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N-Acetyltransferase Mpr1 Confers Freeze Tolerance on *Saccharomyces cerevisiae* by Reducing Reactive Oxygen Species

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***N*-Acetyltransferase Mpr1 of *Saccharomyces cerevisiae* can reduce intracellular oxidation levels and protect yeast cells under oxidative stress. We found that yeast cells exhibited increased levels of reactive oxygen species during freezing and thawing. Gene disruption and expression experiments indicated that Mpr1 protects yeast cells from freezing stress by reducing the intracellular levels of reactive oxygen species. The combination of Mpr1 and L-proline could further enhance the resistance to freezing stress. Hence, Mpr1 as well as L-proline has promising potential for the breeding of novel freeze-tolerant yeast strains.**

Key words: *N*-acetyltransferase Mpr1, freeze tolerance, oxidative stress, reactive oxygen species, *Saccharomyces cerevisiae*.

Abbreviations: AZC, L-azetidine-2-carboxylate; DCFDA, 2′,7′-dichlorofluorescin diacetate; ROS, reactive oxygen species.

We previously discovered, in budding yeast *Saccharomyces cerevisiae* Σ1278b, novel genes required for resistance to the L-proline analogue L-azetidine-2-carboxylate (AZC) (*1*). Intriguingly, the *MPR1* and *MPR2* (sigma 1278b gene for proline-analogue resistance) genes were present on chromosomes XIV and X, respectively, of the Σ1278b background strains, but were absent in the sequenced laboratory strain S288C. Although there is a one amino acid change at position 85 between *MPR1* and *MPR2*, the two genes play similar roles in AZC resistance. The *MPR1* gene encodes an *N*-acetyltransferase that detoxifies AZC (*2*). AZC is transported into the cells via L-proline transporters. There it causes the misfolding of proteins into which it is incorporated competitively with L-proline, thus inhibiting cell growth in both prokaryotic and eukaryotic cells. We believe that the *MPR1*-encoded protein (Mpr1) converts AZC into *N*-acetyl AZC and consequently that *N*-acetyl AZC does not replace L-proline during the biosynthesis of proteins (*2*). Homologous genes encode similar acetyltransferases in *S. paradoxus* (*3*) and fission yeast *Schizosaccharomyces pombe* (*4*). Further, genomic PCR analysis showed that most of the *S. cerevisiae* complex species have sequences highly homologous to that of *MPR1* (*3*, *5*). These results suggest that *MPR1* is a "yeast-specific gene" that is widely present in yeast strains.

However, it is unlikely that AZC is a natural substrate of Mpr1 because AZC is only found in some plant species (*6*, *7*). In our search for the physiological function of Mpr1 (*5*), we found that *mpr1*-disrupted cells were hypersensitive to oxidative stress, including H_2O_2 and heat-shock treatments, and contained increased intracellular levels of reactive oxygen species (ROS). In contrast, expression of *MPR1* leads to a decrease in the ROS level and an increase in cell viability after oxidative treatment. These results indicate that Mpr1 plays a crucial role in protecting yeast cells under oxidative stress by reducing the ROS level. We consider that Mpr1 acetylate unidentified intermediate(s) involved in ROS generation in the cytoplasm via an oxidative event. It is also possible that yeast Mpr1 acts as a backup system for antioxidant enzymes.

Recently, the involvement of oxidative stress in freeze-thaw injury to yeast cells was analyzed using mutants defective in antioxidant functions. Park *et al*. have reported that superoxide anions and free radicals were generated in yeast cells during aerobic freezing and thawing (*8*, *9*). Also, an oxidative burst during freezing and thawing has been considered to lead to oxidative damage to many cellular molecules including proteins, lipids and DNA through the generation of ROS (*10*–*12*). These results showed that oxidative stress causes serious injury to yeast cells during freezing and thawing, in addition to physical damage caused by ice nucleation and dehydration (*13*, *14*). Thus, we report here the role of Mpr1 in freezing stress. We found that yeast cells exhibited increased levels of ROS during the freeze-thaw process and that Mpr1 is involved in resistance to freezing stress by reducing the intracellular ROS.

