Gene Dosage Effect of L-Proline Biosynthetic Enzymes on L-Proline Accumulation and Freeze Tolerance in Saccharomyces cerevisiae

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We have previously reported that L-proline has cryoprotective activity in Saccharomyces cerevisiae. A freeze-tolerant mutant with L-proline accumulation was recently shown to carry an allele of the PRO1 gene encoding γ-glutamyl kinase, which resulted in a single amino acid substitution (Asp154Asn). Interestingly, this mutation enhanced the activities of γ-glutamyl kinase and γ-glutamyl phosphate reductase, both of which catalyze the first two steps of L-proline synthesis and which together may form a complex in vivo. Here, we found that the Asp154Asn mutant γ-glutamyl kinase was more thermostable than the wild-type enzyme, which suggests that this mutation elevated the apparent activities of two enzymes through a stabilization of the complex. We next examined the gene dosage effect of three L-proline biosynthetic enzymes, including Δ1-pyrroline-5-carboxylate reductase, which converts Δ1-pyrroline-5-carboxylate into L-proline, on L-proline accumulation and freeze tolerance in a non-L-proline-utilizing strain. Overexpression of the wild-type enzymes has no influence on L-proline accumulation, which suggests that the complex is very unstable in nature. However, co-overexpression of the mutant γ-glutamyl kinase and the wild-type γ-glutamyl phosphate reductase was effective for L-proline accumulation, probably due to a stabilization of the complex. These results indicate that both enzymes, not Δ1-pyrroline-5-carboxylate reductase, are rate-limiting enzymes in yeast cells. A high tolerance for freezing clearly correlated with higher levels of L-proline in yeast cells. Our findings also suggest that, in addition to its cryoprotective activity, intracellular L-proline could protect yeast cells from damage by oxidative stress. The approach described here provides a valuable method for breeding novel yeast strains that are tolerant of both freezing and oxidative stresses.
 MATERIALS AND METHODS

Yeast and bacterial strains. The S. cerevisiae strains used in this study are described in Table 1. Strain MB329-17C was derived from a cross between S288C and Σ1278b (34). An L-azetidine-2-carboxylic acid (AZC)-resistant mutant strain, FH515, with higher levels of intracellular L-proline was isolated from strain MB329-17C after ethyl methanesulfonate mutagenesis (31). In this study, put1 gene disruptant strain INVDput1 was constructed from strain INVSc1 (Invitrogen, Carlsbad, Calif.), which is the wild-type strain with an S288C background. E. coli strain DH5α [F− λ− phoA ΔZΔMΔ5 ΔlacZYA argF169 deoR recA1 endA1 hsdR17 (rK+/M−)] was used to construct the expression plasmids for the yeast genes.

Plasmids. Yeast episomal plasmids pAD4, pTV3, and pUV2 (supplied by J. Nikawa) (24), all of which are S. cerevisiae-E. coli shuttle vectors containing the bacterial ampicillin resistance gene and the LEU2, TRP1, and URA3 genes, respectively, were used for complementing the auxotrophic markers and for expressing the PRO1, PRO2, and PRO3 genes, respectively, in S. cerevisiae. Plasmid pAD4 contains the S. cerevisiae ADH1 promoter and terminator regions. Plasmid pCH33 (supplied by S. Harashima) was used for disruption of the PUT1 gene.

Culture media. The media used for growth of S. cerevisiae were SD (2% glucose, 0.67% Bacto Yeast Nitrogen Base without amino acids; Difco Laboratories, Detroit, Mich.) and YPD (2% glucose, 1% Bacto Yeast Extract, 2% Bacto Peptone). The SD medium contains ammonium sulfate (0.1%) as the nitrogen source. When appropriate, required supplements were added to the media for auxotrophic strains. Yeast strains were also cultured on SD agar plates containing an L-proline analogue, AZC (Sigma Chemical Co., St. Louis, Mo.). The E. coli recombinant strains were grown in Luria-Bertani medium (26) containing ampicillin (50 μg/ml). When necessary, 2% agar was added to solidify the medium.

