APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2003, p. 6527–6532 Vol. 69, No. 11
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.11.6527–6532.2003

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

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Received 16 June 2003/Accepted 2 September 2003

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 Δ^1-pyrroline-5-carboxylate reductase, which converts Δ^1-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 Δ^1-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.

Frozen-dough technology has recently been used in the baking industry to supply oven-fresh bakery products to consumers. Many freeze-tolerant yeasts have been isolated from natural sources and have also been constructed by conventional mutation techniques (9, 10, 15, 19, 20). However, the mechanism of freeze tolerance is not well understood, and a baker's yeast that provides good leavening qualities for both sweet- and lean-thawed doughs after frozen storage has not yet been developed.

We previously investigated the cryoprotective effects of amino acids on freezing stress in the yeast *Saccharomyces cerevisiae* and found that L-proline, known as an osmoprotectant (4, 7), has cryoprotective activity that is nearly equal to that of glycerol or trehalose (17, 31). *S. cerevisiae* synthesizes L-proline from L-glutamate catalyzed by three enzymes, γ-glutamyl kinase (γ-GK; the *PRO1* gene product), γ-glutamyl phosphate reductase (γ-GPR; the *PRO2* gene product), and Δ^1-pyrroline-5-carboxylate reductase (P5CR; the *PRO3* gene product), although the rate-limiting step has not yet been determined (Fig. 1). On the other hand, L-proline is converted to L-glutamate within mitochondria by the action of two enzymes, proline oxidase (the *PUT1* gene product) and P5C dehydrogenase (the *PUT2* gene product) (Fig. 1). We also showed that there is a positive correlation between intracellular L-proline levels and resistance to these stresses in *S. cerevisiae*, although there is strain-to-strain variability in freezing tolerance (17, 31, 32).

We therefore isolated a mutant, derived from L-proline analogue-resistant mutants of *S. cerevisiae*, which exhibited both L-proline accumulation and freeze tolerance (31). The mutant was recently found to carry an allele of the *PRO1* gene encoding γ-GK, to have a single amino acid replacement of Asp by Asn at position 154, and to show a prominent increase in both γ-GK and γ-GPR activities (18). In *Escherichia coli*, the γ-GK and γ-GPR enzymes are believed to form a heterodimer to channel the unstable intermediate, γ-glutamyl phosphate, in vivo (1). As reported by Smith et al. (29), γ-GK activity was undetected unless purified γ-GPR had been added to the in vitro assay system. In a moth bean P5C synthetase, which exhibited both γ-GK and γ-GPR activities, a leucine zipper sequence is present in each of the enzymatic domains. Leucine zippers may function intramolecularly to maintain the structure of the two domains of the *Vigna* P5C synthetase, and homodimer or heterodimer formation may occur through the zippers to allow close association between originally separate domains (12). Tomenchok and Brandriss (33) reported that the *S. cerevisiae PRO1* gene complemented the *E. coli proB*-deleted strain, which lacks γ-GK activity, and suggested that *S. cerevisiae* γ-GK can complex with *E. coli* γ-GPR. Our results also suggested that yeast γ-GK and γ-GPR together form a complex to function with each other in vivo (18).

Thus, we report here the gene dosage effect of *PRO1*, *PRO2*, and *PRO3* in the pathway of L-proline biosynthesis on the intracellular L-proline level and freeze tolerance. In addition, a possible mechanism for L-proline accumulation is discussed.

