Cloning of Stress Tolerance Gene in *Torulaspora delbrueckii* No. 3110

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A cryptic plasmid (pSRY1) was found in *Torulaspora delbrueckii* No. 3110. A plasmid vector (pSRY21) was constructed by ligating it to pPRI-RIM-C ORF with a cycloheximide resistance gene (RIM-C). The transformation efficiency of the strain No. 3110 with pSRY21 by the protoplast-PEG method was increased by 3000-fold by treatment of the protoplasts with polylysine [poly(e-L-lysine)].

Previously we have reported a mutant of No. 3110, T1, which can neither assimilate nor accumulate trehalose. The mutant was salt- and temperature-sensitive. We cloned a DNA fragment from No. 3110, which, when introduced, complemented these mutant characters of T1. These results suggested an important role of metabolism of trehalose for the stress tolerance.

Key words: polylysine; cycloheximide; stress tolerance; trehalose; transformation

*Torulaspora delbrueckii* No. 3110 is a salt-tolerant and highly ethanol-producing yeast. The strain constitutively accumulated trehalose intracellularly and assimilated exogenous trehalose. We obtained a mutant, T1, not able to assimilate exogenous trehalose or accumulate trehalose. T1 was salt sensitive as to growth rate and ethanol productivity. The intracellular accumulation and metabolism of trehalose was suggested to be important for the expression of salt tolerance.

For a further genetic study, we here developed an efficient transformation procedure by using polylysine [poly(e-L-lysine)]. Polylysine, which can bind DNA with strong and noncovalent force, has been used in some DNA transformation experiments in cooperation with other materials, such as glycoprotein–polylysine conjugate, transferrin–polylysine conjugate, and lipopolylysine.

Materials and Methods

Strains and plasmids. *Torulaspora delbrueckii* No. 3110 was isolated from a natural source as a highly ethanol-producing yeast. Strain T1 was isolated as a trehalose non-accumulating and non-assimilating mutant of the strain No. 3110. Plasmid pPRI-RIM-C ORF was constructed by Takagi et al.⁶

Polylysine-mediated transformation. Yeast strains were cultivated in 100 ml of MY medium [0.3% malt extract (Difco), 0.3% yeast extract (Difco), 0.5% Polypepton (Nihon Pharmaceutical Co.), 1% glucose, pH 5.5] for 16 h at 28°C in a 500-ml Erlenmeyer flask. One ml of the culture was transferred into a fresh 100 ml of MY medium and incubated for 5 h at 28°C. After they were washed and collected, cells were suspended in SED buffer (1 ml sorbitol, 50 mM diithiothreitol, 25 mM EDTA, pH 8.0), kept at 30°C for 10 min, and suspended in 20 ml of 1 M sorbitol. One ml of zymolyase 20T (Seikagakukogyo Co.) was added to the cell suspension and the mixture was incubated at 30°C for 20 min. Protoplasts were harvested, washed with, and suspended in 20 ml of STC buffer (1 M sorbitol, 10 mM Tris, 10 mM CaCl₂, pH 8.0). Ten µl of a 10-fold concentration of polylysine (Sigma, molecular weight: from 1000 to 4000 unless designated) and then ten µl of DNA solution were added to 80 µl of the protoplast suspension. After 15 min one ml of PEG solution (1 M sorbitol, 20% PEG 8000, 10 mM Tris–HCl, 10 mM CaCl₂, pH 8.0) was added to the suspension and it was kept for 10 min at room temperature. Harvested protoplasts were suspended in SOS (1 M sorbitol and 10 mM CaCl₂ in MY medium) and incubated at 30°C for 2 h. In development of polylysine-mediated transformation of No. 3110, cells were laid on a selective MY plate containing 1 M sorbitol and 30 mg/liter cycloheximide by a soft agar method and incubated at 28°C for 5 days.

Construction of a plasmid. Digestion with restriction enzymes (Takara Shuzo Co.) and ligation with a T4 ligase (Takara Shuzo Co.) were done by the methods recommended by the manufacturer. Plasmid pSRY1 was prepared from total DNA of No. 3110 by the CsCl-ultracentrifugation method.

Cloning of tna (trehalose non-assimilating) gene. Total DNA of the strain No. 3110, which was prepared by the CsCl-ultracentrifugation method, was digested with SpII and ligated with the SpII-digested pSRY21. The polylysine-mediated transformation was done with the ligated DNA and a recipient strain T1. Transformed cells were laid on a selective YNB plate (0.67% Yeast Nitrogen Base (Difco), 1% trehalose, 1 M sorbitol, 30 mg/liter cycloheximide, 2% agar) by a soft agar method, and incubated at 28°C for 5 days. Plasmid DNA, pTNA, from a cycloheximide-resistant and trehalose-assimilating transformant, was prepared by the CsCl-ultracentrifugation method.

Results and Discussion

Construction of a host-vector system

A 5.5-kb low copy number plasmid (pSRY1) was found in the strain No. 3110. The physical map of the plasmid is shown in Fig. 1.

The drug sensitivity of No. 3110 was measured using a plate assay for some antibiotics widely used in yeast genetics, such as G418, (minimal inhibitory concentration was 500 mg/liter), methotrexate (more than 100 mg/liter), chloramphenicol (more than 500 mg/liter), and cycloheximide (10 mg/liter). The strain was most effectively sensitive to cycloheximide.

