Mem. Fac. Sci. Shimane Univ.,
26, pp. 85–94 Dec. 25, 1992

A Role of Na^+/H^+ Antiporter on Growth of *Escherichia coli*: L-form RK4

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(Received September 10, 1992)

Escherichia coli, L-form NC7 was able to grow over pH range 6.2–8.8 in a complex medium supplemented with 0.34 M-NaCl (NaPY medium) or 0.34 M-KCl (KPY medium). On the other hand, variant strain, L-form RK4 derived from L-form NC7 was able to grow at acidic pH range in KPY medium, but not at all under alkaline pH conditions in both the media. When the NC7 was treated with amiloride or harmaline, inhibitor of Na^+/H^+ antiporter, the similar growth inhibition as those of the RK4 was observed in both the media. The Na^+/H^+ antiporter activity on everted membrane vesicles from the RK4 was markedly lower than that from the NC7. However, when the RK4 was incubated in alkaline buffer (pH 8.15) significant alkalization of the intracellular pH was not observed. When melibiose, one of substrate for Na-coupled transport system, was added into KPY medium, Na^+ was actively transported with melibiose and accumulated in the RK4 cells. In addition, cell swelling and lysis of the RK4 occurred when the cells were incubated in 0.34 M NaCl solution. Therefore, these findings strongly suggest that inability of growth of the RK4 in NaPY medium due mainly to insufficient activity of the Na^+/H^+ antiporter in response to extrusion of the exess Na^+ accumulated into the RK4 cells.

Introduction

Mycoplasma and L-forms derived from various bacterial species are well known as organisms without rigid cell walls. Swelling of Mycoplasma cells in hypotonic solution of NaCl is due to the inward diffusion of NaCl and water as a result of colloid-osmotic and Gibbs-Donnan force caused by intracellular nondiffusable micromolecules (Macknight & Leaf, 1977). In previous paper, it was suggested that cell swelling of L-form NC7 was induced by increase in osmotically active Na^+ enterring into cell and that regulation of the cell volume depends on extrusion of Na^+ from the cells (Onoda *et al.*,

The abbreviations used are: DCCD, dicyclohexylcarbodiimide; DTE, dithioerythritor; DMO, 5,5′-dimethyl-2,4-oxiazolidinedione; MA, methylamine; TPP, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Na_i^+, intracellular Na^+; H_o^+, extracellular H^+; $[Na^+]_o$, extracellular concentration of Na^+; $[Na^+]_i$, intracellular concentration of Na^+; pH_i, intracellular pH value; pH_o, extracellular pH value; $\Delta\phi$, transmembrane electrical gradient; $\Delta\tilde{\mu}H^+$, transmembrane proton electrochemical gradient.

1991). In addition, we proposed that L-form NC7 regulates its intracellular volume by extrusion of Na^+ through the combined operation of a DCCD-sensitive H^+-ATPase and an Na^+/H^+ antiporter. A variant strain, L-form RK4 was isolated from *E. coli* (Onoda, unpublished data); L-form NC7 lacking rigid cell wall and being osmotically fragile. The L-form RK4 is able to grow in PY medium containing 340 mM of KCl, but not 340 mM of NaCl. Many bacteria utilize H^+ and Na^+ gradient as major intermediates in energy transduction (Mitchell, 1966; Unemoto *et al.*, 1990). The exchange of Na_i^+ for H_o^+, *via* Na^+/H^+ antiporter, is essential to maintain $\Delta\tilde{\mu}H^+$ for growth and Na^+/solute symport activity (Booth, 1985; Krulwich, 1983; Dibrov, 1991).

Although the growth of L-form RK4 is inhibited in the presence of NaCl of high concentrations, little is known about its physiological role. In this paper, we have examined the roles of Na^+ for growth of the L-form RK4.

Materials and Methods

Strains and growth conditions

The L-form NC7 derived from *Escherichia coli* K12 strain 3301 (Onoda *et al.*, 1987) and the L-form RK4 which was spontaneously isolted from the revertant RNC7-1 (Onoda *et al.*, 1987) were used in this experimet. The media used in this study were KPY and NaPY medium. The KPY medium contained 1% of peptone, 0.5% of yeast extract, 0.34 M KCl, 10 mM glucose and 100 U/ml of penicillin G. The NaPY medium contained 1 mM $CaCl_2$ was same as KPY medium, except that NaCl was used instead of KCl.