Disruption of the PUT1 genes. The enzymes used for DNA manipulation were obtained from Takara Shuzo (Kyoto, Japan) and were used under the conditions recommended by the supplier. Conventional techniques were used for DNA manipulation and transformation as described previously (24). The DNA fragment containing the Candida glabrata HIS3 gene was amplified by PCR with plasmid pCgHIS3 and oligonucleotide primers 5′-TGG CAA TTC TCT TTC GGC AAT GGC TTT CCG GTT ACC ACG CGT TGT AAA ACG ACG GCC AGT-3′ and 5′-TAA GCC TGA CGA CGA CAA GCC ACT TTA CCA CGA ATT TAG GCA CAG GAA ACA GCT ATG ACC-3′ (the underlining indicates the sequences 322 bp upstream of the ATG initiation codon and 287 bp downstream of the TGA termination codon of the PUT1 gene, respectively). The unique amplified band of 2.0 kb containing the C. glabrata HIS3 gene was purified and then integrated into the PUT1 locus in strain INVSc1 by transformation. The resultant put1 disruptant, INVDput1, was selected from among several His+ transformants, and the correct disruption was verified by chromosomal PCR analysis.

Construction of plasmids for expression of the PRO1, PRO2, and PRO3 genes.

![Diagram](image-url)

**FIG. 1.** Biosynthesis and metabolism of L-proline in *Saccharomyces cerevisiae*. Genes encoding particular enzymes are shown in parentheses.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or strain (plasmids)</th>
<th>Background and/or description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB329-17C</td>
<td>α trp1 ura3-52 put1-54 PRO1</td>
<td>S288C and Σ1278b</td>
</tr>
<tr>
<td>FH515</td>
<td>α trp1 ura3-52 put1-54 mutated PRO1</td>
<td>MB329-17C, L-Proline-accumulated mutant</td>
</tr>
<tr>
<td>INVSc1</td>
<td>a his3ΔΔ1 leu2 trp-289 uri3-52</td>
<td>INVSc1, wild type</td>
</tr>
<tr>
<td>INVSc1</td>
<td>a his3ΔΔ1 leu2 trp-289 uri3-52 put1::CgHIS3</td>
<td>INVSc1, put1 disruptant, Leu Trp Ura</td>
</tr>
<tr>
<td>INV-WT</td>
<td>INVSc1 (pAD4, pTV3, pUV2, pHV1)</td>
<td>INVSc1, put1 disruptant</td>
</tr>
<tr>
<td>INV-Dput1-WT</td>
<td>INV-Dput1 (pAD4, pTV3, pUV2)</td>
<td>High-copy PRO1</td>
</tr>
<tr>
<td>INV-Dput1-W1</td>
<td>INV-Dput1 (pAD-WTPRO1, pTV3, pUV2)</td>
<td>High-copy mutated PRO1</td>
</tr>
<tr>
<td>INV-Dput1-M1</td>
<td>INV-Dput1 (pAD-D154NPRO1, pTV3, pUV2)</td>
<td>High-copy mutated PRO1, PRO3</td>
</tr>
<tr>
<td>INV-Dput1-W12</td>
<td>INV-Dput1 (pAD-WTPRO1, pTV-PRO2, pUV2)</td>
<td>High-copy mutated PRO1, PRO2</td>
</tr>
<tr>
<td>INV-Dput1-M12</td>
<td>INV-Dput1 (pAD-D154NPRO1, pTV-PRO2, pUV-PRO3)</td>
<td>High-copy mutated PRO1, PRO2, PRO3</td>
</tr>
<tr>
<td>INV-Dput1-M2</td>
<td>INV-Dput1 (pAD-D154NPRO1, pTV-PRO2, pUV-PRO3)</td>
<td>High-copy PRO2</td>
</tr>
<tr>
<td>INV-Dput1-W2</td>
<td>INV-Dput1 (pAD4, pTV-PRO2, pUV2)</td>
<td>High-copy PRO2</td>
</tr>
<tr>
<td>INV-Dput1-W3</td>
<td>INV-Dput1 (pAD4, pTV3, pUV-PRO3)</td>
<td>High-copy PRO3</td>
</tr>
</tbody>
</table>

**TABLE 1.** Yeast strains used in this study

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*Note: The table is a simplified representation of the provided information and may not capture all the details presented in the original document.*
in *S. cerevisiae*. To overexpress the wild-type and mutated *PRO1* genes, a DNA fragment of the open reading frame was prepared by PCR with genomic DNA from strains MB329-17C and FH515 and oligonucleotide primers 5′-ACC CAA GCT TGT GTC AGT GGC ACA G-3′ and 5′-ACC CGA GCT CCG AAG ATT TTA ACG GAT CAC A-3′ (the underlining indicates the positions of *HindIII* and *SacI*, respectively). The unique band of 1.9 kb amplified from genomic DNA of MB329-17C and FH515 was digested with *HindIII* and *SacI* and then ligated to the large fragment of pAD4 digested with *HindIII* and *SacI* to construct pAD-WTPRO1 and pAD-D154NPRO1, respectively. The nucleotide sequences of the wild-type and mutated *PRO1* genes were confirmed by DNA sequencing.