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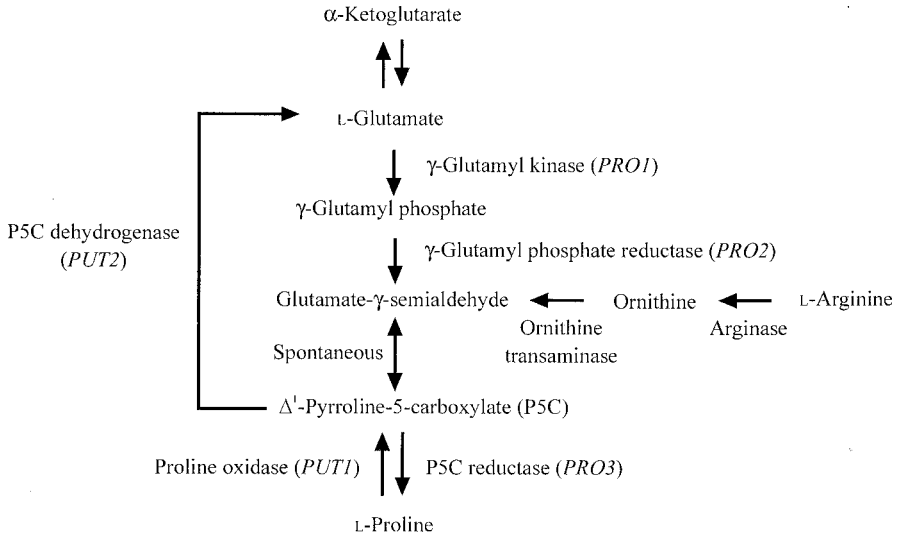

FIG. 1. Biosynthesis and metabolism of L-proline in *Saccharomyces cerevisiae*. Genes encoding particular enzymes are shown in parentheses.

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^- *Φ80lacZΔM15* Δ(*lacZYA argF*)*U169 deoR recA1 endA1 hsdR17*(r_k^- m_k^+) *supE44 thi-1 gyrA96*] 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 pCgHIS3 (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′-TTG GAA TTT CCT 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 CTA CCG 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

TABLE 1. Yeast strains used in this study

Strain	Genotype or strain (plasmids)	Background and/or description
MB329-17C	α *trp1 ura3-52 put1-54 PRO1*	S288C and Σ1278b
FH515	α *trp1 ura3-52 put1-54* mutated *PRO1*	MB329-17C, L-Proline-accumulated mutant
INVSc1	**a** *his3-Δ1 leu2 trp1-289 ura3-52*	S288C, His^- Leu^- Trp^- Ura^-
INVDput1	**a** *his3-Δ1 leu2 trp1-289 ura3-52 put1*::*CgHIS3*	INVSc1, *put1* disruptant, Leu^- Trp^- Ura^-
INV-WT	INVSc1 (pAD4, pTV3, pUV2, pHV1)	INVSc1, wild type
INVDput1-WT	INVDput1 (pAD4, pTV3, pUV2)	INVSc1, *put1* disruptant
INVDput1-W1	INVDput1 (pAD-WTPRO1, pTV3, pUV2)	High-copy *PRO1*
INVDput1-M1	INVDput1 (pAD-D154NPRO1, pTV3, pUV2)	High-copy mutated *PRO1*
INVDput1-W12	INVDput1 (pAD-WTPRO1, pTV-PRO2, pUV2)	High-copy *PRO1*, *PRO2*
INVDput1-M12	INVDput1 (pAD-D154NPRO1, pTV-PRO2, pUV2)	High-copy mutated *PRO1*, *PRO2*
INVDput1-W123	INVDput1 (pAD-WTPRO1, pTV-PRO2, pUV-PRO3)	High-copy *PRO1*, *PRO2*, *PRO3*
INVDput1-M123	INVDput1 (pAD-D154NPRO1, pTV-PRO2, pUV-PRO3)	High-copy mutated *PRO1*, *PRO2*, *PRO3*
INVDput1-W2	INVDput1 (pAD4, pTV-PRO2, pUV2)	High-copy *PRO2*
INVDput1-W3	INVDput1 (pAD4, pTV3, pUV-PRO3)	High-copy *PRO3*