Preparation of everted membrane vesicles

The L-form cells were harvested and washed twice with solution A containing 500 mM KCl, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$. The washed cells were resuspended in solution B (the same as solution A, except that 500 mM sucrose was added instead of 500 mM KCl) and washed twice with the same solution. Then, the cells were suspended in 10 mM Tris-HCl buffer (T-buffer) (pH 7.2 or 8.0) containing 500 mM choline-Cl or KCl, 5 mM $MgCl_2$, 0.5 mM DTE, and washed once with the same buffer. The cells were passed through a French pressure cell (150 kg/cm^2). Unbroken cells were removed by low speed centrifugation (5,000 *g* 10 min) and the supernatant was centrifuged at 541,000 *g* for 30 min. Resultant precipitate was resuspended in T-buffer and washed once, and subjected to prepare everted membrane vesicles.

Fluorescence assay

Cation/proton antiporter activities were estimated from the energy-linked quenching of quinacrine fluorescence. Assay were performed in the T-buffer containing 1 μ M quinacrine and 200 to 300 μg/ml of everted membrane vesicles. Quenching was initiated by addition of Tris-lactate (final concentration of 10 mM). The magnitude of

dissipation of pH by adding each substrate and the magnitude from the intensity before addition of cations to the intensity recovered by addition of 5 μM CCCP were expressed as ΔF and F, respectively. The antiporter activity was evaluated as $\Delta F/F$.

Assay of Na^+/H^+ antiporter activity in whole cell

The Na^+/H^+ antiporter activities were measured in whole cells by Li^+ extrusion from Li^+ loaded cells. The L-form cells grown in a KPY medium were harvested and washed twice with 20 mM-MOPS-KOH buffer (pH 7.0) containing 200 mM LiCl and 200 mM $CaCl_2$, and resuspended into the same buffer. The cell suspension was standing on ice bath for an hour. Then the cells were collected by centrifugation and resuspended in 20 mM-MOPS-NaOH (pH 7.0) or HEPES-NaOH (pH 8.2) containing 400 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 20 mM-glucose. At appropriate intervals, 1 ml of the cell suspension was took out and centrifuged at 50,000 rpm for 5 min. Sonication was carried out after addition of 1 ml of distilled water to the pellet, and the internal Li^+ concentration was determinated using Hitachi 170–40 flame photometer.

Measurement of $\Delta\psi$ and pH_i

pH_i and membrane potential, $\Delta\psi$, were estimated from the distribution across the cell membrane of [^{14}C] DMO, [^{14}C] MA and [^{3}H] TPP as described previously (Onoda *et al.*, 1992). To calculated the concentration of isotopes within the cells, the intracellular space [10 μl/mg protein] was estimated by determining the distribution voluem of [^{14}C] inulin and ^3H_2O (Rottenberg, 1979).

Protein determination

Protein was determinated by using Bradford protein assay (Bio-Rad). Bovine serum albumin was used as a standard.

Chemicals

CCCP and amiloride were obtained from Sigma. Harmaline and quinacrine were purchased from Nacalai Tesque. [^{14}C] MA, [^{14}C]DMO and [^{14}C] inulin carboxyl were products of American Radio Labeled Chem. Inc. [^{3}H]TPP and [^{3}H] water were supplied by Amersham Corp. All other reagents used were of analytical grade.

Results

Growth characteristics of L-form NC7 and RK4

Growth of the L-form RK4 was compared with the parental L-form NC7 in NaPY and KPY medium (Fig. 1). In KPY medium, no significant difference was observed on growth of NC7 and RK4. The RK4 grew at almost same rate as the NC7 in KPY medium, but the growth of the RK4 was markedly inhibited in NaPY medium. Further, significant difference between the growth rate on the NC7 and the RK4 was

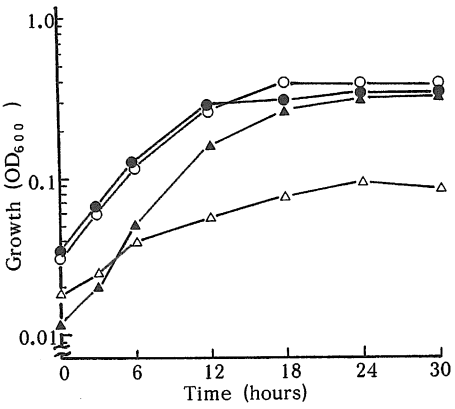

Fig. 1. Effects of salts on growth of the NC7 and the RK4. The NC7 (○, ●) and RK4 (△, ▲) cells were cultured in K^+ containing (KPY) (●, ▲) or Na^+ containing (NaPY) medium (○, △) at 32°C and the OD_{600} nm was measured at the times indicated.