To overexpress the *PRO2* and *PRO3* genes, a DNA fragment of the open reading frame was prepared by PCR with genomic DNA from strain INVSc1 and oligonucleotide primers 5′-CCA ACT GCA GTT GTG GGC TTG C-3′ and 5′-ACC CGA GCT CCT GGA AGC TTC CGC C-3′ for the *PRO2* gene and 5′-CCA ACT GCA GCC TAC AAA AGG AGC AGA TC-3′ and 5′-ACC CGA GCT CTT ATC GGA CGG ACC G-3′ for the *PRO3* gene (the underlining indicates the positions of *PstI* and *SacI*, respectively). The unique amplified bands corresponding to 1.7 and 1.1 kb, respectively, were digested with *PstI* and *SacI* and then ligated to the *PstI* and *SacI* sites of pAD4 to construct pAD-PRO2 and pAD-PRO3, respectively. The nucleotide sequences of the *PRO2* and *PRO3* genes were confirmed by DNA sequencing. The 4.0- and 3.4-kb *SacI* fragments from pAD-PRO2 and pAD-PRO3 were then ligated into the *BamHI* site of pTV3 and pUV2 to construct pTV-PRO2 and pUV-PRO3, respectively. These genes were placed under the control of the *S. cerevisiae* *ADH1* promoter and terminator in the resultant plasmids. These plasmids were then introduced into strain INVSc1.

**Enzyme assay.** To determine the activities of γ-GK (EC 2.7.2.11), strains MB329-17C and FH515 were grown in 50 ml of SD medium at 30°C for 30 min to 2 h and then terminated by the addition of 1 ml of stop solution (50 mM Tris base, and the enzyme plus water). The reaction was carried out at 37°C for 30 min to 2 h and then terminated by the addition of 1 ml of stop solution (55 g of FeCl₃·6H₂O, 20 g of trichloroacetic acid, 21 ml of 12 N HCl per liter). Precipitated proteins were removed by centrifugation, and the absorbance at 535 nm was recorded against a blank identical to the one mentioned above but lacking ATP. The amount of γ-glutamyl hydroxamate was measured from the absorbance at 535 nm by comparison with a standard curve of γ-glutamyl hydroxamate (Sigma). One unit of activity was defined as the amount of enzyme required to produce 1 µmol of γ-glutamyl hydroxamate per h.

**RESULTS**

Asp154Asn mutant γ-GK had increased stability. We have previously shown that the allele of *PRO1* enhanced the activities of both γ-GK and γ-GPR, which together may form a complex in vivo (18). It has been suggested that the Asp154Asn substitution in the γ-GK protein causes a stabilization of the complex, which leads to an elevation in the apparent activities of the two enzymes. Therefore, we first examined the thermostabilities and optimum temperatures of the wild-type and Asp154Asn mutant γ-GK enzymes in the crude extracts prepared from strains MB329-17C and FH515 (Fig. 2). The mutant γ-GK had a residual activity that was 1.3- to 1.4-fold
greater than that of the wild-type enzyme under all of the conditions tested (Fig. 2A). The optimum temperature of the mutant \( \gamma \)-GK also increased to 45°C, whereas the wild-type enzyme had maximal activity at 35°C and a remarkable drop in activity was observed when the enzyme was assayed at 45°C (Fig. 2B). These results indicate that the Asp154Asn mutant \( \gamma \)-GK becomes more stable than the wild-type enzyme.

However, these characteristics of \( \gamma \)-GPR enzymes were not analyzed at temperatures above 30°C, because some unexpected contaminants in the crude extracts perturbed the reverse reaction of \( \gamma \)-GPR by a phosphate-dependent reduction of NADP\(^+\) with glutamate-\( \gamma \)-semialdehyde (derived from equilibrium with P5C) as the substrate.