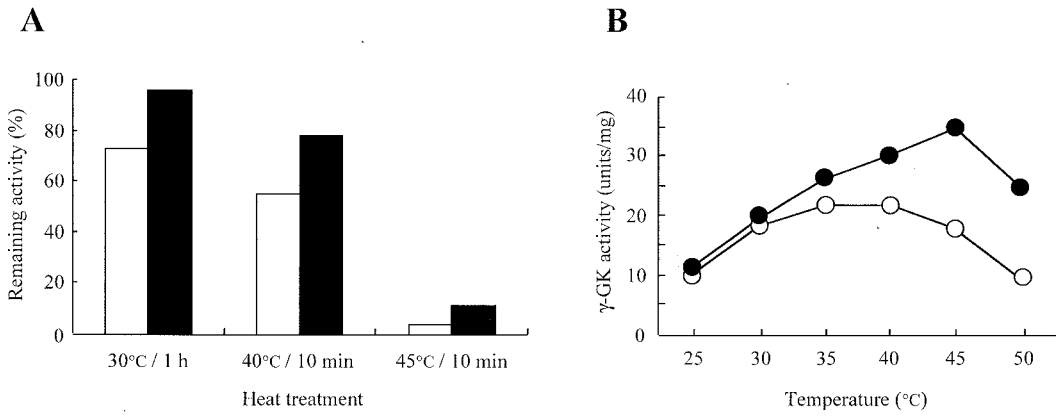

FIG. 2. The Asp154Asn mutant γ-GK is highly thermostable compared with the wild-type enzyme. (A) Thermostability of the wild-type (open bars) and Asp154Asn mutant (filled bars) γ-GK. γ-GK activity remaining after incubation at the indicated conditions was determined at 37°C and was expressed as a percentage of the original activity. The variations in the values were less than 5%. (B) Temperature dependence of the relative enzymatic activity of the wild-type (open circles) and Asp154Asn mutant (filled circles) γ-GK. The values are means of results from three independent experiments. The variations in the values were less than 5%.

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 TTG GTC AGT GGC ACA G-3′ and 5′-ACC CGA GCT CGA AGG ATT TTA ACG GAT CAC A-3′ (the underlining indicates the positions of *Hin*dIII and *Sac*I, respectively). The unique band of 1.9 kb amplified from genomic DNA of MB329-17C and FH515 was digested with *Hin*dIII and *Sac*I and then ligated to the large fragment of pAD4 digested with *Hin*dIII and *Sac*I 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 GCG TTG GGT C-3′ and 5′-ACC CGA GCT CCT TGA AGC TTC CGC C-3′ for the *PRO2* gene and 5′-CCA ACT GCA GCG TAC AAA AGG ACA AGA TC-3′ and 5′-ACC CGA GCT CTT ATC GGA CCG ACG G-3′ for the *PRO3* gene (the underlining indicates the positions of *Pst*I and *Sac*I, respectively). The unique amplified bands corresponding to 1.7 and 1.1 kb, respectively, were digested with *Pst*I and *Sac*I and then ligated to the *Pst*I and *Sac*I 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 *Bam*HI fragments from pAD-PRO2 and pAD-PRO3 were then ligated into the *Bam*HI 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 INVDput1.

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 24 h with shaking. The whole-cell extracts were prepared by vortexing the cells with glass beads. An ammonium sulfate precipitate (70% saturation) of the extracts was then desalted with a PD-10 column (Amersham Pharmacia Biotech, Buckinghamshire, England) and used as the enzyme source. Protein concentrations were determined using a Bio-Rad (Hercules, Calif.) protein assay kit with bovine serum albumin as the standard protein.

The γ-GK activity was assayed by the procedure of Smith et al. (29), and the reaction mixture contained the following in a final volume of 0.25 ml at pH 7.0: 50 mM L-glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM hydroxylamine-HCl, 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 $FeCl_3$-$6H_2O$, 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.

