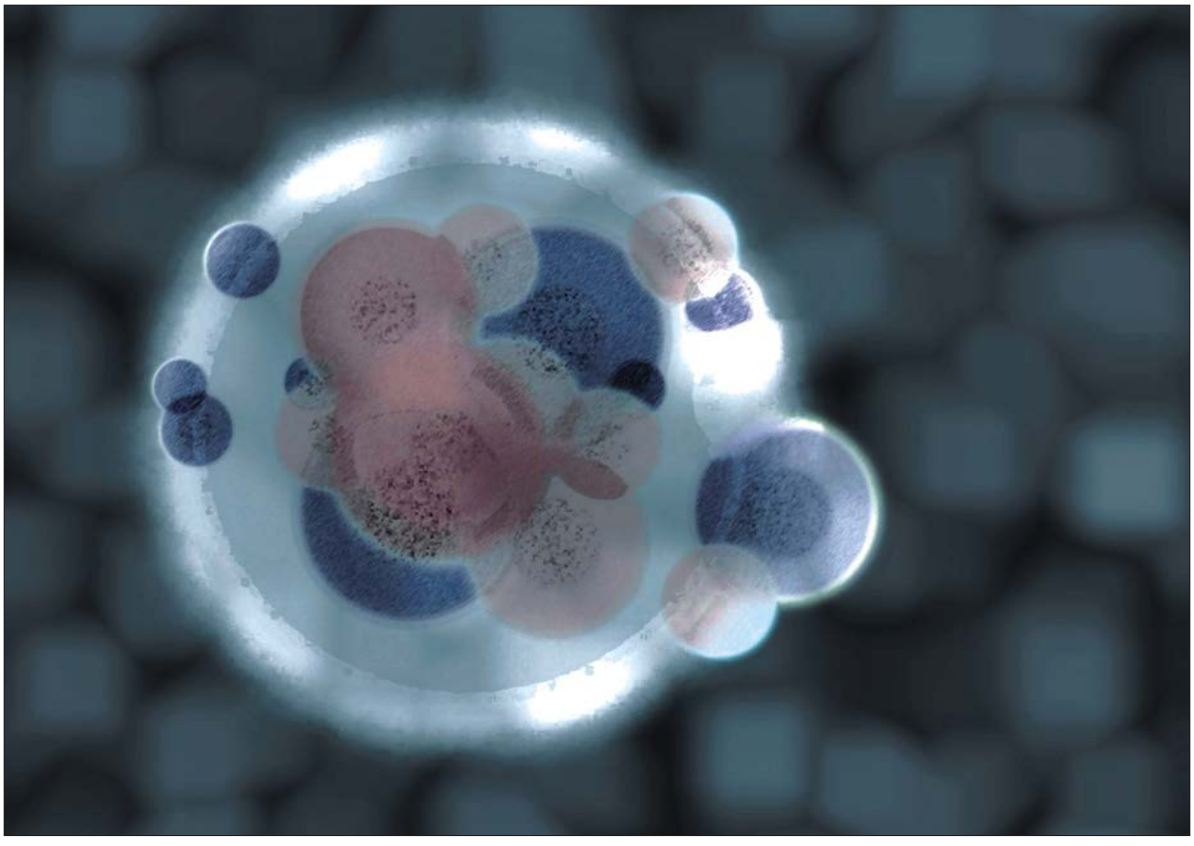

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Utilization of Proline in *Escherichia coli* K-12 at Different Osmolarities

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Abstract: Utilization of proline in *Escherichia coli* K-12 was analyzed at different osmolarities. Under the proline-base cultivation, sufficient growth of strain K-12 accompanied with high amount of proline uptake was observed in the presence of less than 0.5 M NaCl, where growth time lag was recognized. Distinct decrease in growth yield was observed in the presence of 1 M NaCl despite the sufficient accumulation of proline. Activities on respiration supported the result of growth. Analyses of L-[5-^{3}H]proline uptake in the cell cytoplasm suggested that proline in cells was incorporated into hot TCA insoluble fraction in the absence of NaCl, but it was efficiently utilized as compatible solute in the presence of high concentrations of NaCl. These data suggested that proline itself functions as compatible solute rather than the participation of metabolization in cells under high osmolarity.

