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Influence of NaCl on the Growth and Metabolism of *Halomonas salina*

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Abstract In this research, we examined the effect of NaCl on the growth, energy metabolism, and proton motive force of *Halomonas salina*, and the effect of compatible solutes on the bacterium growing in the high salinity environment. *H. salina* was isolated from seawater and identified by 16s-rDNA sequencing. The growth of *H. salina* was not enhanced by the addition of external compatible solutes (choline and betaine) in the high salinity environment. The resting cells of *H. salina* absorbed more glucose in the presence of 2.0 M NaCl than in its absence. *H. salina* did not grow in the medium with either KCl, RbCl, CsCl, Na_2SO_4, or $NaNO_3$, in place of NaCl. The optimal concentration of NaCl for the growth of *H. salina* ranged from 1.4 M to 2.5 M, and the growth yield was decreased in the presence of NaCl below 1.4 M and above 2.5 M. The activity of isocitrate dehydrogenase, pyruvate dehydrogenase, and malate dehydrogenase of *H. salina* was not inhibited by NaCl in *in vitro* test. The proton translocation of *H. salina* was detected in the presence of NaCl only. These results indicate that NaCl is absolutely required for the normal growth and energy metabolism of *H. salina*, but the bacterial growth is not enhanced by the compatible solutes added to the growth medium.

Key words: *Halomonas salina*, compatible solute, proton translocation, salinity environment, cyclic voltammetry, halophilic bacterium

Halophilic organisms living in the saline environments such as salt lakes, coast lagoons, man-made saltern, and salted foods are challenged by two stress factors: the high salt (inorganic ion) concentration and the low water potential [16]. A nonadapted organism exposed to such an environment must cope with its cytoplasmic water, which has higher water potential than the water of the surrounding environment. There are various species belonging to the genus *Halomonas* capable of growing in the presence of more than 3 M NaCl. The halophilic bacteria have been reported to produce and accumulate the compatible solutes that confer protection against the deleterious effect of the low water activity [11, 21]. Cánovas *et al.* [2, 3] reported that the external compatible solute, choline, added to the growth medium of *H. elongata* extended the salinity range from 2.0 M to at least 3.5 M NaCl and the optimal salinity from 1.75 M to 2.5 M NaCl, and that externally provided betaine, choline, or choline-O-sulfate (but not proline, ectoine, or proline betaine) enhanced the growth of *H. elongata* on 3.0 M NaCl, and betaine and choline stimulated the growth of *H. elongata* DSM 3043 over the entire range of salinity. Grammann *et al.* [12] reported that an ectoine synthesis-defective mutant of *H. elongata* tolerated elevated salt concentrations only in the presence of external compatible solutes. The compatible solute functions as an osmoregulatory agent and is produced by the gene expressed by osmotically induced response [18]. We also found that the halophilic bacteria could grow without NaCl and were dependent on the compatible solutes for growing under a high salinity environment [1, 5, 6, 9]. However, *Halomonas salina* used in the above research did not grow without NaCl and its growth was not enhanced by the addition of the external compatible solutes. In the present study, to solve the question of why *H. salina* cannot grow in the absence of NaCl or in the presence of less than 0.7 M NaCl, we examined the effect of NaCl on the growth, enzyme activities, glucose absorption, and proton translocation of *H. salina*.

Materials and Methods

Reproducibility of Results

All experiments were repeated three times with identical or similar results.

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Organism and Growth

The *Halomonas salina* was isolated from seawater and identified by the forward and reverse sequencing of 16s-rDNA, which was purified from the PCR product of genomic DNA. The bacterium was grown in the defined medium containing 10 g/l glucose, 2 g/l KH_2PO_4, 2 g/l NH_4Cl, 150 g/l NaCl, and 2 ml/l trace mineral stock solution which contained 0.01 g/l $MnSO_4$, 0.01 g/l $MgSO_4$, 0.01 g/l $CaCl_2$, 0.002 g/l $NiCl_2$, 0.002 g/l $CoCl_2$, 0.002 g/l $SeSO_4$, 0.002 g/l WSO_4, 0.002 g/l $ZnSO_4$, 0.002 g/l $Al_2(SO_4)_3$, 0.0001 g/l $TiCl_3$, and 0.002 g/l $MoSO_4$. KH_2PO_4 was added to the medium after autoclave, and pH was adjusted to 6.8. Culture was incubated at 30°C with vigorous shaking (200 strokes).