MATERIALS AND METHODS

Strains and Plasmids—The yeast haploid strains and expression plasmids used in this study are listed in Table 1. The *S. cerevisiae* strains with a Σ1278b background were the wild-type strain L5685 supplied by G. Fink and *mpr*-disrupted strain LD1014ura3 (*5*). We also used *S. cerevisiae* strains with a S288C background lacking the *MPR* genes, wild-type strain CKY8 supplied by C. Kaiser and *put1*-disrupted strain INVDput1 (*15*).

Yeast plasmids episomal pMH1 (*1*) and integrative pI-MPR were used for expression of the *MPR1* gene. Plasmid pI-MPR was constructed by cloning of the 5.2-kb *Kpn*I fragment containing *MPR1* from pMH1 into the *Kpn*I site of pRS406 harboring the *URA3* gene. The linearized

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Table 1. **Yeast strains and plasmids used in this study.**

Strain or plasmid	Genotype	Background and/or description
L5685	*a ura3-52 trp1 MPR1 MPR2*	Σ1278b, wild type
LD1014ura3	*a ura3-52 trp1 mpr1::URA3 mpr2::TRP1 ura3*	L5685, *mpr1 mpr2* double disruptant, 5-fluoroorotic acid–resistant mutant (*5*)
CKY8	*α ura3-52 leu2-3,112*	S288C, wild type
INVDput1	*a his3-Δ1 leu2 trp1-289 ura3-52 put1::CgHIS3*	S288C, *put1* disruptant (*15*)
pYES2	2μ origin *URA3*	Episomal plasmid, Invitrogen (Carlsbad, Calif.)
pRS414	*CEN6 TRP1*	Centromeric plasmid, Stratagene (La Jolla, Calif.)
pRS415	*CEN6 LEU2*	Centromeric plasmid, Stratagene
pMH1	*MPR1* in pYES2	Episomal plasmid, High-copy *MPR1* (*1*)
pI-MPR	*MPR1* in pRS406 (*URA3*)	Integrative plasmid pRS406 (Stratagene), One-copy *MPR1*,This study
pAD-D154NPRO1	$pro1^{D154N}$ in pAD4 (2μ origin *LEU2*)	Episomal plasmid, High-copy mutated *PRO1* (*15*)
pTV-PRO2	*PRO2* in pTV3 (2μ origin *TRP1)*	Episomal plasmid, High-copy *PRO2* (*15*)

pI-MPR cut with *Stu*I in *URA3* of pRS406 was introduced for integration of *MPR1* to the *URA3* locus of strain LD1014ura3. *E. coli* strain DH5α [F^-λ-Φ80*lacZ*Δ*M15* Δ(*lacZYA argF*)*U169 deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *supE44 thi-1 gyrA96*] was used to construct plasmid pI-MPR. The 2μ-based high-copy-number plasmids pAD-D514NPRO1 and pTV-PRO2 were used for expressing the mutated *PRO1* and wild-type *PRO2* genes, respectively (*15*). Plasmids pYES2, pRS414, and pRS415, which contain the *URA3*, *TRP1*, and *LEU2* genes, respectively, were used for complementing the auxotrophic markers.

Culture Media—The media used for the growth of *S. cerevisiae* were a synthetic minimal medium SD (2% glucose, 0.67% Bacto-yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI, USA]) and a nutrient medium YPD (2% glucose, 1% yeast extract, 2% peptone). The SD medium contained ammonium sulfate (0.5%) as the nitrogen source. When appropriate, required supplements were added to the media for auxotrophic strains. The *E. coli* recombinant strains were grown in Luria-Bertani medium (0.5% yeast extract, 1% tryptone, 1% NaCl) containing ampicillin (50 μg/ml). For solid media, 2% agar was added.

Measurement of the Intracellular Oxidation Level—The level of intracellular oxidation induced in a cell during freezing and thawing was measured with the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate (DCFDA) (Molecular Probes, Eugene, OR, USA). This probe is trapped inside the cells after cleavage of the diacetate by an intracellular esterase (*16*). It then becomes susceptible to attack by radical species, a more fluorescent compound being produced (*17*).