Key words: *Escherichia coli* K-12, different osmolarities, proline, growth, respiration, incorporation

INTRODUCTION

Many species of bacteria respond to increase an osmotic pressure by accumulating osmoregulatory solutes, so called compatible solutes, up to high intracellular concentrations for coping with external high salinity. Such osmoregulatory solutes as K^+, glutamate, proline, ectoine, glycine betaine and so on in bacteria have been extensively reported (Galinski, 1993). Among them, proline is one of the unique compatible solutes that are utilized not only as osmoprotectant but also as substrate of metabolization in the cells.

To transport proline, *Escherichia coli* has three independent proline transporters, PutP, ProP and ProU. The PutP system, which is not regulated by the osmolarity (Grothe *et al*., 1986), is required for the transport of proline when it is used as a carbon or nitrogen source. The ProP and the ProU systems operate for the accumulation of proline and/or glycine betaine to high levels, which makes the cells possible to adaptation with high osmotic conditions (May *et al*., 1986). The *proP* encodes a constitutive low affinity transporter system ProPand this expression is stimulated several fold during osmotic upshock (May *et al*., 1986). The *proU* encodes a binding protein dependent on high affinity transporter system ProU, which is induced by elevated osmolarity (Barron *et al*., 1987).

In *E. coli*, proline is first oxidized to Δ-1-pyrroline-5-carboxylate (P5C) and then P5C is converted to L-glutamate, those of which are carried out by proline dehydrogenase and P5C dehydrogenase, respectively (Frank and Ranhand, 1965). These two enzymatic activities are performed by a single multifunctional protein PutA encoded by the *putA* gene (Menzel and Roth, 1981).

Recovery of bacterial cellular activities such as cell division, transport and respiration under high osmolarity is carried out by the accumulation of compatible solutes. In our previous study, *E. coli* K-12 was shown to be highly adaptive on the external high osmolarity as a result of the accumulation of compatible solute of proline (Nagata *et al*., 2002). In addition, the presence of proline led to the sufficient levels on growth and respiration activity of *E. coli* incubated under high salinity (Nagata *et al*., 2005). However, we have not yet confirmed the function of proline as the sources for metabolization in non-halophilic bacteria at high osmolarity. In this study, we analyzed the utilization of proline on the growth and respiration of *E. coli* K-12 at different osmolarities.

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MATERIALS AND METHODS

Organism and culture conditions: *E. coli* K-12 IFO 3301 utilized in this study was precultured in nutrient medium (pH 7.0) consisting of 5 g L^{-1} dry yeast extract D-3 (Nihon Pharm. Co., Tokyo, Japan), 10 g L^{-1} Polypepton (Nihon Pharm. Co.) and 2 g L^{-1} NaCl. Cell culture by inoculating pre-culture in modified Davis minimal medium (pH 7.5) which contains 50 mM Na_2HPO_4-NaH_2PO_4 (NaP_i) buffer, 0.1 g L^{-1} $MgSO_4$, 1.0 g L^{-1} $(NH_4)_2SO_4$, 1 mM KCl and 0.5 g L^{-1} sodium citrate. 10 mM proline was supplemented as carbon source. The culture was performed in 10 mL medium in 30 mL flask or 6 mL medium in 18×180 m/m test tube with 120 strokes at 30°C. Growth was determined by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer DU 640 (Beckman Instruments, Fullerton, CA, USA).

Measurement of respiratory activity: Oxygen uptake by the intact cells at the late exponential phase of growth in nutrient medium for 16 h was measured by the conventional volumetric technique using O_2 Up Tester (Taitec Co., Saitama, Japan) at 37°C (Nagata *et al*., 2002). The volumetric vessels contained modified Davis medium (pH 7.0) with 1 mM proline. The reaction in the above mixture containing a total volume of 4 mL was initiated by the addition of cells. If necessary, various concentrations of NaCl were added to the reaction mixture. Respiratory activities were expressed as μL of oxygen consumed per min per mg cell dry weight.