Intracellular contents of L-proline and freeze-tolerance test. In a 500-ml flask, yeast cells were grown to the stationary phase in 50 ml of SD medium at 30°C for 48 h with shaking. For the determination of intracellular L-proline, 5 ml of cell suspension (approximately 5×10^8 cells) was removed and the cells were washed twice with 0.9% NaCl and suspended in 0.5 ml of distilled water. The 1.5-ml microcentrifuge tube containing cells was transferred to a boiling-water bath, and intracellular amino acids were extracted by boiling for 10 min. After centrifugation (5 min at 15,000 × *g*), each supernatant was subsequently quantitated with an amino acid analyzer (L-8500A; Hitachi Co., Tokyo, Japan). L-Proline content was expressed as a percentage of dry weight.

For the freeze-tolerance test, 0.1 ml of cell suspension (approximately 10^7 cells) was stored at −20°C. Under these conditions, it took about 1 h until the cells were frozen, assuming that the cooling rate was low (approximately 0.5 to 1.0°C/min). Samples of the frozen cells were thawed at room temperature for 15 min, serial dilutions in distilled water were prepared, and aliquots were plated on YPD plates. After incubation at 30°C for 2 days, the survival rates were expressed as percentages, which were calculated as follows: [(number of colonies after freezing at −20°C)/(number of colonies before freezing)] × 100.

H_2O_2 tolerance test. Yeast cells were cultured to the exponential growth phase (optical density at 600 nm of 1.0) in 5 ml of SD medium at 30°C with shaking and were exposed to 3 mM H_2O_2 for various times. Before addition of H_2O_2 and at intervals thereafter, 0.1 ml of the culture was removed and diluted in distilled water and aliquots were plated on YPD plates. After incubation at 30°C for 2 days, the survival rates were expressed as percentages, which were calculated as follows: [(number of colonies after addition of H_2O_2)/(number of colonies before addition of H_2O_2)] × 100.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *PRO1*, *PRO2*, *PRO3*, *PUT1*, and *C. glabrata HIS3* genes are M85293, U43565, M57886, M18107, and U31470, respectively.