In 500-ml flasks, the *S. cerevisiae* strains were grown to the stationary phase in 50 ml of SD medium at 30°C with shaking. Yeast cells were resuspended in 40 ml of SD (optical density at 600 nm of 1.0) containing 10 μM DCFDA and then incubated at 30°C for 15 min. The cells were then washed, resuspended in 500 μl of distilled water, and 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 30°C for 5 min and then disrupted with glass beads in a vortex mixer for 5 min. Cell extracts (50 μl) were mixed with 450 μl of distilled water, and then the fluorescence was measured with λ_{EX} = 490 nm and λ_{EM} = 524 nm using a fluorescence spectrophotometer (F4500; Hitachi, Tokyo, Japan). The value of λ_{EM} = 524 nm was normalized as to protein in the mixture.

Freezing Stress Tolerance Test—Yeast cells were cultured to the stationary phase in 50 ml of SD medium at 30°C for 48 h with shaking and then resuspended in 40 ml of SD (optical density at 600 nm of 1.0) in a similar manner to as above, and 0.1 ml aliquots of the cell suspension were stored at –20°C. Before freezing, to confirm the expression of *MPR1*, approximately 10^6 cells of each strain and serial dilutions in distilled water were spotted onto SD plates containing 0.1 mg of AZC/ml (Fig. 1). Samples of the frozen cells were thawed at 30°C for 5 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, calculated as follows: [(number of colonies after freezing at –20°C)/(number of colonies before freezing)] × 100.

Intracellular Contents of L-Proline—Yeast cells were grown to the stationary phase in 50 ml of SD medium at 30°C for 48 h with shaking, 5 ml of the cell suspension was removed, and the cells were suspended in 0.5 ml of distilled water. A 1.5-ml microcentrifuge tube containing the cells was transferred to a boiling-water bath, and then 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). Intracellular L-proline contents were expressed as percentages of dry cell weight.

RESULTS AND DISCUSSION

ROS Is Generated during the Freeze-Thaw Process in Yeast Cells—We first examined the changes in ROS levels in yeast cells during the freeze-thaw process. As shown in Fig. 2A, crude extracts from strain L5685 with the Σ1278b background showed a 2-fold increase in fluorescence after freezing at –20°C for 12 days. The same experiment was performed with strain CKY8 with the S288C background. The mean fluorescence in CKY8 cells was significantly increased (almost 3-fold) after freezing at –20°C for 8 days (Fig. 3A). These results indicate that the freeze-thaw