Determination of proline: To determine the uptake of proline by intact cells, we measured their disappearance from the incubation mixture that contained 1 mM proline in modified Davis minimal medium, pH 7.5. The reaction at 30°C was initiated by the addition of the cells, in which the cell density (OD_{600}) was adjusted to 5.0 finally. If necessary, NaCl and 1 mM proline were added to the incubation mixture. At the same time, amounts of proline in the medium was also measured accompanied with the cell growth. At designated time intervals, an appropriate volume of the incubation mixture was centrifuged at 12,000×g for 5 min to obtain a cell-free supernatant.

For the measurements of free proline modified method of Whatmore *et al.* (1990) was used; 0.5 mL supernatant sampled was reacted with 0.5 mL of 6% aqueous 5-sulphosalicylic acid, 0.5 mL acid ninhydrin and 0.5 mL acetic acid at 100°C for 60 min. Acid ninhydrin was prepared by dissolving 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M H_3PO_4. Samples were then plunged into ice and mixed with 50% ethanol. Proline concentration was determined by measuring the absorbance at 520 nm using a spectrophotometer DU 640 (Beckman Instruments).

Uptake and incorporation of ^{3}H-proline by intact cells: Uptake and incorporation of proline by whole cells were measured by using L-[5-^{3}H]proline (962 GBq/mmol; Amersham Pharmacia Biotec UK Ltd., UK) as a substrate. The cells grown in LB medium were harvested and washed twice and re-suspended with modified Davis minimal medium. Uptake and incorporation of proline by whole cells was aerobically carried out at 30°C in the reaction medium which contained modified Davis minimal medium supplemented with appropriate amount of NaCl and 1 mM L-[^{3}H]proline (9.62 KBq/mmol). The cell suspension (final concentration was 0.289 mg dry weight/mL corresponding to OD_{600} =1.0) was added to the reaction medium except for L-[^{3}H]proline and then the reaction was initiated by the addition of L-[^{3}H]proline. To determine the uptake of proline, 0.5 mL of the reaction mixture was filtrated through GF/F glass microfibre filter (Whatman) at designated time intervals. The cells on the filter were washed twice with 3 mL of the reaction medium without labeled proline. The radioactivity remaining on the filters was determined by liquid scintillation spectrometer (Tri-Carb 2001 TR, Perkin Elmer, USA). To determine the incorporation of L-[^{3}H]proline by intact cells, a method of Bobier *et al.* (1972) was used with minor modification. At designated time intervals, 0.5 mL of the reaction medium was injected into 0.5 mL of 10% trichloroacetic acid (TCA). The TCA treated mixture was heated at 90°C for 20 min, cooled and filtrated through GF/F glass microfibre filter. The precipitates on the filter were washed twice with 3 mL of 5% TCA. The radioactivity remaining on the filters was determined as described above.

RESULTS AND DISCUSSION

Growth analysis: Under the proline-base culture as carbon source, *E. coli* K-12 attained to the similar growth as that of glucose-base culture described in our previous paper (Nagata *et al*., 2005), although the occurrence of a little growth lag time and the reduction of growth rate were recognized. Effect of NaCl on the growth of *E. coli* K-12 was examined in the proline-base medium. As shown in Fig. 1, strain K-12 showed the adaptation towards the wide range of NaCl concentrations. At 0-0.5 M NaCl, sufficient growth was observed, although time lag of growth became longer in proportion to the increase of NaCl concentration in medium. Distinct decrease in growth yield was observed in the presence of 1 M NaCl, where growth of strain K-12 was not permitted at least during 100 h of incubation (data not shown).

