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Effects of K^+ and Na^+ on growth, respiration, and amino acid uptakes of an alkalophilic *Bacillus* ASSC–2

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A *Bacillus,* ASSC–2, belonging to alkalophilic bacterium is known to grow at pH range of 6.5 to 10.5 (Oshima *et al.,* 1987). The growth rate at neutral pH range was accelerated by addition of KCl to the growth medium. On the contrary, the growth rate at alkaline pH range was accelerated by addition of NaCl. Similar stimulative effect of salt to each pH also was observed in *NADH oxidase.* Incorporation of leucine into whole cells fractions was equally accelerated at pH 7 and 9. Higher concentration of K^+ is absolutely required in growth of the bacteria at neutral pH, but not alkaline pH, compared with other conventional neutrophilic bacteria. Effects of potassium ions to growth of alkalophilic bacteria were discussed.

Introduction

Alkalophilic bacteria can grow well at alkaline pH range and have unique characteristics in order to grow at alkaline pH. Na^+ requirement is one of the important characteristics of these bacteria. For example, Na^+ is essential for its maintenance of intracellular pH through Na^+/H^+ antiporter (Krulwich *et al.*, 1979), solute transport (Horikoshi and Akiba, 1982) and flagellar rotation (Hirata *et al.,* 1979).

Recently, Koyama & Nosoh (1985) reported that when culture pH of the alkalophilic *Bacillus,* YN–2000, was changed from 10.2 to 7.5, the growth stopped and the addition of KCl to the culture was necessitated for continuation of the growth. We have recently found that the growth rate at neutral pH of an alkalophilic *Bacillus,* ASSC–2, was enhanced by the addition of KCl to the medium. This finding excited the authors to examine the effect of K^+ on the some physiological properties of the bacterium because even in the neutral pH many alkalophilic bacteria required Na^+ for its growth (Horikoshi and Akiba, 1982). In this paper, the effects of K^+ and Na^+, on the growth, respiration and leucine uptake in this bacterium are reported.

Materials and Methods

Strain and growth condition

Alkalophilic *Bacillus,* ASSC–2, isolated from an alkaline hot spring (Oshima *et al.,* 1987) and *Bacillus subtilis,* RIMD, were used throughout this experiment. These strains were usually grown in NB-medium contained 10 g of Erlich meat extract and 10 g

of peptone per liter. The pH of the medium was adjusted by the addition of NaOH for *B. subtilis* or Na_2CO_3 for ASSC-2, and then cells were grown aerobically at 37°C. P-medium contained 6.057 g of tris-(hydroxymethyl)aminomethan (Tris), 1 g of $MgCl_2$ and 5 g of polypeptone per liter.

Measurement of the respiration activity

Cells grown in the NB-medium were harvested and washed three times with buffer A contained 50 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$, 150 mM NaCl and 10 mM KCl. The cells were resuspended in the same buffer and the respiration activity was measured by the YSI-type 53 oxygen electrode.

Preparation of membrane vesicles

Inverted membrane vesicles were prepared by the following procedure. Cells in the mid-logarithmic phase of growth were harvested and washed three times with buffer A containing with 0.2 M sucrose. Cells were resupended in the same buffer and lysozyme (final concentration 50 μg/ml) was added. Then, the suspension was incubated for 20 min at 37°C. During this incubation many protoplasts were produced. The protoplasts were harvested by centrifugation (20,000×g), and osmotically lysed by resuspending in buffer B contained 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$ and then sonicated for 1 min. When the salt-loaded vesicles were prepared, 50 mM of salt was added to the buffer B. After removal of unbroken cells by low speed centrifugation (500×g for 10 min), the membrane vesicles were collected by high speed centrifugation (20,000×g for 30 min).

Measurement of NADH oxidase activity

NADH oxidase activity was assayed by measuring the decrease in absorbance at 340 nm at 30°C. The reaction mixture contained 20 mM Tris-HCl, 134 mM NADH and appropriate amount of enzyme in a final volume of 2 ml. The reaction was started by addition of NADH.