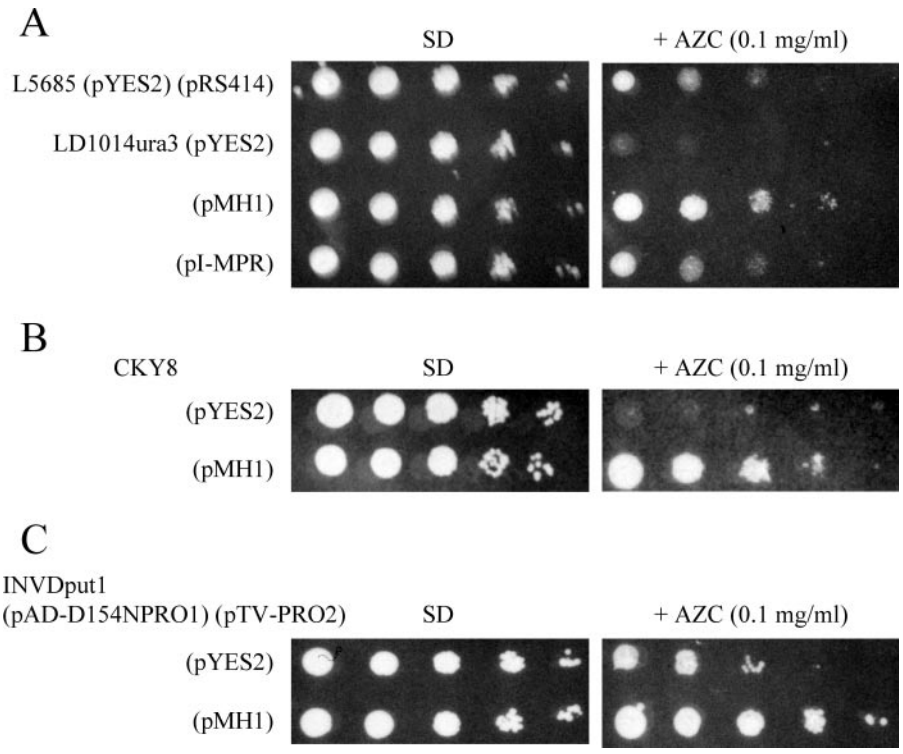

Fig. 1. **The growth phenotypes on AZC-containing medium of *S. cerevisiae* strains expressing *MPR1*.** After cultivation in liquid SD medium at 30°C for 48 h with shaking, approximately 10^6 cells of each strain and serial dilutions of 10^{-1} to 10^{-4} (from left to right) were spotted onto SD plates containing 0.1 mg of AZC/ml.

process (freezing stress) represents toxicity through intracellular ROS generation in addition to deleterious damage to the cell membrane and functional proteins caused by ice crystals or dehydration (*13*, *14*). It was recently reported that the highest levels of free radicals after freezing at –20°C for 2 h and thawing at 4°C for 40 min were detected in yeast cells lacking superoxide dismutases encoded by *SOD1* and *SOD2* directly by electron paramagnetic resonance spectroscopy (*8*). Oxidative damage occurring during freezing and thawing is probably initiated in the cytoplasm by an oxidative burst of superoxide radicals formed from oxygen and electrons leaking from the mitochondrial electron transport chain (*8*). Interestingly, the levels of ROS of strain L5685 possessing the *MPR* genes (Fig. 1A) were approximately 50% lower than those observed in strain CKY8 lacking the *MPR* genes (Fig. 3A). This finding suggests that Mpr1 may be involved in reduction of ROS generated by freezing stress.

We also observed that prolonged storage of the cells at –20°C caused a gradual increase in the intracellular oxidation level in both strains (Figs. 2A and 3A). The reason for the ROS accumulation remains unclear; however, it is possible that during the aerobic freezing performed in this study the generation of ROS, specifically the superoxide anion, is proportional to the amount of O_2 available, and the electrons leaked from the respiratory chain or other enzymatic reactions since the generation of superoxide anion is a first-order reaction with respect to the concentration of O_2 or the electrons leaked (*18*). Hence, restriction of the availability of O_2 by freezing the cells anaerobically might decrease the generation of ROS, although an anaerobic freezing test in which the cell suspension was deoxygenated with argon before freezing (*9*) was not performed in this study.

Mpr1 Reduces the Intracellular Level of ROS under Freezing Stress—We previously found that Mpr1 is involved in yeast cell growth and reduces the ROS level under oxygen stress, including H_2O_2 and heat-shock treatments (*5*). We therefore examined the effect of Mpr1 on the levels of intracellular ROS generated in the freeze-thaw process. After freezing at –20°C for 12 days, the ROS level in *mpr*-disrupted strain LD1014ura3 was 50% higher than that in wild-type strain L5685 (Fig. 2A). However, high and low expression of *MPR1* in strain LD1014ura3 caused by introducing plasmids pMH1 and pI-MPR, respectively, significantly reduced the ROS level, which was virtually unchanged from that in strain L5685 (Fig. 2A).

On the other hand, when high-copy-number plasmid pMH1 harboring *MPR1* was introduced into strain CKY8 with the S288C background, the transformant showed a 40% reduction in the fluorescence rate after freezing at –20°C for 8 days as compared with that of strain CKY8 carrying only the vector (Fig. 3A). These results indicate that Mpr1 reduces the intracellular level of ROS during exposure to freezing stress, in agreement with those obtained after H_2O_2 and heat-shock treatments (*5*). As an antioxidant mechanism, Mpr1 is supposed to reduce the intracellular oxidation level by acetylating the toxic metabolite(s) involved in ROS generation. Oxidative stress caused by the freeze-thaw process in a similar manner to in the cases of H_2O_2 and heat-shock treatments probably leads to the accumulation of an unidentified intermediate, which may be a cellular substrate of Mpr1. It is therefore proposed that Mpr1 reduces the intracellular levels of ROS, although the pathway through which the intermediate induces ROS production is unknown.