Determination of Optimal Concentration of NaCl

The optimal concentration of NaCl for the bacterial growth was determined by the serial culture containing NaCl from 0.0 M to 3.5 M at intervals of 0.35 M.

Effect of External Compatible Solutes on the Growth

Betaine or choline chloride at 2 mM, which were used as the external compatible solutes, was added to the serial culture containing NaCl from 0.0 M to 3.5 M at intervals of 0.35 M before inoculation [2, 3].

Effect of NaCl on Glucose Absorption

The resting cell was used to test the effect of NaCl on the glucose absorption by *H. salina* in a short time. The resting cell was aseptically prepared from 16-h-old culture of *H. salina* by centrifugation at 5,000 ×*g* and 4°C for 30 min, and resuspended in 50 mM phosphate buffer without or with 2.0 M NaCl, respectively. The optical density of bacterial suspension at 660 nm was adjusted to 5.0. The reaction was started by the addition of final 18 mg/ml glucose, and the bacterial suspension was incubated at 30°C with mild shaking at 100 strokes. After the reaction, the bacterial culture was centrifuged at 10,000 ×*g* for 30 min and filtrated with membrane filter (pore sized, 0.22 μM), which was used as a sample for analysis. The glucose absorption by *H. salina* was determined by the difference of glucose concentration in bacterial suspension before and after the incubation. Glucose was analyzed by HPLC (YoungLin system M925 pump, Seoul Korea) equipped with RI detector (RI750F model) and Aminex HPX-87H ion-exchange column (Bio-Rad, Burlington, U.S.A.). The concentration was calculated using a standard calibration curve that was previously prepared.

Activity Staining of Crude Enzymes

Native gel electrophoresis was used to resolve three enzymes in the TCA cycle, including pyruvate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase. Thus cell-free extract was resolved by native polyacrylamide gel electrophoresis, while the gel was soaked in 50 mM Tris-HCl (pH 7.5) containing substrates (100 mM each of pyruvate, isocitrate, and malate), NAD^+ (2 mM), phenazine methosulfate (0.5 mg/ml), and methyl thiazolyl tetrazolium (1 mg/ml). Then, the mixture was incubated at 30°C for 60 min. The NAD^+ is reduced to NADH by coupling with oxidation of the pyruvate, isocitrate, and malate by the enzymes located on the acrylamide gel, and phenazine methosulfate and methyl thiazolyl tetrazolium are converted to water-insoluble complex (formazan) coupled to oxidized NADH to NAD^+. The water-insoluble formazan is fixed in acrylamide gel and emerges as a band with dark blue color [27].

Influence of Other Salts on Bacterial Growth

Two molar $NaNO_3$, Na_2SO_4, KCl, RbCl, and CsCl as the substitute for NaCl were individually added to the growth medium. The inoculum was cultivated in a medium with 2.0 M NaCl for 24 h, and the cultures in the substitute was incubated for 72 h to ensure adaptation of bacterial cell to new growth condition with other salts.