observed when those strains were grown in NaPY and KPY media under various pH conditions. The NC7 was able to grow at pH range of 6.2 to 8.8 in NaPY and KPY media. On the other hand, the RK4 was unable to grow at pH values above 8.0 in both medium. It is widely accepted that Na^+/H^+ antiporter activity is required for growth of *Escherichia coli* at alkaline pH (McMollow *et al.*, 1989) and Na^+/H^+ antiporter activity-deficient mutant (Ishikawa *et al.*, 1987) no longer grow in alkaline medium. To determine whether the growth repression of the RK4 observed at alkaline pH is caused by the insufficient activity of Na^+/H^+ antiporter, the growth of NC7 was tested in the

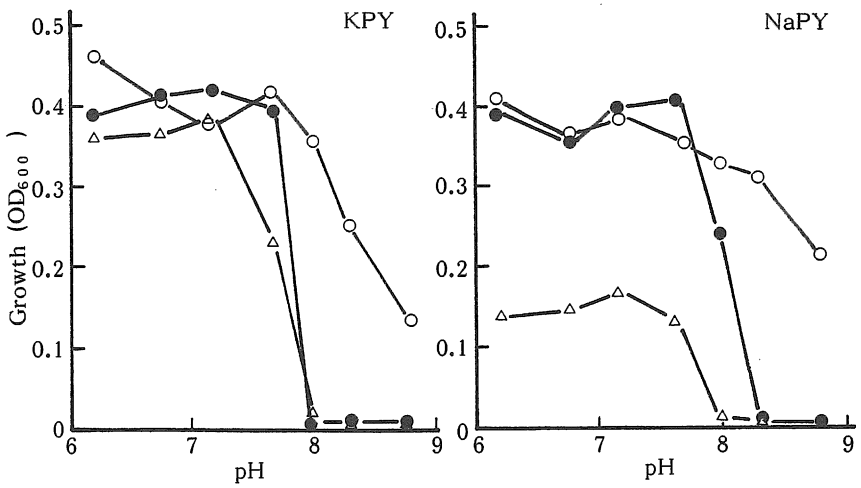

Fig. 2. Effect of pH on growth of the NC7 and the RK4. The NC7 (○, ●) and the RK4 (△) cells were cultured at 32°C in KPY and NaPY media with the indicated initial pH values adjusted by 50 mM MES-KOH or NaOH for pH 6.2 to 7.2, HEPES-KOH or NaOH for pH 7.0 to 8.0, and Tris-HCl for pH 8.0 to 8.8. The OD_{600} nm was measured after 48 h incubation. Amiloride was added at final concentration of 1 mM (●).

presence of 1 mM amiloride, one of the potent inhibitor of the Na^+/H^+ antiporter. At pH values of 6.0 to 7.7, the NC7 grew well in KPY medium in the presence of amiloride, but at pH values over 7.8, the growth was completely prevented by amiloride (Fig. 2). The growth properties of the RK4 in KPY medium are similar to those of the NC7 in the presence of amiloride.

Na^+/H^+ antiporter activity of the L-form NC7 and RK4

The results described above suggested that the marked repression of the growth of the RK4 observed at NaPY and especially alkaline KPY media may be caused by the low Na^+/H^+ antiport activity. In order to determine the activity of cation/proton antiporter(s) on the NC7 and RK4, we prepared the everted membrane vesicles and effects of pH on the antiporter activities were estimated by measuring the fluorescence quenching of quinacrine as descbied in MATERIALS AND METHODS. Three different antiporter systems (K^+/H^+, Na^+/H^+ and Ca^{2+}/H^+ antiporters) were observed in the everted membrane vesciels prepared from the RK4 and NC7 (data not shown). Although all three cation-proton antiporter systems of the NC7 and the RK4 were similar in pH-activity relationships, the $\Delta F/F$ values of the Na^+/H^+ antiporter of the RK4 were markedly lower (loss of about 50%) than the values of the NC7 over all pH values tested (Fig. 3). Further, we tested the effect of Na concentration on kinetic parameters of Na^+/H^+ antiport systems. Fig. 4 shows the Lineweaver-Burk plot on activities of Na^+/H^+ antiporter. The K_m value was found to be 550 μM for Na in both the membrane vesicles, while the V_{max} was significantly greater for the NC7 than for the RK4.