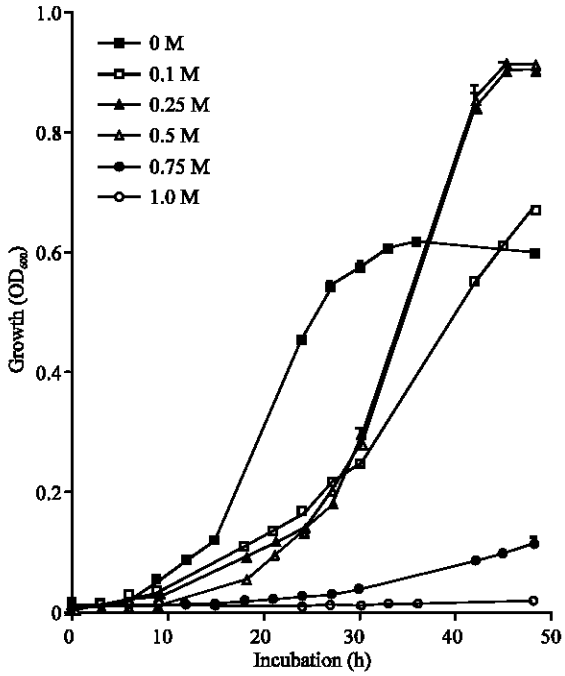

Fig. 1: Effect of NaCl concentrations on the growth of *E. coli* K-12. The cells were grown in modified Davis minimal medium with 10 mM proline at 30°C. Cell growth was determined by measuring the OD_{600} as a function of external NaCl concentrations. The values are cited from OD_{600} during 48 h of cultivation, those of which are the averages±standard deviations from three separate incubations

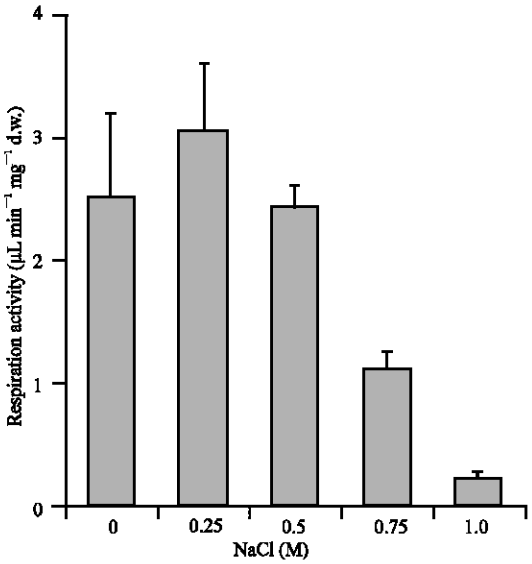

Fig. 2: Effect of NaCl concentrations on the respiration of *E. coli* K-12. The measurements of respiration in the presence of 0-1 M NaCl were initiated by the addition of the cells into modified Davis minimal medium with 1 mM proline as a substrate. The values are the averages±standard deviations from three separate incubations

Respiratory activity: The respiratory activity of strain K-12 in the presence of 1 mM proline as respiratory substrate was reduced with increase of external NaCl concentrations. As shown in Fig. 2, respiratory activity was markedly reduced under 0.5-0.75 M NaCl conditions. Extremely low activity of respiration in strain K-12, however, was still observed under 1 M NaCl condition. Respiratory profiles of strain K-12 described above were in good accordance with those of cell growth as shown in Fig.1.

Behavior of proline in cells: Uptake of proline and K^+ in initial response of *E. coli* K-12 under various salinities was examined. High amounts of proline uptake in the cells were observed under 0.25 and 0.5 M NaCl conditions after 60 min of incubation, while low ability of proline accumulation in the strain K-12 was observed in the absence and presence of 1 M NaCl (Fig. 3A). Present results on uptake rates of proline at different salinities were consistent with those of cell growth as shown in Fig. 1. Under the cultivation of 0 M and 1 M NaCl conditions, 1 mM of proline in medium was gradually accumulated in the cells, although uptake rate was slower in the presence of 1 M NaCl compared with that in the absence of NaCl (Fig. 3B).

Using [^{3}H]-labeled proline, uptake of proline was directly examined in the presence of 0, 0.5 and 1 M NaCl. Isotope tracing data showed that the proline uptake supported the analytical result shown in Fig. 3. The highest amount of proline accumulated in the cells was observed in the presence of 0.5 M NaCl after 60 min of incubation (Fig. 4A). In the absence and presence of 1 M NaCl, smaller rates of proline uptake were observed in comparison with that of 0.5 M NaCl. The fate of proline taken up in the cells was traced by the incorporation analysis, which clearly indicates that the higher portion of proline was incorporated into hot TCA insoluble fraction in the absence of NaCl in comparison with those in the presence of 0.5 and 1 M NaCl, as shown in Fig. 4B.