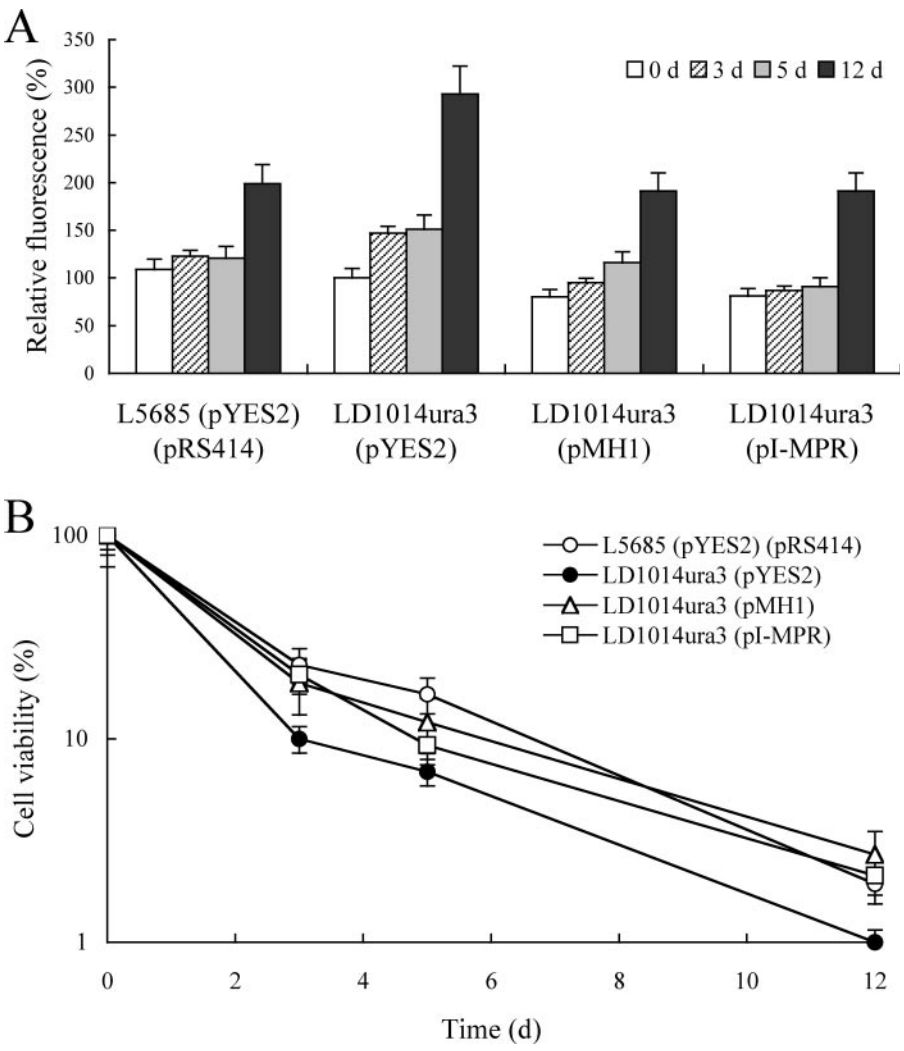

Fig. 2. **Effect of Mpr1 on *S. cerevisiae* strains with the Σ1278b background under freezing stress.** The detailed conditions for the experiments are given in the text. (A) Intracellular oxidation levels after freezing at –20°C for the times indicated. The intensity of fluorescence of *mpr*-disruptant LD1014ura3 carrying pYES2 was relatively taken as 100%. (B) Time course of the cell viability of each strain after freezing at –20°C for the times indicated. The strains used were the wild-type L5685 carrying pYES2 and pRS414 (open circles), LD1014ura3 carrying pYES2 (filled circles), LD1014ura3 carrying pMH1 (open triangles), and LD1014ura3 carrying pI-MPR (open squares). Results indicate the means and standard deviation for three independent experiments.