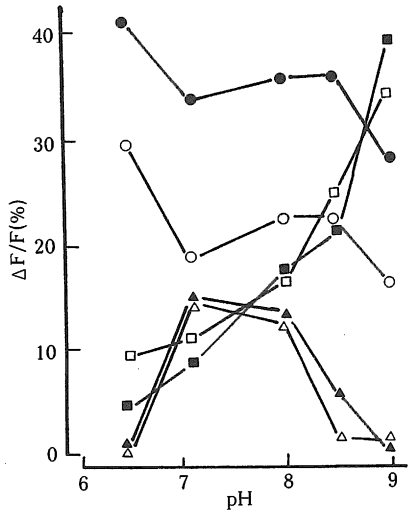

Fig. 3. Effect of pH on the antiporter activities of the NC7 and the RK4. The K^+/H^+ (□, ■), Na^+/H^+ (○, ●) and Ca^{2+}/H^+ (△, ▲) antiporter activities in the membrane vesicles prepared from the NC7 (●, ■, ▲) and the RK4 (○, □, △) were measured with following buffer systems: 50 mM MOPS-Tris for pH 6.5 to 7.0, and 50 mM Tris-HCl for pH 7.0 to 9.0. The cations were added successively in the order of KCl, NaCl and $CaCl_2$ at final concentration of 10 mM, 10 mM and 1 mM, respectively. The antiporter activities were evaluated as $\Delta F/F$ as described in Materials and Methods.

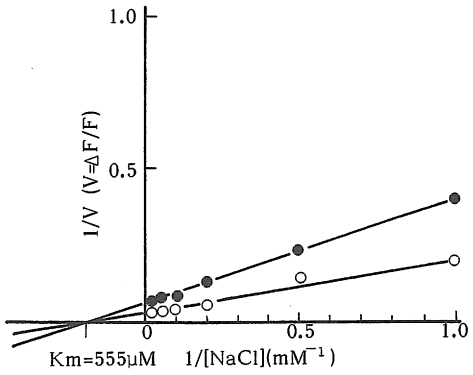

Fig. 4. Lineweaver-Burk plots of Na^+/H^+ antiporter against Na^+ concentration. The antiporter activities of the NC7 (○) and RK4 (●) at pH 7.2 were measured at various concentrations of NaCl.

Role of Na^+/H^+ antiporter activity on the growth of RK4

In order to understand the role of deficient Na^+/H^+ antiporter activity on the growth of the RK4 in the NaPY and alkaline KPY medium, we observed the effect of harmaline, a strong potent inhibitor of Na-dependent process involving Na^+/H^+ antiporter, on growth of the NC7 in NaPY and KPY media (Fig. 5). At neutral pH (pH 7.2), the NC7 grew well in KPY medium containing up to 500 μM harmarine, but no growth was observed in the presence of the drug above 200 μM at alkaline pH (pH 8.0). In the NaPY medium, inhibition of the growth were increased with increasing in the concentration of harmarine not only at alkaline pH but also at neutral pH. Although these results were somewhat differ from the results obtained by using amiloride as an inhibitor for Na^+/H^+ antiporter, these results suggest that the activity of the Na^+/H^+ antiporter is necessary for the growth of the NC7 in NaPY medium even at neutral pH. Thus, we postulated a possibility that the growth inhibition of the RK4 is owing to the deficiency of intracellular pH homeostasis activity and/or extrusion activity of intracellular Na. First, we measured the intracellular pH of the RK4 at

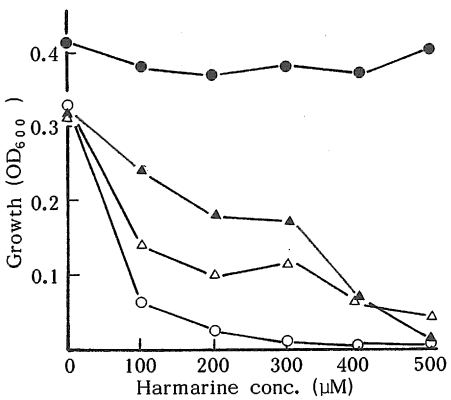

Fig. 5. Effect of harmaline concentration on the growth of the NC7. The cells were cultured in KPY (○, ●) and NaPY (△, ▲) media at pH 7.2 (●, ▲) and 8.0 (○, △). The OD_{600} nm was measured after 48 h cultivation.