Measurement of leucine uptake

Leucine uptake was measured by a filtration method. The reaction mixture contained 20 mM Tris-HCl pH 7 or 9, cell suspension (0.1 mg dry weight/ml), chloramphenicol (0.2 mg/ml) and 5 μM [^{3}H]-leucine. The reaction mixture was preincubated for 2 min at 37°C and the reaction was started by addition of [^{3}H]-leucine. At suitable intervals, 0.1 ml of samples were filtrated through membrane filter (pore size 0.45 μm, Millipore Corp.). Then, the filter was washed 2 times with 5 ml of reaction mixture excepted leucine and radio activity of the filters were measured.

Chemicals

L-[^{3}H]-leucine (137 Ci/m mole) was purchased from Amersham. Peptone and

Erlich meat extract were obtained from Kyokuto Pharmaceutical Industrial Co. Polypeptone was obtained from Daigo Nutrient Chemical Co., Ltd. Lysozyme was purchased from Nagase Biochemiclas Ltd. NADH was purchased from Shigma Chemicals. All other reagents used in this study were of analytical grade.

Results

Effects of salts on the growth of ASSC-2

Effects of NaCl and KCl on growth of ASSC-2 under various pH value were tested. At range of alkaline pH, the growth rate of ASSC-2 was accelerated by the addition of NaCl to the medium. On the contrary, the growth rate at neutral pH was accelerated by the addition of KCl, excepted NaCl (Fig. 1A). The same experiment was carried out using *B. subtilis* as a control experiment, but no significant difference of growth rate at each pH was obtained between ions of NaCl and KCl (Fig, 1B). It was reported that alkalophilic *Bacillus* required Na^+ for its growth even at neutral pH as well as alkaline pH (Horikoshi & Akiba, 1982). Therefore, it is interesting that KCl was required for the growth of *Bacillus,* ASSC-2, at neutral pH. To examine effects of other mono-

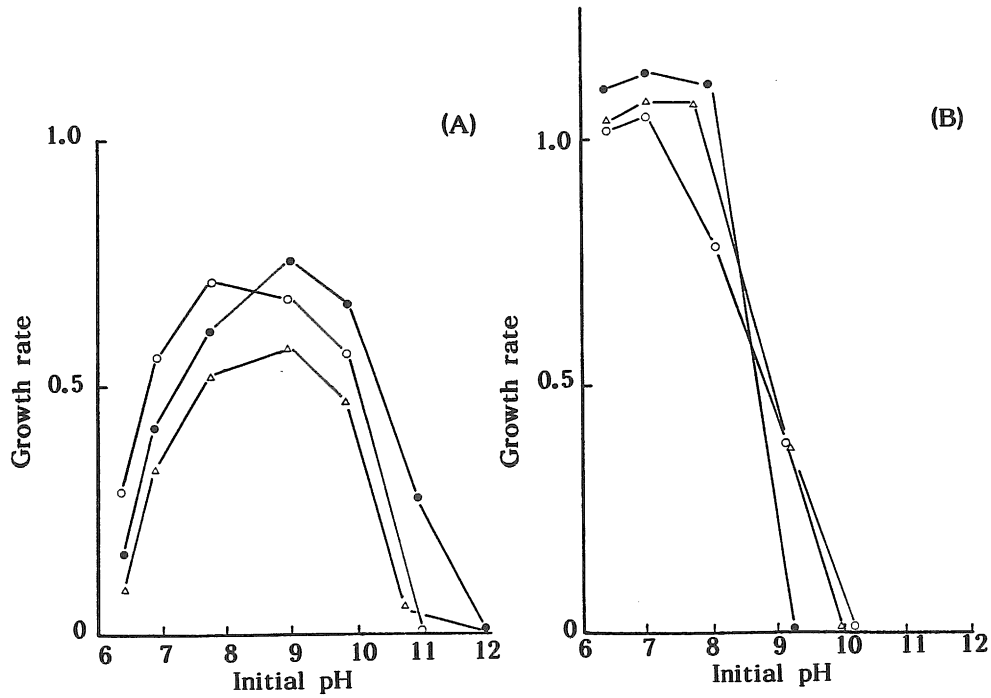

Fig. 1. Effects of NaCl and KCl on the growth rates of ASSC-2 (A) and *B. subtilis* (B) at various pH. 50 mM NaCl or KCl was added to the NB-medium and growth rate was measured. Symbols: △-△; No addition, ○-○; with 50 mM KCl; ●-●, with 50 mM NaCl.