It is thought that proline accumulated in the cells was utilized as sources for metabolization when they were incubated under low osmolarities. On the other hand, proline added to the culture medium with high osmolarity is thought to perform the role as compatible solute for the growth of *E. coli* K-12 (Nagata *et al.*, 2002, 2005). Thus, it is interesting to examine whether the strain K-12 could utilize proline not as the nutrient but as the adaptational tool under high osmolarity. The strain K-12, however, could not grow under 1 M NaCl condition despite the

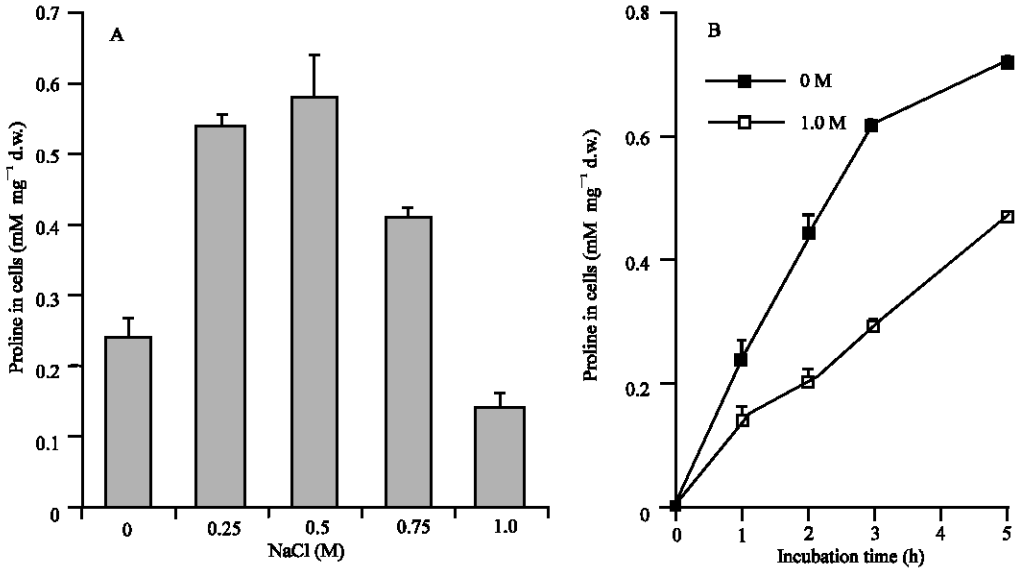

Fig. 3: Proline uptake of *E. coli* K-12 as an instant response. The cells were incubated in modified Davis minimal medium with 1 mM proline for 1 h under various salinities (A). In addition, the incubation of the cells was carried out in modified Davis minimal medium with 1 mM proline in the absence and presence of 1 M NaCl (B) as a function of incubation time, in which final cell density was adjusted to 5.0 of OD_{600}. Analytical method of external proline was described in materials and methods. The values are the averages±standard deviations from three separate incubations