Overexpression of mutant \( \gamma \)-GK and wild-type \( \gamma \)-GPR causes \( L \)-proline accumulation. Enhancement of the apparent \( \gamma \)-GK and \( \gamma \)-GPR activities due to substitution of one amino acid in the \( \gamma \)-GK protein leads to oversynthesis of \( L \)-proline in yeast cells. Therefore, because the rate-limiting step has not been determined yet in \( S. cerevisiae \), one might expect that the increase of enzyme activities involved in the \( L \)-proline biosynthetic pathway due to gene dosage caused the accelerated conversion of \( L \)-glutamate to \( L \)-proline (Fig. 1). We then constructed four high-copy-number plasmids for the \( S. cerevisiae \) PRO1 encoding \( \gamma \)-GK (the wild type and the Asp154Asn mutant), PRO2 encoding \( \gamma \)-GPR, and PRO3 encoding P5CR as described in Materials and Methods. In these plasmids, pAD-WTPRO1, pAD-D154NPRO1, pTV-PRO2, and pUV-PRO3, each gene was expressed under the control of the \( ADH1 \) promoter in \( S. cerevisiae \).

Strain INVDput1, which lacked the proline oxidase required for \( L \)-proline utilization, was transformed with these plasmids or with the vector only as controls. The Leu\(^+\), Trp\(^+\), and Ura\(^+\) transformants were cultivated in SD medium, and the cellular \( L \)-proline levels were examined (Fig. 3). In agreement with the results of our previous report (32), the put1-disrupted strain INVDput1 accumulated higher \( L \)-proline levels (0.10% of the dry weight) than did the control strain INV-WT (<0.05%). \( L \)-Proline content was virtually unchanged in the strains which overexpress the wild-type enzymes (INVDput1-W1, INVDput1-M12, INVDput1-M123, INVDput1-W2, and INVDput1-W3).

In contrast, strain INVDput1-M1 carrying the mutated PRO1 gene showed a prominent fourfold increase in \( L \)-proline content (0.40%), probably due to an increase in enzyme activity. It is noteworthy that when the wild-type \( \gamma \)-GPR was co-overexpressed with the mutant \( \gamma \)-GK, the \( L \)-proline level in strain INVDput1-M12 was approximately 1.5-fold that of strain INVDput1-M1. However, the gene dosage effect of PRO3 on \( L \)-proline accumulation was not significantly observed in strain INVDput1-M123.

Overproduction of \( L \)-proline is believed to dilute the toxic \( L \)-proline analogue AZC, which is incorporated into proteins competitively with \( L \)-proline (28). We examined the growth of
yeast strains on SD agar plates containing toxic AZC (Fig. 3). Strains INVDput1-M1, INVDput1-M12, and INVDput1-M123 clearly showed AZC resistance, whereas the rest of the strains were sensitive to AZC. Also, the increased l-proline level reflects greater resistance to AZC. These results indicate that overexpression of mutant γ-GK and wild-type γ-GPR and disruption of the proline oxidase gene are effective for l-proline accumulation in yeast cells.

**Yeast strains with l-proline accumulation showed higher tolerance to freezing stress.** To test the freeze tolerance, yeast strains were cultured in liquid SD medium. As shown in Fig. 4, in proportion to the cellular l-proline level, strains INVDput1-M1, INVDput1-M12, and INVDput1-M123 exhibited increased cell viability compared with the rest of the strains when the cell suspensions were exposed to freezing at −20°C for 1 day. Prolonged storage of the cells at −20°C caused a gradual loss of freeze tolerance in all of the strains, although a significant cryoprotective effect was observed (data not shown). These results are consistent with the finding that there is a positive correlation between intracellular l-proline levels and resistance to freezing stress in S. cerevisiae (17, 18, 31, 32).

**Intracellular l-proline protects yeast cells from damage by oxidative stress.** The processes of freezing and thawing are known to result in oxidative stress to cells (22). In particular, free radicals and reactive oxygen species are generated and cause oxidative damage to cellular components (23). Also, elevated l-proline in plants has been shown to reduce the levels of free radicals in response to osmotic stress (11). We therefore compared yeast cell viabilities after the addition of 3 mM H₂O₂ to liquid SD medium. Wild-type strain INV-WT contained a trace amount of l-proline (<0.05% of the dry weight), and its cell viabilities at 2 and 4 h after the addition of H₂O₂ decreased dramatically (24.7 and 3.11%, respectively).