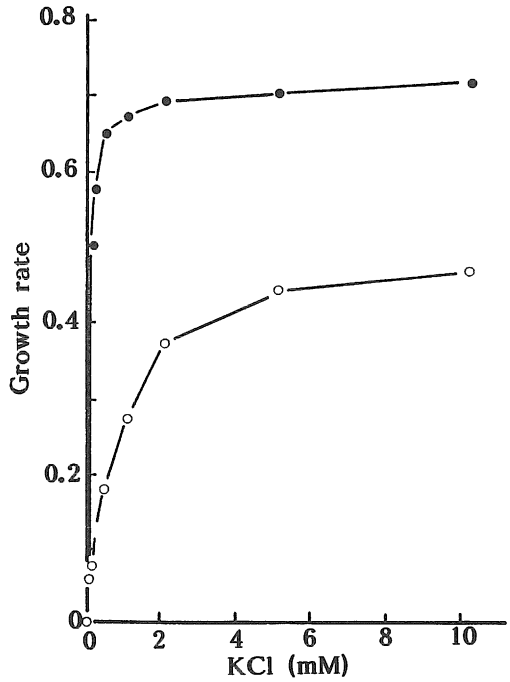

Fig. 2. Effects of KCl concentration on the growth rates of ASSC-2 and *B. subtilis* at pH 7. Cells were inoculated into the P-medium containing various amounts of KCl, and the growth rate was measured at 37°C. Symbols: ○-○; ASSC-2, ●-●; *B. subtilis*.

Table 1. Effects of various salts on the growth of ASSC-2 and *B. subtilis*

Salt	ASSC-2	ASSC-2*	*B. subtilis*
None	0.061	0.072	0.596
LiCl	0.051	0.104	0.572
NaCl	0.060	0.115	0.572
KCl	0.722	0.687	1.944
RbCl	0.687	N.D.**	1.867
CsCl	0.071	0.104	0.052
NH_4Cl	0.053	0.062	0.509
Choline-Cl	0.053	0.068	0.698
KNO_3	0.674	0.696	1.772
K_2SO_4	0.686	0.689	1.508

Each bacterium was inoculated in the P-medium containing 50 mM of salt. At 6 h after inoculation, A_{600} was measured.
*Precultured at pH 7 in P-medium containing with 50 mM KCl.
**Not determined.

valent cations for the growth of this bacterium at neutral pH, various cations were added and the growth (A_{600}) was measured at pH 7 (Table 1). It was found that the NB-medium contained contaminating amount of 141 mM-Na^+ as NaCl and 7.7 mM-K^+ as KCl. P-medium, therefore, was used in this experiment. ASSC-2 grew only when KCl or RbCl was present, whereas none of the other cation tested had any effect for growth at this pH. Further, similar results were obtained when cells precultured in the P-medium containing 50 mM KCl at pH 7 were tested under the same condition. Comparing with the result in NB-medium (Fig. 1B), the growth of *B. subtilis* was stimulated by the addition of KCl or RbCl to P-medium, but the extent of increasement (three fold) against the control was smaller than the case of ASSC-2 (ten fold). In addition, the effect of concentration of KCl on the growth rate was measured. As shown in Fig. 2, ASSC-2 required higher concentration of KCl at pH 7 than *B. subtilis* did and the calculated Km for the growth rate of ASSC-2 and *B. subtilis* were 1.041 mM and 0.07 mM, respectively.