Electrochemical Test of Bacterial Absorption of Glucose

The bacterial cells were harvested and washed twice with 50 mM Tris-Cl buffer (pH 7.5) by centrifugation (at 5,000 ×*g* and 10°C for 30 min), and were resuspended in the same buffer containing 200 mM neutral red and incubated at 4°C for 30 min. The incubated cells were washed three to five times with 50 mM Tris-Cl buffer (pH 7.5) by centrifugation (at 5,000 ×*g* and 4°C for 30 min) to completely remove neutral red unbound to bacterial cells. The optical density of the bacterial suspension at 660 nm was adjusted to 5.0. The cyclic voltammogram of bacterial cells modified with neutral red was obtained by using the glassy carbon (diameter 5 mm, Electrosynthesis) as a working electrode, platinum wire as a counter-electrode, and Ag/AgCl as a reference electrode in 25 mM Tris-Cl buffer (pH 7.5) in the absence and in the presence of 2.0 M NaCl and 2.0 M KCl. Cyclic voltammetry was performed using a potentiostat (model CV50W, BAS, U.S.A.) linked to the data acquisition system under complete anaerobic condition. Prior to use, the electrodes were cleaned with an ultrasonic cleaner. The scanning rate was adjusted to 50 mVs^{-1} over the range of -0.2 volt to -0.8 volt. For the test of electron transfer between electrode and bacterial cell, final 100 mM glucose was added to the bacterial suspension just before the cyclic voltammetry. The transition of cyclic voltammogram was observed and compared during 40 cycles.

Measurement of Proton Translocation

Proton translocation was measured under oxygenic atmosphere. The proton translocation in the cell suspensions was measured as described by Fitz and Cypionka [8]. The cells were harvested by centrifugation at 5,000 ×*g* and 20°C for 30 min, washed twice with 100 mM KCl, and resuspended in KKG solution (pH 7.1), containing 100 mM KSCN,

150 mM KCl, and 1.5 mM glycylglycine. The bacterial suspension (5.0 OD at 660 nm) was preincubated in KKG without or with 2.0 M NaCl for 30 min at 30°C. The pH electrode (Toyo glass electrode) was placed in the bacterial suspension of reactor connected to a recorder for conversion of pH variation to the recordable signal. The measurement was started by the addition of 0.05 volumes of glucose to the bacterial suspension in KKG without or with 2.0 M NaCl and the fast acidification of the bacterial suspension was recorded.

RESULTS AND DISCUSSION

Effect of Compatible Solutes and Different Salts

The external compatible solutes have been reported to enhance the osmoprotection of the halophilic bacteria in the high salinity environment [1, 4, 6, 7, 9], and they function as a protectant against the deleterious effect of the low water activity [11]. However, as shown in Fig. 1, the growth of *H. salina* was not enhanced by the addition of the external compatible solutes, choline and betaine, and *H. salina* did not grow in the medium without NaCl or with NaCl below 0.7 M. The highest and lowest concentrations of NaCl for the growth of *H. salina* were 3.5 M and 0.7 M, respectively. It was quite unexpected to observe the above growth of the halophilic bacteria, since the osmoprotection system depends on the compatible solutes. Because of the result in Fig. 1, we tested the effects of other salts such as 2.0 M of Na_2SO_4, $NaNO_3$, KCl, RbCl, and CsCl as the osmoregulatory agent on the growth of *H. salina*, and the growth of *H. salina* was measured after 72 h of cultivation. As shown in Table 1, *H. salina* did not grow in the medium with the above salts in place of NaCl. However, *Micrococcus varians* has been reported to grow in 1.5 to 2.0 M LiCl, RbCl, or CsCl in the presence of 60 mM Na^+ [13], and *H. elongata* and *H. halophilus* have been shown to grow well on $NaNO_3$ [20, 21] and Na_2SO_4 [25, 26], respectively. On the basis of these results, it is quite possible that NaCl might absolutely be required for the energy metabolism of *H. salina* in addition to the osmoregulation. Ono *et al.* [21] tested the thermal stability of ectoine synthase isolated from *Halomonas elongata* grown under condition with 3 M NaCl, 3 M KCl, and without salts at different temperatures, and reported that the stabilizing effect of NaCl was 1.5, 4, and 10 times higher than that of KCl at 15°C, 30°C, and 40°C, respectively. According to earlier reports, the other salts are not toxic to the halophilic bacteria, but act as an osmoregulatory agent or a signal for synthesis of compatible solutes for some halophilic bacteria, similar to NaCl [13, 20, 21, 22, 25]. Consequently,