Table 1. Intracellular pH and membrane potential of the NC7 and the RK4

Strain	pH_o	pH_i	$\Delta\psi$ (mV)
NC7	6.80	7.20	−45.8
	8.15	7.95	−67.2
RK4	6.80	7.20	−48.7
	8.15	7.97	−78.5

Data are means of at least three determinations.

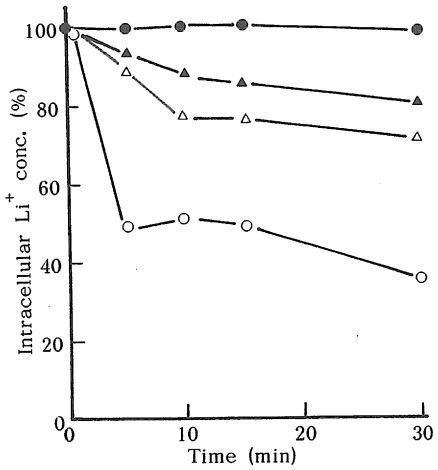

Fig. 6. Li^+ extrusion from Li^+ loaded cells. Li^+ loaded cells of the NC7 (○, ●) or the RK4 (△, ▲) were suspended in 20 mM MOPS-NaOH (pH 7.0) containing 400 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 20 mM glucose. At the times indicated, cells were collected and Li^+ concentrations were measured as described in Materials and Methods. Control experiments (●, ▲) were carried out in the presence of CCCP (10 μM).

external pH (8.15). Though the RK4 could not grow at external pH of 8.15, pH_i value of the RK4 was same as that of the NC7 (Table 1). Next, we determined the Na^+-extrusion activity of the RK4 in the Na^+ containing buffer. As described in MATERIALS AND METHODS, Na^+-extrusion activity was expressed as Li^+-extrusion activity from Li^+-loaded cells of the RK4. Fig. 6 shows that the NC7 could extrude Li^+, whereas the RK4 not. Further, it is expected that Na^+-load in the cells was achieved by addition of melibiose because melibiose is transported into the cells by Na^+/meliboise symporter. When melibiose was added to the KPY medium increase in intracellular Na^+ concentration was observed. As shown in Fig. 7, Na^+ was taken up more in the RK4 than the NC7 cells. Furthermore, the NC7 grew well in the presence of melibiose, while that of the RK4 was strongly suppressed (Fig. 8).

Effects of Na on swelling and lysis of the L-form NC7 and RK4

In order to test a possibility that the growth repression of the RK4 is owing to accumulation of Na^+, the change of absorbance were monitorred when NC7 and RK4 were suspended in the buffer contianed 340 mM-KCl or -NaCl (Fig. 9). The no change

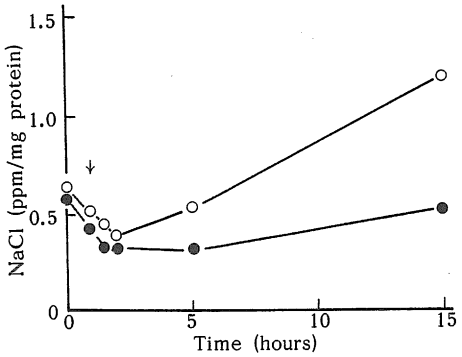

Fig. 7. Effect of melibiose on the intracellular concentration of Na^+. The NC7 (●) or the RK4 (○) cells were inoculated in glucose-omitted KPY medium and an hour after inoculation, as indicated by the arrow, melibiose was added at final concentration of 10 mM. At the time indicated, 1 ml of the cell suspension was took out and centrifuged at 50,000 rpm for 5 min. Sonication was carried out after addition of 1 ml of distilled water to the pellet, and the internal Na^+ concentration was determinated using Hitachi 170–40 flame photometer.