To insert a cycloheximide resistance gene, a β-lactamase gene, and the replication origins (ARS of *Saccharomyces cerevisiae* and the ori of *Escherichia coli*), BamHI-digested pSRY1 was ligated with the BlII-digested pPRI-RIM-C ORF (harboring a cycloheximide resistance gene, RIM-C) (Fig. 1). The constructed plasmid (pSRY21) could replicate and functioned for the resistance gene in No. 3110 cells, but the transformation efficiency by the protoplast PEG-mediated method⁹ was low (0.3 colonies/µg of DNA).

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![Diagram of plasmid pSRY21](image)

**Fig. 1.** Construction Scheme of the Vector Plasmid pSRY21. A BamH1-digested pSRY1 and a BgII-digested pPR1-RIM-C ORF were ligated.

**Fig. 2.** Effects of Polylysine on the Transformation Frequency of No. 3110.

Polylysine-treated cells were transformed with one μg of pSRY21 as described in Materials and Methods. Colony number of the cycloheximide-resistant strains was counted.

A. Pretreatment and posttreatment. Symbols: ○, polylysine-treated before the addition of the plasmid DNA; ●, treated after the addition.

B. Molecular weight of polylysine. Symbols: ○, molecular weight 1000–4000; □, 4000–15,000; △, 15,000–30,000.

C. Effects of Ca²⁺. Transformation was done in the presence of 10 mM (○) and absence of CaCl₂ (●).

**Development of polylysine-mediated transformation in No. 3110**

The effect of polylysine on the transformation efficiency of No. 3110 was examined. Concentrated polylysine solution was added to the protoplast suspension just before the addition of the DNA. About 10 mg/liter of polylysine gave the highest transformation efficiency (Fig. 2A). Higher concentrations caused cell death and lower concentrations did not stimulate the transformation efficiency. The addition of plasmid DNA before the polylysine treatment resulted in a less efficiency.

We examined the effects of the molecular weight of polylysine on the transformation efficiency. When higher molecular weight polylysine was used, a larger amount was necessary for the full effect (Fig. 2B). Polylysine might function on the level of numbers of molecules rather than the numbers of cationic charges.

Transformation in the absence of Ca²⁺ required a higher concentration of polylysine (Fig. 2C). Ca²⁺ and polylysine were synergistic and probably their function seemed to be similar. Polylysine might possibly be attached to the cell membrane and neutralize the negative electricity. Polylysine on the cell membrane might adsorb the negatively charged DNA plasmid and stimulate its intake into the cell.

**Cloning of tna gene**

For a genetic study of the mechanism of stress tolerance, we cloned a DNA fragment in No. 3110, which complemented the mutant character (tna: trehalose non-assimilating) of T1.

By using the plasmid pSRY21 and the polylysine-
mediated transformation system, we obtained the plasmid pTNA, which had 4.0-kb SphI-digested DNA in the SphI site of pSR21 and conferred trehalose-assimilating ability on the mutant T1. A physical map of the cloned region is given in Fig. 3.

The intracellular concentration of trehalose in the transformant was measured (Table), showing that the transformant accumulated trehalose, though less than No. 3110.

Two BamHI-digested sub-fragments (1.7 and 2.3-kb) of the 4.0-kb DNA were ligated respectively with the larger fragment of BamHI- and SphI-digested pSR21. The former conferred trehalose-assimilating ability on T1.

### Stress tolerance of the transformant

We did an ethanol fermentation test under two stress conditions [salt stress (1 M NaCl) and temperature stress (38°C)] and under a control condition (28°C without NaCl) (Fig. 4). The mutant T1 produced ethanol at about 60% of the level of No. 3110 under the control conditions, and fermentation rate was strongly inhibited under two stress conditions. The transformant, T1(pTNA), produced ethanol salt- and temperature-tolerantly. But in less aerobic conditions (70 rpm, 150 ml) fermentation was inhibited, a phenomenon not seen in No. 3110 and T1.

The plasmid pTNA complemented the defect of trehalose metabolism and stress sensitivity of the mutant T1. The results strongly suggest the important role of trehalose metabolism for stress tolerance.

<table>
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<tr>
<th>Table</th>
<th>Concentration of Intracellular Trehalose</th>
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<tr>
<td>Strain</td>
<td>Intracellular trehalose (% of dry cell weight)</td>
</tr>
<tr>
<td>No. 3110</td>
<td>10.5</td>
</tr>
<tr>
<td>T1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>T1 (pTNA)</td>
<td>4.8</td>
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Concentration of intracellular trehalose was measured by the method described elsewhere. \(^\text{13}\)

Fig. 4. Ethanol Fermentation.

Ethanol fermentation tests of the strains were done by a method described elsewhere. \(^\text{13}\) Aerobic conditions as well as a less aerobic one was tested. A: 150 ml of medium in a 250-ml Erlenmeyer flask with rotary shaking at 70 rpm. B: 75 ml of medium at 100 rpm. Two stress conditions (\(\square\), at 38°C; \(\Delta\), in initial addition of 1 M NaCl at 28°C) and a control condition (\(\bigcirc\), at 28°C) were tested.

Fig. 3. Physical Map of the Cloned Region.

The thick bar shows the region conferring trehalose-assimilating ability on T1.
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References