Mpr1 Protects Yeast Cells from Freezing Stress—We further compared yeast cell viability after exposure to freezing at –20°C. As we previously reported (*15*, *19–22*), the freezing stress conditions used in this study caused a significant decrease in the survival rate of yeast cells on prolonged storage of the cells. The cell viability of *mpr*-disruptant LD1014ura3 after exposure to freezing for 3 and 5 days fell to 40% of that of wild-type strain L5685 (Fig. 2B). We observed that expression of *MPR1* restored the survival rate of LD1014ura3 cells, the level being close to that in strain L5685. As shown in Fig. 2, however, the fluorescence and the cell viability of the strain harboring pMH1 (high-copy) were almost the same as those of the strain harboring pI-MPR (low-copy). Although a gene dosage effect of *MPR1* on AZC resistance was clearly shown (Fig. 1A), low expression of *MPR1* in the strain with the Σ1278b background would be adequate to decrease the intracellular level of ROS during freezing and thawing. This phenomenon may be due to the specificity of Mpr1 substrates, AZC and the unknown cellular substrate under oxidative stress conditions.

It may be of interest that the cell viability of strain CKY8 with the S288C background after freezing stress decreased greatly compared to that of strain L5685 with the Σ1278b background, probably due to the lack of the *MPR* genes in the S288C strain (Fig. 3B). CKY8 cells carrying pMH1 showed approximately 2-fold and 5-fold higher survival rates after freezing for 4 days and 8 days, respectively, as compared with those of CKY8 cells harboring the vector only. These results showed that expression of Mpr1 in yeast cells resulted in higher tolerance to freezing stress. This indicates that Mpr1 protects yeast cells from freezing stress by reducing ROS.

As shown in Fig. 3, however, there is a poor correlation between the relative fluorescence and the cell viability. It is probable that the genetic backgrounds of the *S. cerevisiae* strains used (S288C, Σ1278b, *etc.*) result in the differences in freeze tolerance. Also, physical damage caused by ice crystals or dehydration rather than intracellular ROS generation may cause serious injury to yeast cells during freezing and thawing.

Mpr1 Synergizes with L-Proline for Freezing Tolerance in Yeast Cells—We have previously reported that L-proline exhibits cryoprotective activity in *S. cerevisiae* (*19*). Recently, we also found that intracellular L-proline could protect yeast cells from damage by oxidative stresses including H_2O_2 and heat-shock treatments (*15*). We therefore examined the synergetic effect of L-proline and Mpr1 on the intracellular oxidation levels and cell viability after freezing stress. Strain INVDput1 with the S288C