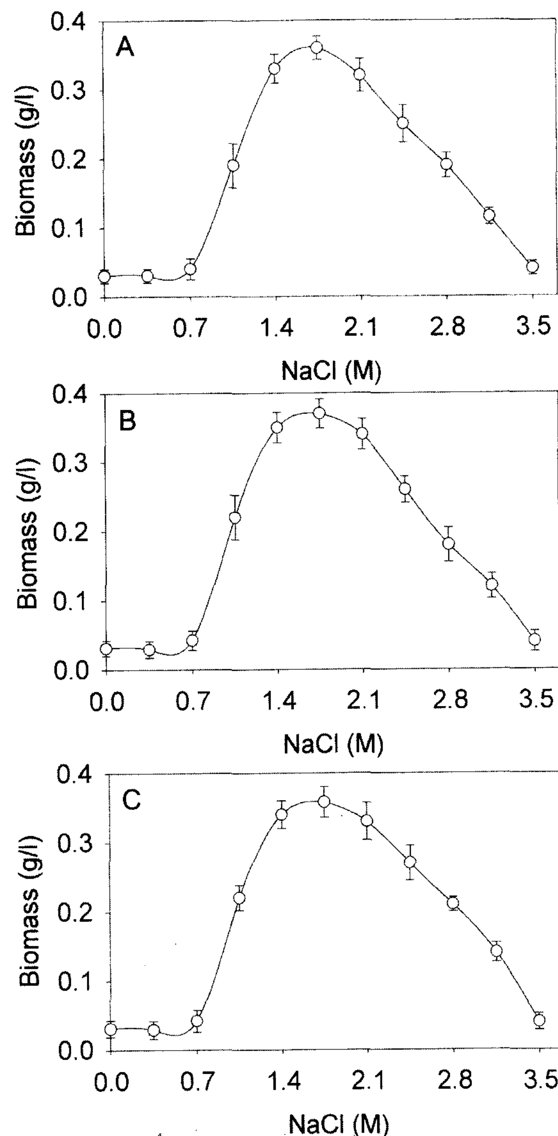

Fig. 1. Effects of the external compatible solutes on the growth of *Halomonas salina*.
The bacterium was cultivated on the defined medium containing glucose, which is the major substrate for the bacterium, without compatible solute (A), with 2 mM betaine (B) and 2 mM choline (C) for 24 h. The experimental data and standard deviations were obtained from the average of three different repeats.

Table 1. Effect of NaCl substitutes on the growth of *Halomonas salina*. A 2.0 M concentration each of KCl, RbCl, and CsCl were used as the substitute of Cl^-, and 2.0 M each of Na_2SO_4 and $NaNO_3$ were used as the substitute of Na^+. The bacterium was cultivated in defined medium with glucose as the carbon source for 72 h.

Salts	NaCl	KCl	RbCl	CsCl	Na_2SO_4	$NaNO_3$
Growth (OD_{660})	2.36±0.081	0.076±0.0041	0.075±0.0029	0.088±0.0028	0.084±0.0032	0.133±0.0043

Table 2. Glucose uptake by the resting cell of *Halomonas salina* in the growth condition with and without NaCl.

Growth condition	Glucose uptake (μg $ml^{-1}g$ $cell^{-1}$)	
	Under 0.0 M NaCl	Under 2.0 M NaCl
0.70 M NaCl	9.17±0.33	11.58±0.35
2.0 M NaCl	46.94±1.54	185.21±7.01

The *Halomonas salina* was grown in the medium with 0.7 M and 2.0 M NaCl, and harvested and washed after 24 h-cultivation. Glucose was added to the bacterial suspension in 50 mM phosphate buffer (pH 7.0), and analyzed before and after 3 h incubation at 30°C. Every growth experiment data are the average of three different repeats, which varied no more than 5%. The glucose was analyzed by HPLC equipped with HPX-87H column and RI detector.

we propose a possibility that *H. salina* may selectively use only NaCl for the osmoregulation, but not use other salts as the signal for substrate transport or osmoregulation [10].