Effects of KCl and NaCl on the respiration activity

Next, we examined the effects of KCl and NaCl on the respiration activity. The

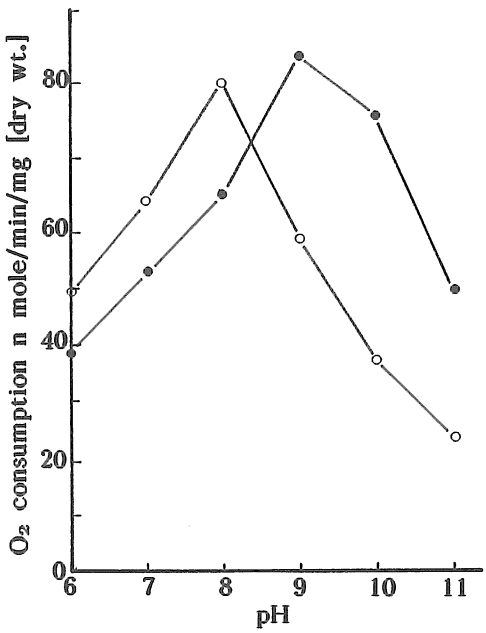

Fig. 3. Effects of NaCl and KCl on the respiration activities at various pH. The respiration activity was measured in the following buffer systems: 50 mM Na_2HPO_4–NaH_2PO_4 or K_2HPO_4–KH_2PO_4 (pH; 6 to 8), $NaHCO_3$–NaOH or $KHCO_3$–KOH (pH; 9 to 11). Symbols: ●–●; Na-containing buffer system, ○–○; K-containing buffer system.

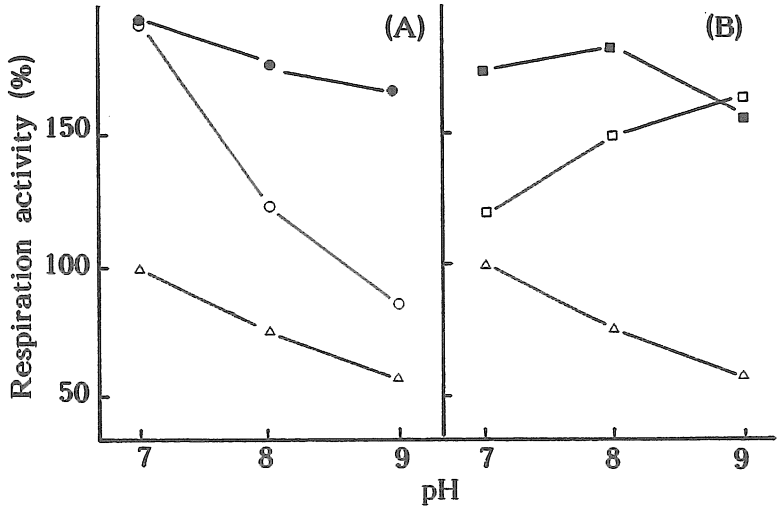

Fig. 4. Effect of K-succinate (A) or Na-succinate (B) on the respiration activity. Respiration activity was measured in 20 mM Tris-HCl. Symbols: □-□; 50 mM sodium-succinate, ■-■; 50 mM sodium succinate+50 mM KCl, ○-○; 50 mM potassium succinate, ●-●; 50 mM potassium succinate+50 mM NaCl, △-△; No addition. 100% of this experiment was 61 nmols O_2/min/mg protein.

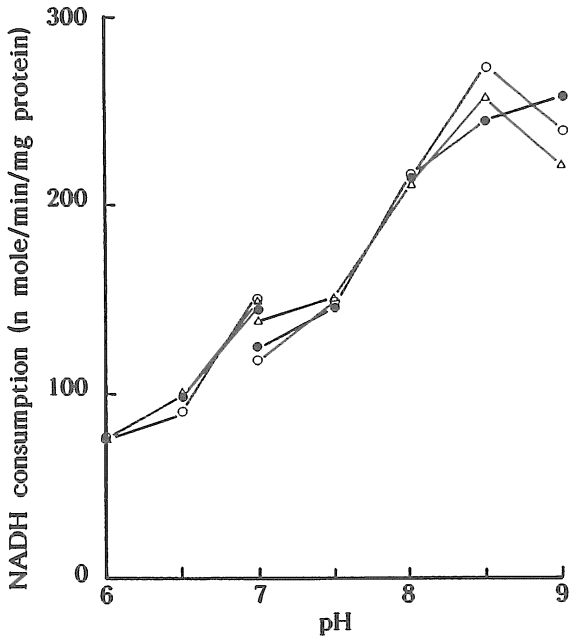

Fig. 5. Effects of NaCl and KCl on the *NADH oxidase* activities at various pH. Buffer systems were 20 mM HEPES-Tris (pH 6–7) and 20 mM Tris-HCl (pH 7–9). Symbols: ●-●; 50 mM NaCl, ○-○; 50 mM KCl, △-△; No addition.

Table 2. Effects of salts on the *NADH oxidase* activity.

