Importance of the Transcription of Proline Transporter ProP Gene in Quick Adaptation of *Escherichia Coli* Cells under High Salinity

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Abstract

The effect of the transcription of proline transporter genes on the mechanism of quick adaptation of *Escherichia coli* cells under high salinity was analyzed. *E. coli* has three independent proline transporters, namely PutP, ProP and ProU. By using moderate salinity stress method, we made three different types of *E. coli* cells which transcript proline transporter genes: (i) PutP gene; (ii) PutP and ProP genes; (iii) PutP, ProP and ProU genes. Although the growth of type (i) *E. coli* cell was inhibited at 1.2 M NaCl culture condition, type (ii) and (iii) cells showed the recovery of growth. The uptake of proline by *E. coli* as compatible solute was in good accordance with those of cell growth. These data showed that the transcription of proline transporter ProP played an important role in *E. coli* cells adapted with 1.2 M NaCl culture conditions rapidly.

Key Words: *Escherichia coli*, High salinity, Adaptation, Proline transporter, ProP

1. Introduction

Non-halophilic bacteria are, in general, not able to grow at higher salinity unless they synthesize compatible solutes in cells or possess the transporters to take them up in the medium. Such osmoregulatory solutes as potassium ion, glutamate, proline, ectoine, glycine betaine in bacteria have been extensively reported 1-3. Growth of *Escherichia coli* was inhibited in the presence of high NaCl, while it was recovered if the cells rapidly accumulate compatible solute, such as proline 2-4. Although *E. coli* did not grow in the presence of 1 M NaCl in chemically defined medium, growth was recovered when proline is present in medium 3. Proline also showed the recovery of the respiration and glucose uptake in *E. coli* cells under 1 M NaCl condition 2,3.

To transport proline in *E. coli*, three independent proline transporters, PutP, ProP and ProU were reported 3. The PutP system, which is not regulated by the salinity, is required for the transport of proline when it is used as a carbon or nitrogen source 6. The ProP and the ProU systems operate by accumulating proline and/or glycine betaine to high levels, which makes the cells possible to adaptation under high salinity 7.

According to the previous reports, it was noted that the short-time incubation of *E. coli* cells in moderate salinity stress solution (0.4-0.6 M NaCl) conferred some salt tolerance under high salinity (> 1 M NaCl) 8, 9. Nagata et al. pointed out that the induction of the salt tolerance appeared to be closely linked to the proline transporters 10. It seems that a proline transporter expressions affect adaptation of high salinity, but the details are unclear. In this study, we attempted to clarify the mechanism for quick adaptation of *E. coli* cells under high salinity mainly on the effect of the transcription of proline transporters.

2. Materials and Methods

2.1 Organism and culture conditions

*E. coli* K-12 IFO 3301 was utilized in this study. This strain was pre-cultured in a nutrient medium 3. Cell growth was initiated by inoculating pre-culture (0.5 %) in a nutrient medium. The incubation was performed with 120 strokes at 37 °C in a 500 mL flask or 12 × 180 m/m test tube with 50 or 4 mL medium, respectively. Growth was determined by measuring the optical density at 600 nm (OD600) using a spectrophotometer PD-303 (Apel, Saitama, Japan).

2.2 Moderate salinity stress

The method of Nagata et al. was used for moderate salinity stress treatment 10. Cells grown in a nutrient medium for 16 h were harvested by centrifugation at 12,000 x g for 5 min and washed with deionized water. Such cells were suspended in saline solution that consisted of Davis minimal...
medium [11] supplemented with 5 mM proline and 0.5 M NaCl (pH 7.0). Cell density (OD$_{600}$) was adjusted to 1.0 in a final volume of 5 ml by the saline solution and short-time incubation was initiated in a 30 ml flask with 120 strokes/min at 37 °C for 1 h.

### 2.3 Detection of the transcription of proline transporter genes

Total RNA was purified from *E. coli* by RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of the RNA was performed by Thermoscript RT-PCR System (Invitrogen, California, USA). Proline transporters, putP, proP, and proU genes were amplified by PCR with Takara Ex Taq Reaction Kit (Takara Bio, Shiga, Japan) and GeneAmp PCR System 9700 (Applied Biosystems, California, USA). Total PCR reaction volume of 30 μl was composed of 3.0 μl 10X Ex Taq Buffer, 3.0 μl dNTP mixture, 2.1 pmol of each primer, 0.8 units Ex Taq and 1.0 μl *E. coli* cDNA solution containing 0.15 μg μl cDNA. Proline transporters specific oligonucleotide primers (Table 1) designed on the basis of known *E. coli* sequence [12] were used in RT-PCR. At the same time, the amplification of gap gene encoding D-glyceraldehyde-3-phosphate dehydrogenase was performed and the product was used as an internal standard for RT-PCR. The gap gene detective primers were based on a previous report [13] (Table 1). The profile of PCR conditions was as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min.