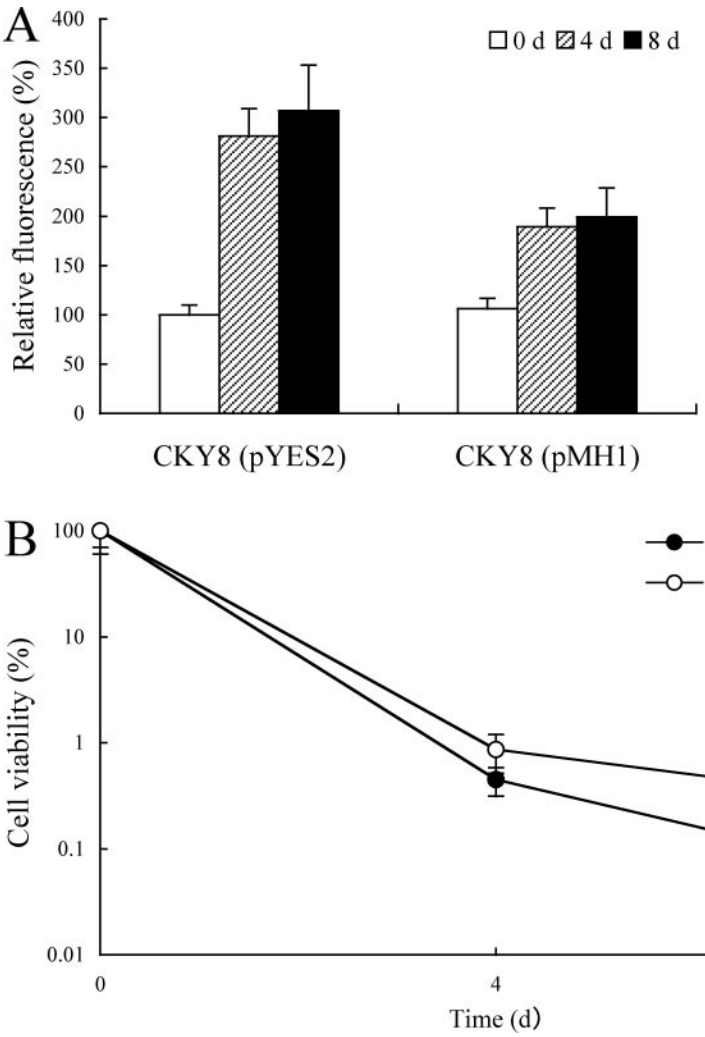

Fig. 3. **Effect of Mpr1 on *S. cerevisiae* strains with the S288C background under freezing stress.** The detailed conditions for the experiments are given in the text. (A) Intracellular oxidation levels after freezing at –20°C for the times indicated. The intensity of fluorescence of the wild-type CKY8 carrying pYES2 was relatively taken as 100%. (B) Time course of the cell viability of each strain after freezing at –20°C for the times indicated. The strains used were CKY8 carrying pYES2 (closed circles) and carrying pMH1 (open circles). Results indicate the means and standard deviation for three independent experiments.

background was constructed by disrupting the *PUT1* gene on the chromosome encoding proline oxidase involved in L-proline utilization (*22*). When high-copy-number plasmids pAD-D154NPRO1 (*15*) with the mutated *PRO1* gene (*pro1*^{D1554N}) encoding the Asp154Asn mutant γ-glutamyl kinase and pTV-PRO2 (*15*) with the wild-type *PRO2* gene encoding γ-glutamyl phosphate reductase were introduced into strain INVDput1, the transformants accumulated higher L-proline levels (0.62% of the dry cell weight) than did the control strain INVDput1 (0.10%). The Asp154Asn mutation was found to enhance the activities of γ-glutamyl kinase and γ-glutamyl phosphate reductase, probably through stabilization of the complex (*15*, *22*).

It should be noted that overexpression of *MPR1* in L-proline–accumulating strain INVDput1 carrying pAD-D154NPRO1 and pTV-PRO2 did not cause increases in the L-proline content of the host cells (0.60% of the dry cell weight). L-Proline is known to dilute AZC, its toxic competitor. Therefore, the L-proline-accumulating strain carrying only the vector was less sensitive to AZC than strain CKY8 carrying only the vector (Fig. 1, B and C). With respect to the oxidation of the fluorescent probe after freezing, an about 30% decrease was observed in L-proline–accumulating cells expressing *MPR1* as compared with in cells harboring only the vector (Fig. 4A). Corresponding to the ROS reduction, the *MPR1*-expressing cells showed an approximately 5- to 18-fold increase in survival rate after freezing as compared with cells harboring the vector only (Fig. 4B). This finding strongly suggested that the combination of L-proline and Mpr1 could further enhance the resistance to freezing stress. Prolonged storage of the cells at –20°C for up to 9 days caused a gradual loss of the freeze tolerance, although a significant protective effect was observed in the *MPR*-expressing cells.

In addition to oxidative damage caused by the generation of ROS, injury to cells due to freezing and thawing can be categorized into two types (*13*, *14*). Low cooling rates lead to osmotic shrinkage of cells. Dehydration then occurs, and biological macromolecules and/or membrane components undergo denaturation. More-rapid freezing does not permit the transport of intracellular water through the membrane and thus impairs the membrane structure or function as ice crystals form in the cells. L-Proline is considered to prevent ice nucleation and dehydration by forming strong hydrogen-bonds with intracellular free-water and to function as a free-radical scavenger that protects cells from oxidative damage (*23*). Therefore, it appears that both Mpr1 and L-proline protect yeast cells independently during freezing and thawing.

