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Effect of moderate salinity stress treatment on the stimulation of proline uptake and growth in *Escherichia coli* CSH4 and its mutants under high salinity

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Activity of proline uptake in *Escherichia coli* CSH4 was inhibited in the presence of 1 M NaCl, while it was recovered if the cells were incubated at 30 °C for 1 h in a moderate salinity stress (MSS) solution which consists of Davis minimal medium with 5 mM proline and 0.5 M NaCl. Then, an attempt was made to examine whether MSS treatment is also effective on the activity restoration of proline uptake and growth under high salinity for *E. coli* CSH4 mutants with different combinations of proP, putA, putP, and proU which are related to the transport and metabolization of proline. After MSS treatment, proline uptake was vigorously occurred for the mutants with proline transporter gene *proP* but not for its deficient ones. For the expression of proline uptake activities of these mutant strains after MSS treatment, PO_4^{3-} in MSS solution is more important than K^+. No growth of strain CSH4 and its mutants without MSS treatment was observed, when cultured in high osmotic medium G (0.8 M NaCl) consisting of 1 mM glycine betaine and Davis minimal medium without potassium phosphate supplemented. After MSS treatment, however, mutant strains lacking *proP* showed sufficient growth in medium G. Cell growth of *proP*$^+$ strains was recognized if MSS treatment was performed in the absence of proline. In conclusion, growth of mutant strains under high-salinity medium G depended on their amount of proline accumulated during MSS treatment, in which K^+ and PO_4^{3-} might play a key role to guarantee their sufficient growth.

[**Key words:** Moderate salinity stress; *Escherichia coli* CSH4 and its mutants; Proline uptake; Cell growth; High salinity]

When bacteria are subjected to an osmotic upshock, rapid outflow of water from the cells is recognized to increase the intracellular osmotic pressure as a quick response (1, 2). To overcome such hyper-osmotic conditions which are detrimental and lethal to any living cells, bacteria remain hydration of water by accumulating osmoregulatory solutes termed as compatible solutes such as K^+, amino acids, zwitterionic organic solutes, and polyols (3) up to high cytoplasmic levels, since they have no interference on the cellular activities (4, 5). Primary response to high osmolarity in *Escherichia coli* is the accumulation of K^+ together with a concomitant increase in the glutamate concentration in the cytoplasm (6). As a secondary response, *E. coli* cells accumulate large amounts of compatible solutes if they are supplied in the medium and simultaneously trehalose is synthesized in the cells (7). However, when the external concentration of NaCl is elevated over 1 M, the ability of osmoregulation in non-halophilic *E. coli* is weakened and ultimately their cellular activities such as division, transport and respiration are inhibited (8). Even if the inhibited cellular activities were recovered by the supplement of compatible solutes (9, 10), they are still lower than the normal levels in the absence of NaCl (11).

According to the previous reports, it was noted that the short-time incubation (30–60 min) of *E. coli* cells under rather mild condition of salinity stress (0.4–0.6 M NaCl) prior to the exposure under high salinity (>1 M NaCl) conferred some osmotic tolerance (12, 13). Ishida et al. (14) pointed out that the induction of the osmotolerance in *E. coli* appeared to be closely linked to the effective accumulation of compatible solutes during this short-time incubation. Interrelation between induction of osmotic tolerance and the role of transporters to take solutes up into cell cytoplasm, however, has not been analyzed until now, so far as we know. In this connection, it seems to be of interest to examine what happened in the activities of these transport and metabolization systems during the induction process to acquire the osmotic tolerance.

Proline has been known as one of the representative compatible solutes distributed in wide range of eubacteria (2, 3, 11). Adjusting the focus on the role of proline in non-halophilic *E. coli* in this study, we examined the effect of moderate salinity stress (MSS) treatment on the restoration of activities. For this purpose, we used *E. coli* CSH4 and its proline related mutants, in which they have

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different combinations of proP (proline transporter (solute/ion co-transporter)), putA (proline dehydrogenase), putP (Na^+/proline symporter) and proU (proline transporter). Thus, an attempt was made to examine whether or not strain CSH4 and its mutants acquired the osmotic tolerance after MSS treatment by tracing the changes on the activities of proline uptake as well as cell growth. We also tried to clarify what kinds of components in MSS solution play an important role for the restoration of their activities.