### 2.4 Determination of proline

To determine the uptake of proline by intact cells, we followed the concentration changes of proline outside the cells in the incubation mixture medium that contained 1 mM proline and centrifuged at 12,000 x g for 5 min producing a cell-free supernatant. For the measurements of free proline in the cell-free supernatant, the method of Sasaki *et al.* was used [14]. Reduction of proline in medium was regarded as accumulation in cells.

### 3. Results and Discussion

#### 3.1 Effect of moderate salinity stress on the transcription of proline transporters

For a functional analysis of the transporter system, the method of moderate salinity stress treatment was done to make different types of *E. coli* cells which transcript proline transporter genes. Cell growth was done after pre-culturing for 1 h in nutrient medium, Davis medium, Davis medium with 0.5 M NaCl, or Davis medium with 0.5 M NaCl and 5 mM proline. RT-PCR analysis showed that transcription of the PutP gene was observed in all pre-culture conditions (Fig.1). The ProP gene transcription was observed in cells pre-cultured in the Davis medium, the Davis medium with 0.5 M NaCl, or the Davis medium with 0.5 M NaCl and 5 mM proline. The ProU gene transcription was observed in cells pre-cultured in the Davis medium with 0.5 M NaCl or the Davis medium with 0.5 M NaCl and 5 mM proline. By using a method of moderate salinity stress treatment, we succeeded in making three different types of *E. coli* cell which transcript proline transporter genes: (i) PutP gene; (ii) PutP and ProP genes; (iii) PutP, ProP and ProU genes.

### Table 1

List of primers used for reverse transcription polymerase chain reaction (RT-PCR).

<table>
<thead>
<tr>
<th>Code</th>
<th>Direction</th>
<th>Sequences (from 5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For PutP gene detection</td>
<td></td>
<td>TAACGCCTTAACATGGCGGG</td>
</tr>
<tr>
<td>PutP-F</td>
<td>Forward</td>
<td>TAACGCCTTAACATGGCGGG</td>
</tr>
<tr>
<td>PutP-R</td>
<td>Reverse</td>
<td>AATGGCGACAAAGGTCAGCC</td>
</tr>
<tr>
<td>For ProP gene detection</td>
<td></td>
<td>CCGAGGTAAGGGCCCCGATAAT</td>
</tr>
<tr>
<td>ProP-F</td>
<td>Forward</td>
<td>TTTCTGATTCGACCGCTTGG</td>
</tr>
<tr>
<td>ProP-R</td>
<td>Reverse</td>
<td>CGAGGTAAGGGCCCCGATAAT</td>
</tr>
<tr>
<td>For ProU gene detection</td>
<td></td>
<td>ATCATGGAATATCCGGGCTC</td>
</tr>
<tr>
<td>ProU-F</td>
<td>Forward</td>
<td>ATCATGGAATATCCGGGCTC</td>
</tr>
<tr>
<td>ProU-R</td>
<td>Reverse</td>
<td>GCTGACACTCGGTGAGATTA</td>
</tr>
<tr>
<td>For gap gene detection</td>
<td></td>
<td>ATCAAGCGGTTTGGCGCTAT</td>
</tr>
<tr>
<td>gapA1</td>
<td>Forward</td>
<td>ATCAAGCGGTTTGGCGCTAT</td>
</tr>
<tr>
<td>gapA2</td>
<td>Reverse</td>
<td>GTTGATAACTTTAGGCCACGCGG</td>
</tr>
</tbody>
</table>

#### Fig.1

Transcription of the *E. coli* K-12 PutP, ProP and ProU genes of the cells cultured in (a) nutrient medium, (b) Davis medium, (c) Davis medium with 0.5 M NaCl and (d) Davis medium with 0.5 M NaCl and 5 mM proline. They were analyzed by reverse transcription polymerase chain reaction (RT-PCR). Amplification of gap gene was performed and the product was used as an internal standard of RT-PCR. PCR products were separated on 1.0 % agarose gel and the bands were visualized using ethidium bromide staining.
The effect of NaCl on the growth of *E. coli* K-12 in the nutrient medium was examined. In the absence of NaCl, sufficient growth was observed. Strain K-12 showed an adaptation towards a wide range of NaCl concentrations. Distinct decrease in growth yield was observed in the presence of 1.2 M NaCl. The impaired growth of *E. coli* in the presence of 1.2 M NaCl, however, was recovered when pre-cultured in the Davis medium, the Davis medium with 0.5 M NaCl, or the Davis medium with 0.5 M NaCl and 5 mM proline for 1 h. The uptake of proline by *E. coli* as a compatible solute was in good accordance with those of cell growth. Rapid uptake of proline was observed for cells pre-cultured in all other three medium, that is, concentration of proline in cells was about 103-115 nmol/mg d.w. at 2 h cultivation.