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

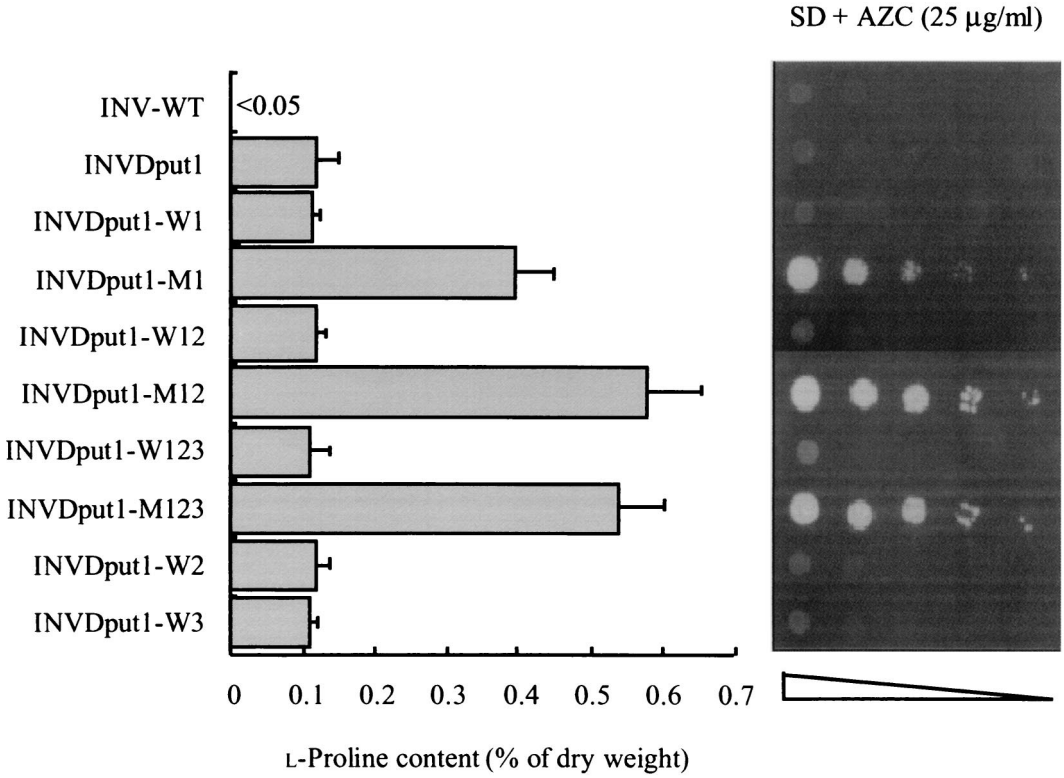

FIG. 3. Intracellular L-proline contents and the growth phenotypes on AZC-containing medium of *S. cerevisiae* strains overexpressing L-proline-biosynthetic enzymes. (Left) Intracellular L-proline content was measured after cultivation in liquid SD medium at 30°C for 2 days. Each bar represents the mean result and standard deviation from five independent experiments. (Right) Approximately 10^6 cells of each strain and serial dilutions of 10^{-1} to 10^{-4} (from left to right) were spotted onto SD plates containing 25 μg of AZC/ml. The plates were incubated at 30°C for 3 days.

greater than that of the wild-type enzyme under all of the conditions tested (Fig. 2A). The optimum temperature of the mutant γ-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 γ-GK becomes more stable than the wild-type enzyme.

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

Overexpression of mutant γ-GK and wild-type γ-GPR causes L-proline accumulation. Enhancement of the apparent γ-GK and γ-GPR activities due to substitution of one amino acid in the γ-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 γ-GK (the wild type and the Asp154Asn mutant), *PRO2* encoding γ-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-WT, INVDput1-W12, INVDput1-W123, 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 γ-GPR was co-overexpressed with the mutant γ-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

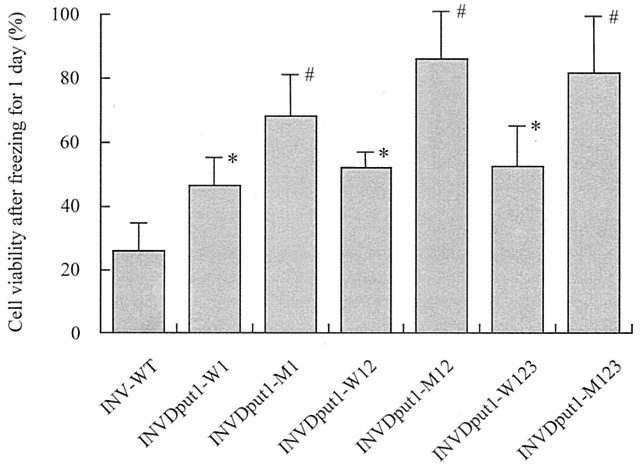

FIG. 4. Freezing stress tolerance of *S. cerevisiae* strains overexpressing L-proline-biosynthetic enzymes. Cell viability was expressed as a percentage of the number of colonies after freezing at −20°C for 1 day relative to the number of colonies before freezing. The total number of cells corresponding to 100% was approximately 10^7. Each bar represents the mean result and standard deviation from three independent experiments. Asterisks indicate significant difference from strain INV-WT by Student's *t* test ($P < 0.05$). Number signs indicate significant difference from strains INV-WT, INVDput1-WT, INVDput1-W12, and INVDput1-W123 by Student's *t* test ($P < 0.05$).

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 H_2O_2 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 H_2O_2 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 H_2O_2 (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 H_2O_2. 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.

ACKNOWLEDGMENTS

We thank S. Harashima (Osaka University, Osaka, Japan) and J. Nikawa (Kyushu Institute of Technology, Fukuoka, Japan) for providing the plasmids. We greatly appreciate the technical assistance provided by Y. Morita and M. Nomura of our laboratory.

This work was supported in part by a grant-in-aid for scientific research to H.T. from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14037262).

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