MATERIALS AND METHOD

Medium and growth conditions In this study, we used *E. coli* CSH4 and its mutant strains (15–17) with different combinations of proP (proline transporter (solute/ion co-transporter)), putA (proline dehydrogenase), putP (Na^+/proline symporter) and proU (proline transporter), which are listed in Table 1. The cells were grown aerobically at 30 °C for 16 h in LB medium which consisted of 5 g/l dry yeast extract D-3 (Nihon Pharmaceuticals, Tokyo, Japan), 10 g/l Polypepton (Nihon Pharmaceuticals) and 2 g/l NaCl (pH 7.0).

To clarify the growth ability of mutant strains, we used high osmotic medium G consisting of 0.8 M NaCl, 1 mM glycine betaine as compatible solute and Davis minimal medium without potassium phosphate (KP_i) supplemented. Composition of Davis minimal medium (18) was 7.0 g/l K_2HPO_4, 3.0 g/l KH_2PO_4, 0.1 g/l $MgSO_4$ $7H_2O$, 1.0 g/l $(NH_4)_2SO_4$, 0.5 g/l sodium citrate and 2.0 g/l glucose (pH 7.0). Glucose added into the medium was sterilized separately by filtration through a 0.2-μm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan).

Growth was monitored by measuring turbidity at 600 nm (OD_{600}) using spectrophotometer (Beckman instruments, Fullerton, CA, USA). If the OD_{600} was over 1.0, samples were diluted to appropriate fold with the corresponding culture medium, since linearity was observed at $OD_{600}<1$.

Procedure of MSS (moderate salinity stress) treatment Cells grown in LB medium for 16 h were harvested by centrifugation (10,000×*g*, 5 min, 4 °C) and washed with deionized water. Such cells were suspended in MSS solution which consisted of Davis minimal medium supplemented with 5 mM proline and 0.5 M NaCl (pH 7.0). In addition, we prepared modified MSS solution (I)–(V) by setting up different combination of following components such as KP_i, sodium phosphate (NaP_i), and proline in original MSS solution; MSS (I), (II), (III), (IV), and (V) was prepared by adding NaP_i in place of KP_i, by subtracting KP_i, by subtracting proline, by adding NaP_i and subtracting proline, and by subtracting both proline and KP_i from MSS solution, respectively (see Table 3).

Density of cell suspension was adjusted to 10.0 (OD_{600}) in a final volume of 5 ml of MSS solution and incubation was initiated in a 30 ml flask with 120 strokes/min at 30 °C for 1 h, unless otherwise noted. The usage of such high cell density in the MSS treatment is to assure the non-occurrence of cell proliferation. Proline added into MSS solution was sterilized separately by filtration described above.

The cells after MSS treatment were harvested by centrifugation (10,000×*g*, 5 min, 4 °C) and the pellets were washed with Davis minimal medium containing 0.5 M NaCl. The cells with and without MSS treatment were called as MSS and non-MSS cells in this text, respectively.

Determination of proline and glycine betaine in cells Proline uptake of MSS or non-MSS cells was carried out in Davis minimal medium containing 2 mM proline and 1 M NaCl at 30 °C during 60 min of incubation. Cell density of the mixture was adjusted to 5.0 (OD_{600}). An aliquot of 1 ml of the mixture was withdrawn and centrifuged (10,000×*g*, 5 min, 4 °C) at designated intervals. The supernatant fractions were used for the analysis of extracellular concentrations of proline. Identification and quantification of proline was performed as described previously (11).

To examine the amounts of glycine betaine, an aliquot of 0.2 ml of culture was sampled at a designated incubation time and washed once with the same volume of Davis minimal medium with 0.8 M NaCl. Both supernatant and pellet fractions, the latter being extracted with 80% ethanol (19), were used for the determination of extracellular and intracellular concentrations of glycine betaine, respectively. Glycine betaine was detected at 210 nm using a high-performance liquid chromatography (HPLC) system (L-5020; Hitachi, Tokyo, Japan) with a Bio-Sil C_8 90-5S reversed phase column (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (19).

Chemicals Chemicals used were of reagent or higher grade.