3.2 Effect of proline transporters on growth and proline uptake

The effect of NaCl on the growth of *E. coli* K-12 in the nutrient medium was examined (Fig. 2). In the absence of NaCl, sufficient growth was observed. Strain K-12 showed an adaptation towards a wide range of NaCl concentrations. Distinct decrease in growth yield was observed in the presence of 1.2 M NaCl. The impaired growth of *E. coli* in the presence of 1.2 M NaCl, however, was recovered when pre-cultured in the Davis medium, the Davis medium with 0.5 M NaCl, or the Davis medium with 0.5 M NaCl and 5 mM proline for 1 h (Fig. 3).

The uptake of proline by *E. coli* as a compatible solute was in good accordance with those of cell growth (Fig. 4). Rapid uptake of proline was observed for cells pre-cultured in the Davis medium, the Davis medium with 0.5 M NaCl, or the Davis medium with 0.5 M NaCl and 5 mM proline. Under the 1.2 M NaCl culture condition, insignificant accumulation of proline was observed in *E. coli* cells pre-cultured in nutrient medium and reached about 6 nmol/mg d.w. at 2 h of incubation. On the other hand, proline was greatly accumulated in cells pre-cultured in all other three medium, that is, concentration of proline in cells was about 103-115 nmol/mg d.w. at 2 h cultivation.
Although growth and proline uptake of type (i) \(E. \text{coli}\) cell was inhibited at 1.2 M NaCl culture condition, type (ii) and (iii) cells showed the recovery of growth and rapid accumulation of proline. These results suggested that ProP played an important role in \(E. \text{coli}\) cells adapted with 1.2 M NaCl culture conditions rapidly. ProP transporter enables the accumulation of high concentration of proline in the cells. ProU transporter was induced by high salinity, but did not participate in quick correspondence. This system may participate in the later sustained adaptation. PutP system did not affect the mechanism of quick adaptation with high salinity.

3.3 Adaptation mechanism of \(E. \text{coli}\) cell to high salinity using proline

The primary response to high salinity in \(E. \text{coli}\) cell is the accumulation of \(K^+\) and glutamate\(^{16}\). \(K^+\) was accumulated through the Kdp and Trk systems\(^5\). When compatible solutes such as proline or betaine present in extracellular environments, \(K^+\) loaded cells release \(K^+\) through the Kef system and deplete glutamate\(^{16}\). The secondary response is accumulation of large amount of compatible solutes through the respective solute transporters\(^2-4\). One of the main compatible solute, proline is accumulated in \(E. \text{coli}\) cells through the salinity regulated proline transporters ProP and ProU. Expression of ProP is stimulated several-fold during saline upshock\(^7\). ProP activity is stimulated by exogenous \(K^+\)\(^{17}\). Expression of ProU is induced by elevated salinity and \(K^+\) accumulated in cells\(^8\). This study showed expression of ProP played an important role to adapt with high salinity rapidly than that of ProU. Proline itself functions as compatible solute rather than the participation of metabolization in cells under high salinity\(^4\). Although proline exists as free amino acid in cells\(^3\), the protection effects to protein or DNA by the free proline is not yet understood.

Acknowledgements

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大腸菌の迅速な高塩環境適応におけるプロリン輸送系タンパク質
ProP 遗伝子の転写の重要性

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要 旨

大腸菌細胞の迅速な高塩環境適応におけるプロリン輸送系タンパク質遺伝子の転写の効果について検討した。大腸菌には PutP、ProP および ProU という 3 種類のプロリン輸送系タンパク質が存在する。温和な塩ストレス処理法により、それらの転写の異なる大腸菌細胞を作製した。PutP のみを転写した大腸菌細胞は 1.2 M NaCl の濃度下において増殖は強く阻害されたが、PutP/ProP、あるいは PutP/ProP/ProU を転写した細胞は、PutP のみを転写した細胞と比較して迅速な増殖を示した。また、浸透圧調整のための補償塩質であるプロリンの細胞内への取込みも、PutP のみを転写した大腸菌細胞では緩やかであったが、PutP/ProP、あるいは PutP/ProP/ProU を転写した細胞では、急速な取込みを示した。以上の結果から、大腸菌細胞の迅速な高塩環境適応にはプロリン輸送系タンパク質遺伝子 ProP の転写が重要である事が示唆された。

キーワード：大腸菌，高塩環境適応，プロリン輸送系タンパク質，ProP

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