Loaded cation	activity (n moles NADH/min)
Choline-Cl	250
NaCl	279
KCl	373
RbCl	331

NADH oxidase activity was measured in Tris-HCl pH 7.0 containing 50 mM Choline-Cl.

optimum pH for the endogenous respiration activities in Na-containing and K-containing buffer systems were pH 9 and pH 8, respectively (Fig. 3). Further, the respiration activity was measured by addition of succinate as a substrate. When K-succinate was added, maximum respiration rate was observed at pH 7 and, by raising the pH, the rate of respiration was gradually declined. On the contrary, when Na-succinate was added, the rate of respiration was gradually declined together with lowering pH. By combination of both the cations in the medium, respiration activity was maintained at rather constant level in range of pH examined (Fig. 4). Furthermore, effects of Na^+ or K^+ on *NADH oxidase* were examined using inverted membrane vesicles. Inverted membrane vesicles were prepared as described in Materials and Methods. Higher activity of *NADH oxidase* was observed at pH 8 to 9, but neither of both the cations added had any influence on the activity (Fig. 5). In addition, we prepared the salt-loaded vesicles and the oxidase activity was again measured. As shown in Table 2, *NADH oxidase* was activated by KCl or RbCl loaded.

Effects of KCl and NaCl on leucine uptake

Leucine uptake was measured as a function of concentration of KCl and NaCl at

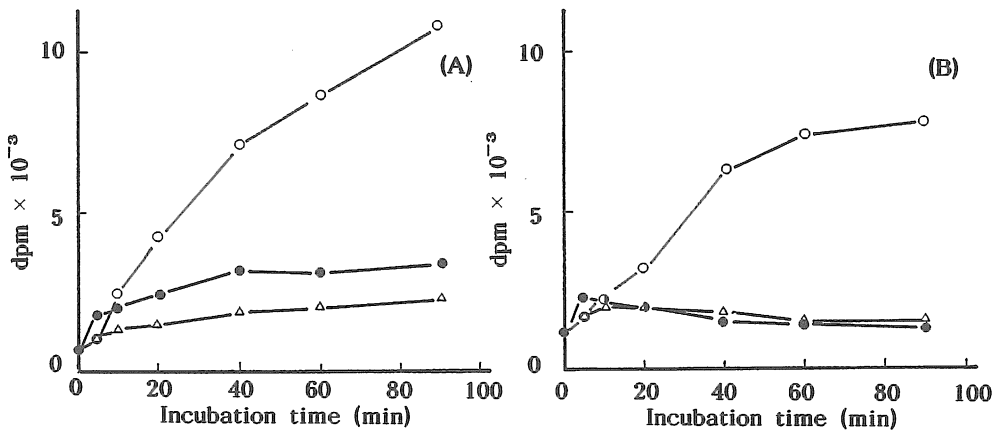

Fig. 6. Effects of NaCl and KCl on the activity of leucine uptake at pH 7 (A) and 9 (B). Symbols; ●–●; 50 mM NaCl, ○–○; 50 mM KCl, △–△; No addition.

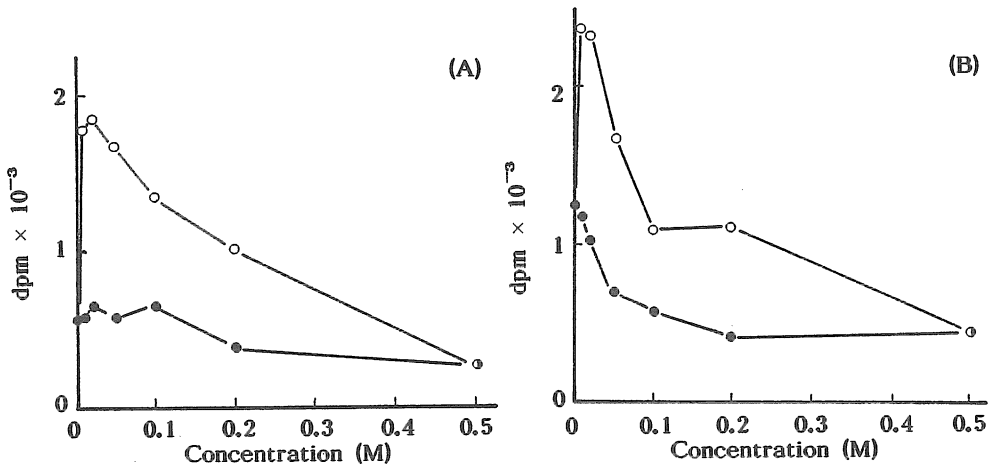

Fig. 7. Effect of concentration of NaCl and KCl on the leucine uptake at pH 7 (A) and 9 (B). Symbols: ●–●; NaCl, ○–○; KCl.