TABLE 1. Strain CSH4 and its mutant strains.

Strain	Genotype	Ref.
CSH4	F⁻ *trp lacZ rpsL thi*	15
RM2	CSH4 △ (*putPA*) *101*	15
JT31	CSH4 *putA1*::Tn5	15
JT34	CSH4 *putP3*::Tn5	15
WG138	CSH4 *proP219*	15
WG170	CSH4 △ (*putPA*) *101 proP219*	15
WG203	CSH4 △ (*putPA*) *101 proP219 proU205*	16
WG207	CSH4 △ (*putPA*) *101 proU205 srl-300*::Tn10	16
WG226	CSH4 *proP219 proU205*	17
WG227	CSH4 *putA1*::Tn5 *proP219 proU205*	17

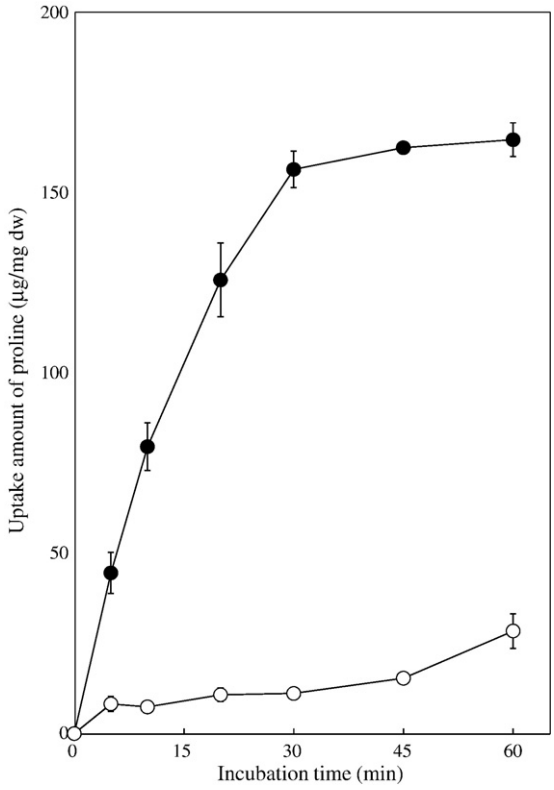

FIG. 1. Effect of MSS treatment on the proline uptake by *E. coli* CSH4 in the presence of 1 M NaCl. Proline uptake was initiated by suspending MSS (closed circles) and non-MSS (open circles) cells in a Davis medium with 2 mM proline and 1 M NaCl. The cells were incubated at 30 °C with 120 strokes/min for 1 h. Amounts of proline were analyzed as described in Materials and methods. The values are the averages ± SD from three independent experiments.

RESULTS AND DISCUSSION

Proline uptake by strain CSH4 and its mutants after MSS treatment An attempt was made to examine whether the uptake activity of proline is affected by treatment of strain CSH4 incubated in MSS solution at 30 °C for 1 h. Strain CSH4 without MSS treatment could not accumulate proline in the presence of 1 M NaCl, but after MSS treatment uptake activity of proline was highly stimulated, that is, proline was taken up into the cell cytoplasm almost linearly until 30 min of incubation (Fig. 1). The amount of proline in the cells resulted in >150 μg/mg dw during 30–60 min of incubation. Present results verified that MSS treatment for strain CSH4 was quite effective for recovery of activity on the proline uptake under high salinity.

Based on the activity enhancement of proline uptake on strain CSH4 by MSS treatment, we examined this procedure is also applicable on the activity restoration of CSH4 mutant strains with different combinations of proP, putA, putP, and proU which are related to transport as well as metabolization of proline (see Table 1). As shown in Table 2, all the mutants without MSS treatment (non-MSS cells) little accumulated proline, at least during 30 min, when they were incubated in the presence of 1 M NaCl at 30 °C. However, when the MSS treatment was carried out for these mutant strains, activities of proline uptake on the *proP*$^+$ strains such as JT34, WG207, JT31 and RM2 were strongly enhanced just like strain CSH4. Simultaneously, the presence of proline dehydrogenase encoded to *putA* gene seemed to support proline uptake, since the amounts of proline accumulated