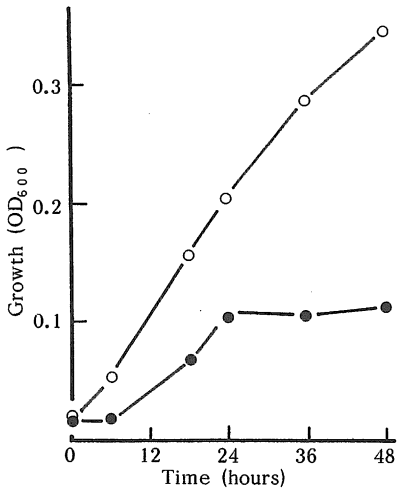

Fig. 8. Effect of melibiose on growth of the NC7 and the RK4. The NC7 (○) or the RK4 (●) cells were cultured in KPY medium (especially glucose was replaced with melibiose) and OD_{600} nm were measured at the times indicated.

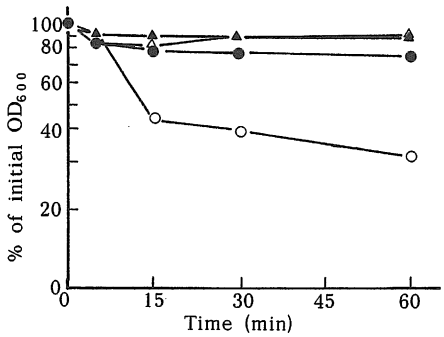

Fig. 9. Effect of NaCl on changes of turbidity of the NC7 and the RK4. The NC7 (△, ▲) or the RK4 (○, ●) cells were harvested by centrifugation and resuspended in the 20 mM MOPS-Tris (pH 7.0) containing with 1% of peptone, 0.2% of glucose and 340 mM of NaCl (○, △) or KCl (●, ▲). At the times indicated the OD_{600} nm were measured.

of absorbance was observed when the NC7 cells were suspended into the KCl or NaCl-buffer. On the other hand, the absorbance on the RK4 cells slightly decreased in the KCl-buffer, but rapidly in the NaCl-buffer. This decrease of absorbance seems to be resulted by a swelling and lysis of the RK4 cells from microscopic observation (data not shown).

Discussion

Growth of the L-form RK4 in NaPY medium was compared with that in KPY medium. The RK4 was able to grow at acidic or neutral pH range in KPY medium, but difficiently in NaPY medium. On the other hand, at alkaline pH_o, the RK4 was unable to grow in both the media. A mechanism that the RK4 is unable to grow at alkaline pH range and especially in NaPY medium was examined.

Many neutrophiles maintain a constant pH_i at around 7.5 over a wide range of pH_o values (Booth, 1985). An Na^+/H^+ antiporter is a carrier that catalyzes the exchange of Na^+ and H^+ across the bacterial cell membrane and have a important role for the pH homeostasis mechanism in *E. coli*. The *E. coli* cells maintain the nonequilibrium transmembrane Na^+ distribution ($[Na^+]_o > [Na^+]_i$) in a wide range of external Na^+ concentrations (Castle, *et al.*, 1986). An inwardly directed Na^+ gradient is supported by the $\Delta\tilde{\mu}H^+$-driven Na^+/H^+ antiporter (West and Mitchell, 1973; Krulwich, 1983). So, $\Delta\tilde{\mu}H^+$ produced by primary proton pumps is utilized for the formation of nonequlibrium transmembrane sodium ion distribution. Ishikawa *et al.*, (1987) reported that mutants of *E. coli* defective in the Na^+/H^+ antiporter grow poorly in alkaline media. The role of Na^+ in cell growth at alkaline pH_o is apparently related to the acidification of the cell interior *via* the Na^+/H^+ antiporter. Na ion is required for the growth of *E. coli* especially at alkaline pH_o (McMorrow *et al.*, 1989).

In this study, the NC7 was able to grow very well at alkaline pH_o in KPY and NaPY media. Amiloride was used as a Na^+/H^+ antiporter inhibitor. When the NC7 was incubated in KPY medium over the pH range 6.2–8.8, growth was inhibited by amiloride at alkaline pH. These results indicated that the Na^+/H^+ antiporter was required for growth of the NC7 under alkaline pH_o. We also found that from the Lineweaver-Burk plotting experiments, the RK4 have a defective Na^+/H^+ antiporter activity (Fig. 4.). When the RK4 and the NC7, respectively, were incubated in NaPY medium with pH_o 8.15 there was no significant difference between both the strains with respect to the pH_i values. In addition, the K^+/H^+ antiporter activity in the NC7 and RK4, which was accelerated with alkaline pH, depended on pH values (Fig. 3). The K^+/H^+ antiporter may function as a regulator of pH_i at alkaline and acidic pH_o.