Table 3. Uptake of ^{3}H-leucine n mole/mg [dry wt]

	pH	
Salt	7	9
None	4.3	6.8
LiCl	4.8	5.6
NaCl	7.4	5.2
KCl	37.8	32.0
RbCl	19.3	32.6
CsCl	11.7	21.7
NH_4Cl	7.5	22.8
Choline-Cl	7.5	3.9
KNO_3	31.8	30.4
K_2SO_4	30.8	28.4

Reaction was carried out for 40 min.

pH 7 and 9 respectively. As shown in Fig. 6, KCl stimulates the leucine uptake at both pH, but NaCl of concentration up to 0.5 M was almost not effective on the uptake even at pH 9 (Fig. 7). Effects of this activations were observed by addition of RbCl or CsCl at both pH and NH_4 at pH 9 (Table 3).

Discussion

In alkalophilic bacteria, many cellular mechanisms such as amino acid and sugar

transport (Horikoshi and Akiba, 1982), respiratory activity (Kitada *et al.*, 1982) and motility (Hirata *et al.*, 1981) are known to be Na^+-dependent. The alkalophilic *Bacillus,* ASSC-2, isolated from alkaline hot spring grows well under alkaline conditions (Oshima *et al.*, 1987), and different from other obligatory alkalophilic bacteria, ASSC-2 can grow to some extents under the neutral pH condition. Horikoshi & Akiba (1982) reported that even at the neutral pH range, many alkalophilic *Bacillus* can grow well when NaCl were added to the culture media. Therefore, we expected that this strain required Na ion at Neutral pH as well as other alkalophilic *Bacillus.* Our results, however, showed that ASSC-2 required K^+ instead of Na^+ for its growth at neutral pH. The growth rate was accelerated by addition of Na^+ only at alkaline pH. Interestingly, the dependencies of K^+ and Na^+ on respiration activity of this bacterium resemble to those cation dependency of the growth rate. So, we think that KCl plays an important role in the respiratory chain and controls the growth of this bacterium at neutral pH. Further, Lewis *et al.*, (1980) reported that respiratory chains of alkalophilic bacteria are different from other bacteria. Thus, we tried to examine the effects of Na^+ and K^+ on the *NADH oxidase* activity. The activities of K^+-loaded vesicles were higher than Na^+- or choline-loaded vesicles. This result suggests that K^+ affects activation of the respiratory chain but it is difficult to think that the growth of ASSC-2, at neutral pH, is completely depended on the respiration activity which is activated by K^+.

In many bacteria, it is well known that K^+ is accumulated into the intracellular spaces to the higher concentration. The cation have roles in the osmoleguration (Epstain & Schultz, 1965), in the activation of some enzymes (Suelter, 1970), in protein synthesis (Lubin & Ennis, 1964) and in the regulation of cytoplasmic pH (Plack & Rosen, 1970). Recently, Ando *et al.*, (1983) reported presence of K-stimulated ATPase in alkalophilic *Bacillus,* A-007. We found that K^+ had no effect on the activity of membrane ATPase of ASSC-2 (data not shown). Koyama & Nosoh (1985) reported that K ion in alkalophilic *Bacillus,* YN-2000, plays an role in maintenance of pH homeostasis of the bacterium in change the culture pH and this effect of K ion was not replaced by other cations. In ASSC-2, K ion may play an important role in pH homeostasis at neutral pH because the requirements of K^+ in its growth and respiration were observed only at neutral pH. It is noteworthy that the optimum concentration of K^+ of ASSC-2 was two to three fold higher than that of the *Bacillus* YN-2000 reported by Koyama & Nosoh (1985). Furthermore, in ASSC-2, these active effect of K^+ is replaced by Rb^+. It is predicted that the activity of K^+ uptake system of the ASSC-2 strain at neutral pH is lower probably than other neutrophilic bacteria. To confirm this possibility, the analysis of K^+ uptake system of this bacterium is now in progress.

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