Membrane Transport and Growth Yield

The substrate transport across the cytoplasmic membrane is one of the metabolic reactions demanding energy consumption and a special signal for activation of transport protein. As shown in Table 2, the glucose absorption by *H. salina* was much higher in the growth conditions with NaCl than that without NaCl. We measured the growth yield of *H. salina* in the condition containing different concentrations of NaCl to test the influence of NaCl on the energy metabolism. As shown in Fig. 2, the growth yield (g cell/M substrate) was proportional to the biomass of the bacteria grown in NaCl ranging from 1.4 M to 2.5 M, but less than the biomass grown in the medium of NaCl below 1.4 M and above 2.5 M NaCl. These results show that *H. salina* spends extra energy when grown in the condition below 1.4 M and above 2.5 M NaCl, and that *H. salina* may use NaCl as the signal or co-substrate for the membrane transport of glucose. The halophilic bacteria are thought to expend the extra energy for the biosynthesis of compatible solutes or the osmoprotectants in the high saline environment, but not in the low saline environment. In the present study, however, *H. salina* did not grow without NaCl or with NaCl below 0.7 M. Theoretically, the growth yield of microorganisms is proportional to the

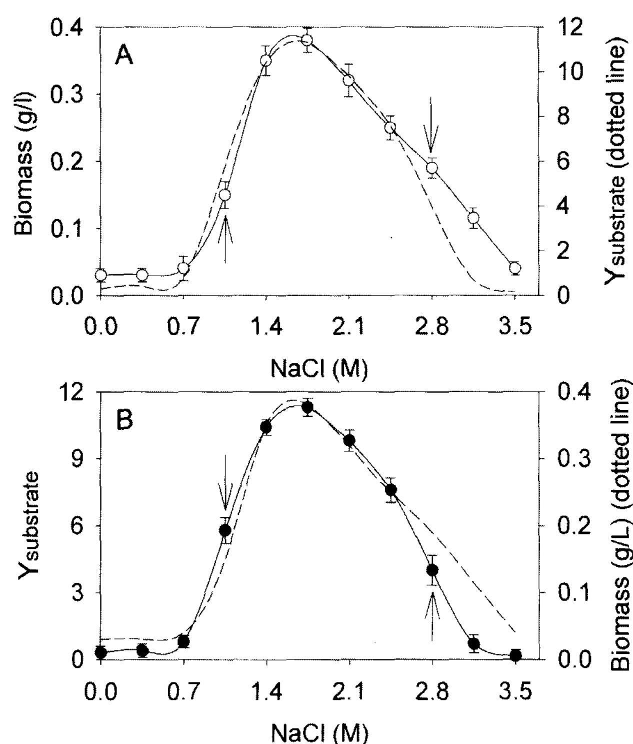

Fig. 2. Growth (A) and $Y_{substrate}$ (growth yield) (B) of *Halomonas salina* on the defined medium containing glucose.
The growth yield of *H. salina* was proportional to the biomass in the condition with NaCl, ranging from 1.4 M to 2.5 M. From this, it can be suggested that both the catabolism for the glucose oxidation and the anabolism for the biosynthesis were not inhibited by NaCl, ranging from 1.4 M to 2.5 M. The bacterium was cultivated for 48 h. The experimental data and standard deviations were obtained from the average of three different repeats.

Fig. 3. Activity staining of isocitrate dehydrogenase, pyruvate dehydrogenase, and malate dehydrogenase, separated from cell-free extract of *Halomonas salina* SK by nativegel polyacrylamide electrophoresis.
The three enzymes in the TCA cycle were not inhibited by NaCl. The gel was incubated in the reaction mixture for 30 min until the band with dark blue color emerged.

growth rates under the normal condition, but less in the presence of inhibitory factors.

Effect of NaCl on Enzyme Activity

The activity of three enzymes engaged in the TCA cycle was compared by the activity staining on the polyacrylamide gel under the condition without or with NaCl. As shown in Fig. 3, the enzyme activities were not inhibited, and the time for the activity band to emerge was not delayed by the addition of NaCl (not shown as data). This shows that the cytoplasmic enzymes of *H. salina* may work under high ionic strength condition such as a saline environment.