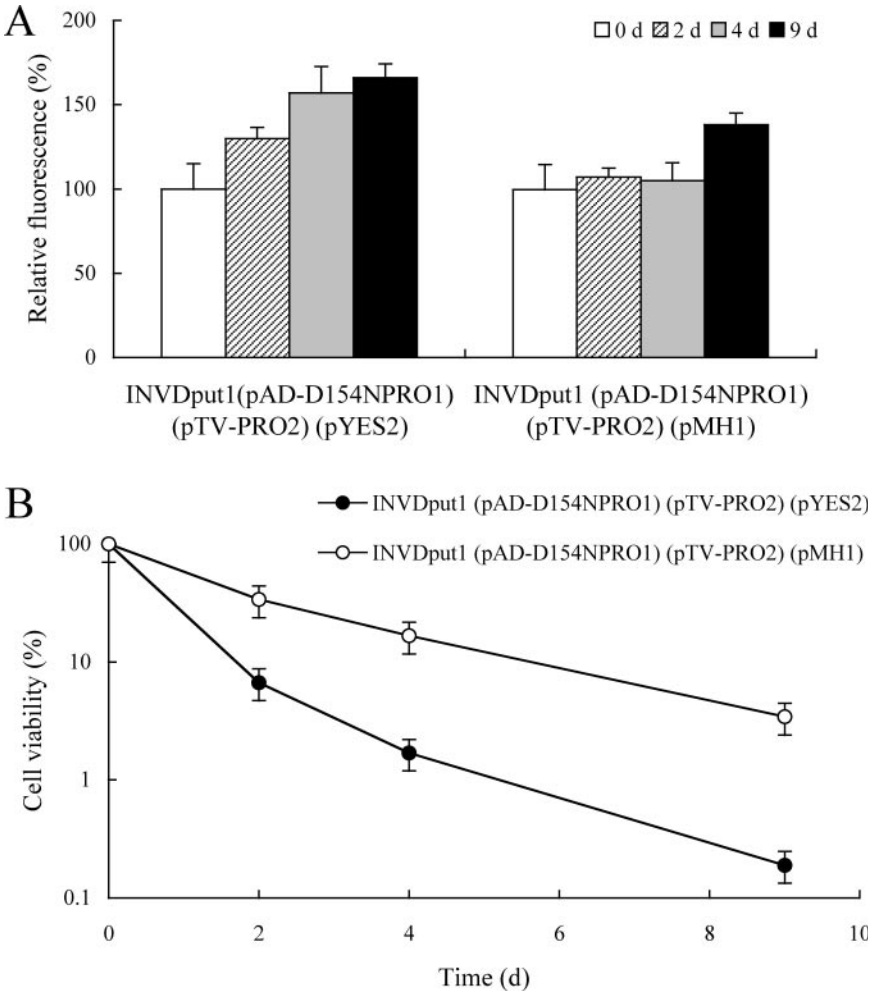

Fig. 4. **Effects of Mpr1 and L-proline on *S. cerevisiae* strains with the S288C background under freezing stress.** The detailed conditions for the experiments are given in the text. (A) Intracellular oxidation levels after freezing at –20°C for the times indicated. The intensity of fluorescence of strain INVDput1 carrying pAD-D154NPRO1, pTV-PRO2 and pYES2 was relatively taken as 100%. (B) Time course of the cell viability of each strain after freezing at –20°C for the times indicated. The strains used were INVDput1 carrying pAD-D154NPRO1, pTV-PRO2 and pYES2 (closed circles), and carrying pAD-D154NPRO1, pTV-PRO2 and pMH1 (open circles). Results indicate the means and standard deviation for three independent experiments.

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 means of gene disruption techniques (*24–27*). Like other stresses, yeast cells may become adapted to freezing stress by means of metabolites such as glycerol and trehalose (*28*, *29*) or synthesis of stress proteins (*30*). Although the gene expression pattern of freezing stress in baker's yeast was recently analyzed (*31*), a baker's yeast that provides good leavening quality for both sweet- and lean-thawed doughs after frozen storage has not yet been developed. Hence, the antioxidant enzyme Mpr1 as well as L-proline could be promising for breeding novel yeast strains that are tolerant to both freezing and oxidative stresses.

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