Na^+ efflux is another important role of the Na^+/H^+ antiporter in the RK4 cells which take up Na^+ *via* an array of Na^+/solute symporters. In *E. coli,* melibiose can enter the cell *via* the lactose transport system or the melibiose carrier, which is induced if the cell grows in the presence of melibiose. The meliboise carrier of *E. coli* can use either Na^+ or H^+ for cotrasport with melibiose (Tsuchia & Wilson, 1978; Wilson & Wilson, 1987). The addition of melibiose to the RK4 causes Na^+ uptake. The defective Na^+/H^+ antiporter of the RK4 was unable to extrude enough Na^+ that was took up into the cells and as a result, accumulation of Na^+ occurred in the cells incubated in NaPY medium. In Mycoplasma, it is reported that Na^+ extrusion systems regulate the osmotic pressure in the cells (Linker & Willson, 1985). The Na^+ accu-

mulation induce water influx into the cells and finally, lead to cell swelling or lysis. Therefore, the osmotic pressure caused by the Na^+ accumulation perhaps may repress growth of the RK4 cells in the presence of high Na^+.

References

Booth, I. R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **48**: 359–378.

Castle, A. M., Macnab, R. M. and Shulman, R. G. (1986) Measurement of intracellular sodium concentration and sodium transport in *Escherichia coli* by ^{23}Na nuclear magnetic resonance. *J. Biol. Chem.* **261**: 3288–3294.

Dibrov, P. A. (1991) The role of sodium transport in *Escherichia coli* energetics. *Biochim. Biophys. Acta.* **1056**: 209–224.

Ishikawa, T., Hama, H., Tsuda, M. and Tsuchiya, T. (1987) Isolation and properties of a mutant of *Escherichia coli* possessing defective Na^+/H^+ antiporter. *J. Biol. Chem.* **262**: 7443–7446.

Krulwich, T. A. (1983) Na^+/H^+ antiporters. *Biochim. Biophys. Acta.* **726**: 245–264.

Linker, C. and Wilson, H. (1985) Cell volume regulation in *Mycoplasma gallisepticum*. *J. Bacteriol.* **163**: 1243–1249.

Macknight, A. D. C. and Leaf, A. (1977) Regulation of cellular volume. *Physiol. Rev.* **57**: 510–573.

McMollow, I. M., Shuman, H. A., Sze, D., Wilson, D. M. and Wilson, T. H. (1989) Sodium/proton antiport is required for growth of *Escherichia coli* at alkaline pH. *Biochim. Biophys. Acta.* **981**: 21–26.

Mitchell, P. (1966) Chemiosmotic coupling in oxidative & photosynthetic phosphorylation. *Biol. Rev.* **41**: 445–502.

Onoda, T., Oshima, A., Nakano, S. and Matsuno, A. (1987) Morphology, growth and reversion in a stable L-form of *Escherichia coli* K12. *J. Gen. Microbiol.* **133**: 527–534.

Onoda, T. Oshima, A. and Tanaka, H. (1991) Regulation of cell volume in *Escherichia coli*: L-form NC7. *Mem. Fac. Sci. Shimane Univ.* **25**: 63–72.

Onoda, T., Oshima, A., Fukunaga, N. and Nakatani, A. (1992) Effect of Ca^{2+} and K^+ on the intracellular pH of an *Escherichia coli* L-form. *J. Gen. Microbiol.* **138**: 1265–1270.

Rottenberg, H. (1979) The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. *Method Enzymol.* **55**: 1747–1755.

Tsuchia, T. and Wilson, T. H. (1978) Cation sugar contrasport in melibiose transport system of *Escherichia coli*. *Membr. Biochem.* **2**: 63–79.

Unemoto, T., Tokuda, H. and Hayashi, M. Primary sodium pumps and their significance in bacterial energetics. In *The Bacteria,* vol. XII, Bacterial Energetics, pp. 33–54. Edited by T. A. Krulwich. San Diego: Academic Press.

West, I. C. and Mitchell, P. (1973) Stoichiometry of lactose-protein transport across plasma membrane of *Escherichia coli*. *Biochem. J.* **132**: 587–592.

Wilson, D. M. and Wilson, T. H. (1987) Cation specificity for sugar substrates of the melibiose carrier in *Escherichia coli*. *Biochim. Biophys. Acta.* **904**: 191–200.