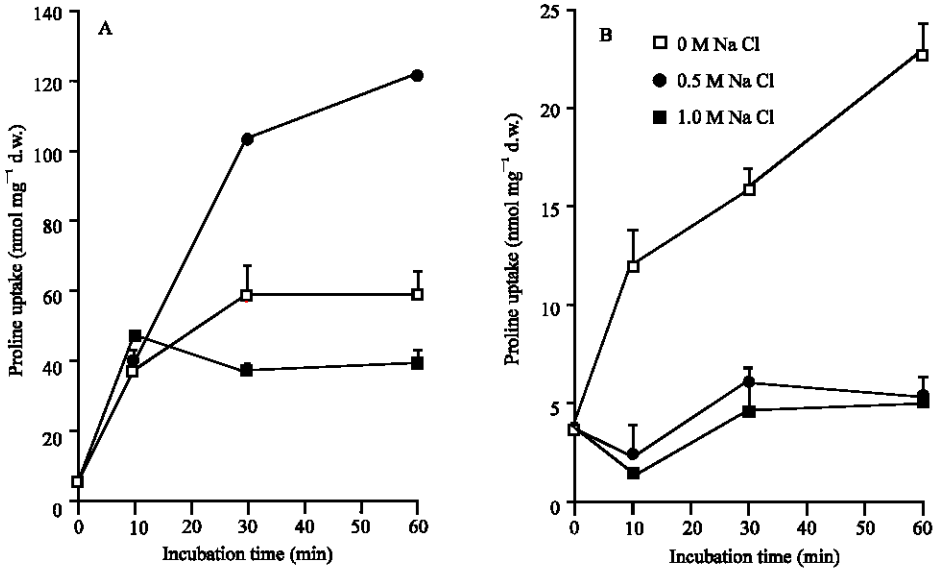

Fig. 4: Effects of NaCl on proline uptake and incorporation in *E. coli* K-12. Proline uptake (A) and incorporation (B) in cells were analyzed using [^{3}H]-labeled proline. Cells were incubated in modified Davis minimal medium with 1 mM proline in the absence (opened squares) and presence of 0.5 M NaCl (closed circles) or 1 M NaCl (closed squares). Details for measurements of uptake and incorporation of proline are given in materials and methods

high amount of proline accumulated in cells (Fig. 1 and 3), which indicates that proline was not metabolized efficiently for cell proliferation. Consequently it may be plausible that free proline functions as compatible solute under high osmolarity. Moreover, under high osmolarities proline was little incorporated in the cells, suggesting that proline was utilized as compatible solute without metabolization like *Lactobacillus acidophilus* IFO 3532

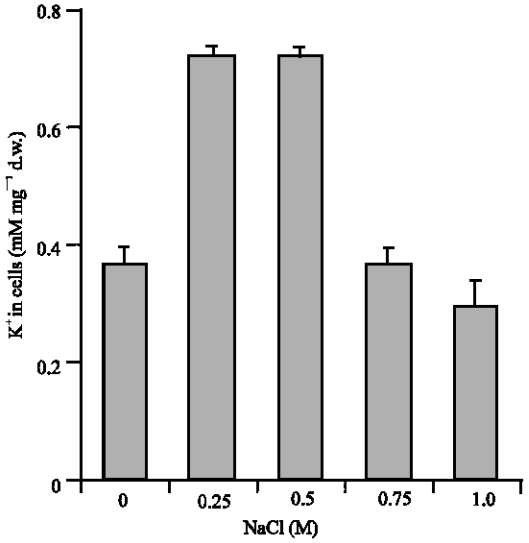

Fig. 5: K^+ uptake of *E. coli* K-12 as an instant response. The cells were incubated in modified Davis minimal medium containing 1 mM proline for 1 h under various salinities, in which final cell density was adjusted to 5.0 of OD_{600}. The values are the averages±standard deviations from three separate incubations

(Jewell and Kashket, 1991). Furthermore, it was reported that the activity of proline dehydrogenase (PutA) to metabolize proline was reduced under high osmolarity (Deutch *et al*., 1989), which might support our present result.

Change of K^+ uptake under proline-base culture condition: During 1 h of cultivation, high amount of K^+ uptake was observed in the presence of 0.25 and 0.5 M NaCl, which is almost similar as those of proline uptake (Fig. 5). In the absence and presence of 1 M NaCl, significant differences of K^+ concentration in medium were not detected between 1 h and 24 h of incubation, although a little reduction was occurred (data not shown).

The absence of NaCl causes high K^+ accumulation in *E. coli* cells grown in the glucose-base cultivation (Nagata *et al*., 2005). Under proline-base condition for growth of strain K-12, on the contrary, large amount of K^+ uptake was not observed. At the present time, it is still unclear why this difference was occurred. In the case of glucose-base condition for growth, K^+ may be closely related to the metabolism of glucose in cells or function towards glucose transporters. In this connection, it seems to be of value to point out that high concentration of K^+ in cells may be needed for cellular activities. Furthermore, difference in the uptake of K^+ was caused by the external high osmolarity. When *E. coli* cells adapt with external high osmolarity, a lot of amount of K^+ is rapidly accumulated in cells when both glucose and proline were existed in medium (Nagata *et al*., 2005). Based on proline alone as nutrients, on the contrary, high level of K^+ was not accumulated under high osmolarity. This result suggested us activation of K^+ transporter need a protection by compatible solute such as proline.

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