Electrochemical Measurement of Glucose Absorption

For measurement of the relationship between the energy metabolism and NaCl, we used the electrochemical method, the cyclic voltammetry. Here, neutral red is an electron mediator capable of binding to the bacterial membrane and can mediate electron transfer across the cytoplasmic membrane [23, 24]. In the cyclic voltammetry for the modified *H. salina* with neutral red, both the lower and upper peak heights were gradually decreased in the condition without NaCl (Fig. 4A) or with KCl (Fig. 4B). The one-directional decrease of the upper and lower peak heights indicates that the neutral red was only electrochemically oxidized and reduced on cathode and anode, respectively. The peak height has to be naturally decreased during the continuous cyclic voltammetry because the bacterial cell has to irreversibly bind to the working electrode. In the present study, the lower peak height was relatively more increased, and the upper peak height was relatively less decreased in the presence of NaCl (Fig. 4C). If the glucose was metabolically oxidized, coupled to reduction of NAD^+ to NADH, under the anaerobic condition, the reduction balance ($NADH/NAD^+$) has to be close to 1.0. In this circumstance, the neutral red can be metabolically reduced, coupled to oxidation of NADH to NAD^+, and the reduced neutral red has to donate the electrons to the anode, by which the lower peak height can be increased a little, thus overcoming the natural decrease of peak height; however, the metabolically reduced neutral red has difficulty in accepting the electrons from the cathode, hence the upper peak height may decrease relatively less (Fig 4C). Based on these results, it is highly likely that *H. salina* cannot absorb glucose under the growth condition without NaCl or with KCl, and that NaCl may function as a signal for the glucose absorption or the operation of electron transport system in the energy metabolism of *H. salina*. The electron transport system is coupled to the proton translocation, which is converted to the proton motive force (PMF is coupled to ATP production).

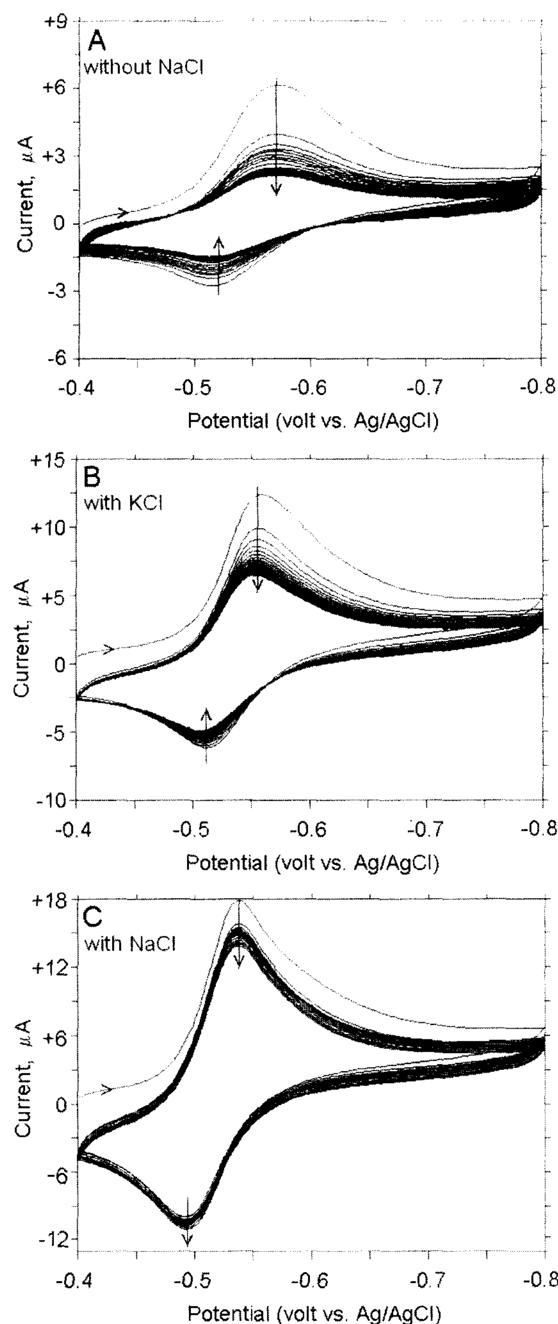

Fig. 4. Cyclic voltammogram of *H. salina* modified with neutral red, which is an electron mediator capable of binding to the cytoplasmic membrane and acts as an electron channel.