In contrast, the typical l-proline-accumulating strain INVDput1-M12 (0.55% of the dry weight) was shown to be much more tolerant of H₂O₂ (cell viabilities of 35.0 and 8.69%, respectively) than was the wild-type strain INV-WT. This finding suggests that a high concentration of l-proline plays a crucial role in protecting yeast cells under oxidative stress.

**DISCUSSION**

To accelerate the conversion of l-glutamate to l-proline, we overexpressed three enzymes (γ-GK, γ-GPR, and P5CR) required for S. cerevisiae l-proline biosynthesis by using high-copy-number plasmids. Interestingly, so long as the wild-type γ-GK was overexpressed, l-proline did not accumulate in the cells overexpressing the wild-type γ-GPR and/or P5CR enzymes. In contrast, it should be noted that a combination of the Asp154Asn mutant γ-GK and the wild-type γ-GPR resulted in an approximately 50% increase in the intracellular content of l-proline. Position 154 in γ-GK may be important for the formation of the γ-GK–γ-GPR complex, and the replacement of Asp154 by Asn may facilitate an intermolecular interaction that stabilizes the complex. This mutation at position 154 in the S. cerevisiae γ-GK is novel in that different mutations in other amino acid residues required for feedback inhibition of γ-GK by l-proline were identified in bacterial and plant genes that have been studied (5, 6, 13, 21, 35). Based on the prediction of the secondary structure by the method of Chou and Fasman (3), Asp154 in the yeast γ-GK protein is believed to be located in a turn-like region between an α-helix (Val147–Phe152) and a β-sheet (Thr157–Thr182), which is probably on the molecular surface. It is possible that mutant γ-GK has a stronger interaction with γ-GPR than does wild-type γ-GK. We now analyze the complex formation or protein–protein interaction of γ-GK and γ-GPR by using a yeast two-hybrid assay. Further, the gene dosage effect of PRO3 on l-proline accumulation was not found (Fig. 3). The PRO3 gene encoding P5CR, which converts P5C into l-proline, is constitutively expressed (2). Also, high expression of the P5CR cDNA in tobacco has not been shown to alter the l-proline level in transgenic plants (30). These results strongly suggest that γ-GK and γ-GPR, not P5CR, are rate-limiting enzymes in l-proline biosynthesis in S. cerevisiae.

Injuries to cells due to freezing can be categorized into two types (8, 16). Low cooling rates cause the osmotic shrinkage of cells. Hydration then occurs, and biological macromolecules and/or membrane components denature. More-rapid freezing does not permit the transport of intracellular water through the membrane and impairs the membrane structure or function as ice crystals form in the cells. Therefore, freezing, desiccation, and osmotic stresses, so-called water stresses, are believed to cause common deleterious damage to the cell membrane and functional proteins (25). It is considered preferable for the natural compounds called cryoprotectants to have the capacity to form strong hydrogen bonds with free water (25).

Using yeast mutants defective in antioxidant functions, Park et al. have reported that superoxide anions formed during the aerobic freezing-thawing process induce oxidative stress and injure yeast cells (23). Osmotic stress also induces free-radical production in plant cells, suggesting that oxidative stress is at least partly responsible for the damage caused to plant cells by...
osmotic stress (11). In general, oxygen-free radicals and other reactive oxygen species could attack vulnerable proteins containing iron sulfur centers (14). It has been proposed that l-proline may act as a free-radical scavenger to protect plants from damage by oxidative stress caused during osmotic stress (11). We found that overexpression of the mutant form of PRO1 could protect yeast cells from oxidative stress during exposure to H2O2. Similar results were obtained when yeast cells were exposed to heat shock treatment at 50°C, which also causes oxidative stress to yeast cells (data not shown). Our findings suggest that, in addition to its cryoprotective activity, l-proline has an important role in reducing the oxidative stress induced during freezing and thawing. l-Proline was found to enhance the stability of proteins and membranes in environments of low water activity or high temperature (4) and to inhibit aggregation during protein refolding. These observations suggest the possibility that l-proline acts as a protein-folding chaperone (27). Hence, l-proline has promising biotechnological potential as a protective agent for industrial microorganisms and enzymes.

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