-loaded cells at pH 7. The Li^+-loaded *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI cells were prepared as described in **Materials and Methods**. Both strains were suspended in T_1'-buffer (pH 7) in the presence (●) or absence (○) of TCS (50 μM) and TPP (250 μM). At the times indicated, cells were collected and intracellular concentration of Li^+ were measured as described in **Materials and Methods**. Initial concentration of internal Li^+ of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI were 425 and 285 nmoles/mg dry wt., respectively.

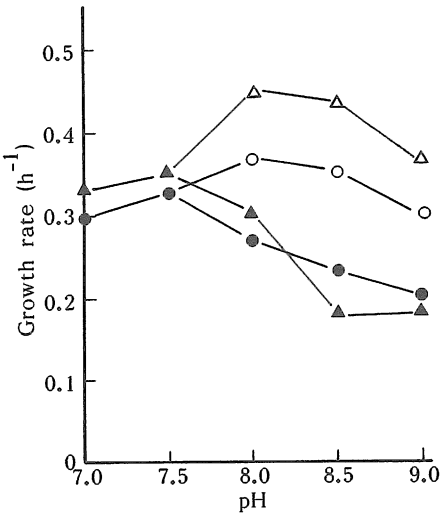

Fig. 8. Effect of amiloride on the growth rate of *Bacillus* ASSC-2KR and *Bacillus* ASSC-2NI at various pH. *Bacillus* ASSC-2KR (○, ●) and *Bacillus* ASSC-2NI (△, ▲) were incubated at 37°C in P-medium containing 5 mM KCl and 5 mM NaCl. (○, △), control; (●, ▲), amiloride (final concentration of 0.2 mM) was added.

even in the low extracellular concentration of K^+ (0.5 mM) was low. On the other hand, the parent strain could not take up at all at the pH. Accordingly, the K^+-transport system of the mutant strain was found to be active at the pH.

Na^+/H^+-antiporter activity of the mutant and parent strains

We previously reported that the Na^+/H^+-antiporter was also active at neutral pH in *Bacillus* ASSC-2 (Oshima & Onoda, 1990). The Na^+/H^+-antiporter activity was demonstrated not only in the parent strains but the mutant strain at pH 7.0 (Fig. 7). As shown in Fig. 4, intracellular pH of the two strains at alkaline pH were maintained constantly. Further, the addition of amiloride, a potent inhibitor of the Na^+/H^+-antiporter (Mochizuki-Oda & Oosawa, 1985), inhibited the growth of the both strains above pH 7.5 (Fig. 8). These results suggest that the Na^+/H^+-antiporter which is necessary to grow at alkaline pH range is active at neutral pH.

Discussion

This work enlarges on our previous studies demonstrating a growth repression of alkalophilic *Bacillus* sp. ASSC-2 at neutral pH (Oshima & Onoda, 1990). The growth repression of *Bacillus* ASSC-2 may be due to a decrease in the K^+-transport activity which read to excess acidification of the intracellular pH under the active Na^+/H^+-antiporter. In order to realize whether the acidification of intracellular pH is responsible for reduced K^+-transport activity or for active Na^+/H^+-antiport system, a mutant, *Bacillus* ASSC-2NI, which is able to grow at neutral pH was selected.

The mutant ASSC-2NI did not exhibit acidification of the internal pH in K^+-limited medium at neutral pH. The mutant strain has much more active respiratory and K^+-transport systems than its parent (Fig. 5, 6) and wild type strains (Oshima *et al.*, 1987b). The respiratory activity of the parent strain was stimulated by addition of K^+, but K^+ had not any effect on the NADH oxidase activity in the parent strain. Further, in the presence of TPP which reduces a membrane potential ($\Delta\psi$) also stimulated the respiratory activity of the parent strain (Table 1). It has been reported that influx of K^+ *via* K^+-transport system results in a depolarization of $\Delta\psi$, and net H^+ extrusion by primary proton pump is accelerated (Kroll & Booth, 1981). Thus, the high respiratory activity in the mutant strain would not be due to the gain of the highly active respiratory system but would dissipation of an excess potential by influx of K^+ through active K^+-transport system.

Although several mutant strains which is able to grow even at neutral pH have been selected from obligately and facultatively alkalophilic *Bacillus* (Krulwich *et al.*, 1979; Krulwich *et al.*, 1982; Koyama *et al.*, 1986), these strains were all non-alkalophilic mutants which had no Na^+/H^+-antiporter activity and was unable to grow at alkaline pH (above 9.0 or 9.5). These results suggests that the capability of growth at neutral pH of the none alkalophilic mutant may be due to deficiency of Na^+/H^+-antiporter.

However, our mutant strain *Bacillus* ASSC-2NI could grow not only at alkaline pH but also at neutral pH in spite of its high activity of Na^+/H^+-antiporter (Fig. 7). The importance of the Na^+/H^+-antiporter in alkalophiles is well established in connection with pH homeostasis in alkaline pH range. However, physiological role(s) of the antiporter activity which seems to be unnecessary for the pH homeostasis at near neutral pHs is remains to clear. It has been reported that solute transport and flageller motor were energized by sodium motive force ($\Delta\tilde{\mu}_{Na^+}$) (Chernyak *et al.*, 1983; Hirota & Imae, 1983; Krulwich *et al.*, 1983, 1988 and Sugiyama *et al.*, 1986). In *Bacillus* ASSC-2, AIB uptake was activated by Na^+ in pH 7 and 9 (data not shown). Since Na^+/H^+-antiporter could convert a $\Delta\tilde{\mu}_{H^+}$ to $\Delta\tilde{\mu}_{Na^+}$, the Na^+/H^+-antiporter may not function at neutral pH as a mechanism for regulation of internal pH but for generation of $\Delta\tilde{\mu}_{Na^+}$.

Our results indicated that the low growth rate of the parent strain at neutral pHs and inability to grow at pH 7.0 in K^+-limited medium are due to insufficinent activity of K^+-transport system.

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