The cyclic voltammetry was performed with bacterial suspension of *H. salina* (OD 5.0 at 660 nm) in Tris-Cl buffer (pH 7.5) containing 200 mM glucose (A), 200 mM glucose plus 2.0 M KCl (B), and 200 mM glucose plus 2.0 M NaCl (C). The arrow marks from outside to inside indicate the current decrease.

Proton Translocation

To test the function of NaCl related to the energy metabolism of *H. salina*, we measured the proton translocation in the condition without and with NaCl. In the bacterial respiration system, the proton motive force is generated by coupling substrate oxidation with O_2 reduction. Therefore, the cells were incubated in the absence of NaCl for 30 min prior to

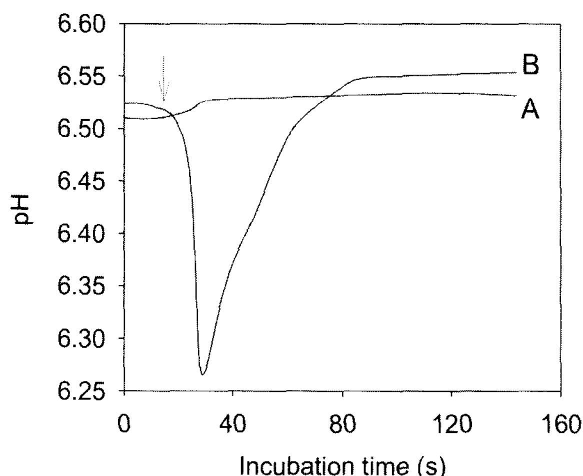

Fig. 5. Proton translocation after addition of glucose to a cell suspension of *Halomonas salina*, which was pre-incubated under the saturated oxygen atmosphere.
Addition of glucose without NaCl (A) and with 2.0 M NaCl (B). Arrow mark indicates time of addition of glucose.

the addition of substrate or substrate plus 2.0 M NaCl, and the proton translocation was measured: Proton translocation is coupled to oxidation of NADH in the electron transport chain, by which the proton motive force is produced. When the NADH is oxidized, coupled to the proton translocation, the pH outside of bacterial cell is suddenly dropped and immediately recovered because the proton is back to the inside of bacterial cell through the ATPase [17]. As shown in Fig. 5, rapid acidification of bacterial suspension was detected immediately after addition of substrate plus NaCl, but not by the addition of substrate. Based on these results, we suggest two possible mechanisms: the primary respiration-driven Na^+ pump coupled to the Na^+/H^+ antiport system in the energy metabolism of *H. salina*, and the membrane transport system activated by the proper concentration of NaCl inside and outside of the cytoplasmic membrane. Ken-Dror *et al.* [14, 15] reported that the movement of Na^+ is directly coupled to the electron transport, and that the uncoupler-insensitive primary Na^+ pump may play an important role in the regulation of intracellular salt concentration [16].

The results described in the present study led us to conclude that, in addition to osmoregulation, NaCl is absolutely required for the metabolisms related to the glucose absorption and electron transport (proton translocation) in addition to the osmoregulation. We were successful to shed some light to the question of why the *H. salina* could not grow in the condition without NaCl; however, we could not find the mechanism of how NaCl works for glucose transport and proton translocation. We are in a process to clarify the mechanisms of how NaCl works for glucose transport and proton translocation, and to develop a new electrochemical method to analyze the function of NaCl-related transport proteins to NaCl.

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