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Characterization of Novel Acetyltransferases Found in Budding and Fission Yeasts That Detoxify a Proline Analogue, Azetidine-2-Carboxylic Acid

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We recently found that budding yeast *Saccharomyces cerevisiae* Σ1278b, but not genome project strain S288C, has a gene conferring resistance to L-azetidine-2-carboxylic acid (AZC), a toxic four-membered ring analogue of L-proline. Also, the gene, designated as *MPR1*, encodes a novel acetyltransferase that detoxifies AZC *via* acetylation. We now report the results of subsequent work. On a homology search with *MPR1*, we detected a gene in fission yeast *Schizosaccharomyces pombe*. This gene, designated as *ppr1*$^+$ (*pombe MPR1*), is responsible for the AZC-resistance of *S. pombe* as judged from the results of gene disruption and overexpression experiments. *Escherichia coli* cells expressing *ppr1*$^+$, like ones expressing *MPR1*, were resistant to AZC and produced an AZC acetyltransferase. We further found that the enzymes encoded by *MPR1* and *ppr1*$^+$ were homodimers, and catalyzed the acetylation of AZC but not any other L-proline–related compounds. Ppr1p was more thermostable than Mpr1p, although Ppr1p had a lower optimum temperature than Mpr1p. The higher AZC acetylation activity of Mpr1p, in comparison to that of Ppr1p, was attributed to the larger k_{cat}/K_m value for acetyl-CoA of Mpr1p than that of Ppr1p.

Key words: *N*-acetyltransferase, L-azetidine-2-carboxylic acid, L-proline analogue resistance, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*.

Abbreviations: AZC, L-azetidine-2-carboxylic acid; NAT, *N*-acetyltransferase; DHP, 3,4-dehydro-DL-proline; PA, L-pipecolic acid; HP, L-hydroxyproline; TAC, L-thiazolidine-4-carboxylic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; TNB, 5-thio-2-nitrobenzoic acid; PCR, polymerase chain reaction; kbp, kilobase pair; NTA, nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

L-Azetidine-2-carboxylic acid (AZC), a toxic four-membered ring analogue of L-proline, is transported into cells *via* proline transporters (*1*, *2*). It causes misfolding of the proteins into which it is incorporated competitively with L-proline, and thereby inhibits growth of both bacterial and animal cells (*3–6*). We recently discovered, on the chromosome of *Saccharomyces cerevisiae* Σ1278b, novel genes involved in the detoxification of AZC. Intriguingly, the genes, *MPR1* and *MPR2* (sigma 1278b gene for L-proline-analogue resistance), both characterized by a single amino acid change at position 85, were present on chromosomes XIV and X of Σ1278b background strains, respectively, but were absent in *S. cerevisiae* strain S288C used for genomic sequence determination (*7*). Gene expression in *Escherichia coli* and enzymatic analysis showed that *MPR1* encodes a novel AZC acetyltransferase, by which L-proline itself and other L-proline analogues are not acetylated (*8*). The *MPR1*-encoded protein (Mpr1p) was considered to be a member of the *N*-acetyltransferase (NAT) superfamily (*9*). We now believe that AZC is converted to *N*-acetyl AZC by Mpr1p in the cytoplasm (Fig. 1), and consequently *N*-acetyl AZC can no longer replace L-proline during the biosynthesis of protein.

To our knowledge, *MPR1* is the first gene found to be present in *S. cerevisiae* strain Σ1278b but absent in other laboratory strains (S288C, *etc*.). Strain Σ1278b is known to have unique genetic features as to nitrogen metabolism, and unique morphological characteristics as to diploid pseudohyphal development and haploid invasive growth (*10–12*). However, aside from its role in the detoxification of AZC, the physiological role of *MPR1* in strain Σ1278b is unclear and perplexing, given that unusual imino acid AZC is only found in several plants belonging to the Lilaceae family (*13*, *14*). The amino acid sequence of the predicted Mpr1p was homologous to that of the fission yeast *Schizosaccharomyces pombe* hypothetical 23.8 kDa protein (*7*). *S. pombe* is highly different from *S. cerevisiae*, and these two yeasts often exhibit distinct differences when carrying out the same cellular functions.

We report here the isolation and analysis of a *S. pombe* homologue of *MPR1*, designated as *ppr1*$^+$ (*pombe MPR1*). This gene is responsible for the AZC resistance of *S. pombe* and encodes an acetyltransferase that detoxifies AZC in a manner similar to that in the case of *MPR1*. We also report comparison of the characteristics of the two novel yeast acetyltransferases purified from *E. coli* transformed cells.

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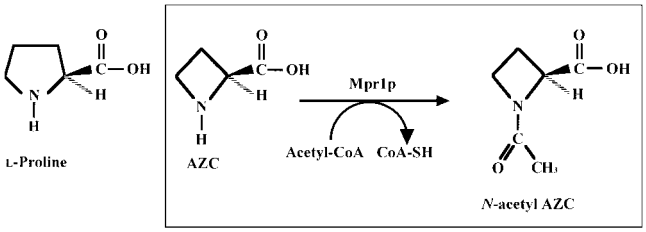

Fig. 1. **Proposed scheme for the AZC acetyltransferase reaction.**

MATERIALS AND METHODS

Materials—The enzymes used for DNA manipulations were obtained from Takara Shuzo (Kyoto). The oligonucleotides used in this study were synthesized by Hokkaido System Science. AZC, 3,4-dehydro-DL-proline (DHP), and L-pipecolic acid (PA) were from Sigma. L-Proline, L-hydroxyproline (HP), L-thiazolidine-4-carboxylic acid (TAC), isopropyl-β-D-thiogalactopyranoside (IPTG), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and acetyl-CoA were from Wako Pure Chemical. Yeast glutamate dehydrogenase, pig heart lactate dehydrogenase, yeast enolase, yeast myokinase, and horse heart cytochrome *c* were from Oriental Yeast.

Strains and Plasmids—The yeast haploid strains used in this study are listed in Table 1. *S. pombe* strain ARC039 was derived from strain L972. *S. cerevisiae* strain CKY263 with a S288C background was used for expression of *ppr1*$^+$. Two yeast high-copy-number plasmids, pAL-SK (supplied by K. Tanaka) and pAD4 (supplied by J. Nikawa), both of which contain the bacterial ampicillin resistance gene and the *S. cerevisiae LEU2* gene, were used to express *ppr1*$^+$ in *S. pombe* and *S. cerevisiae*, respectively. Plasmids pUC18 (Takara Shuzo) and pBSUra4 harboring the *S. pombe ura4*$^+$ gene (supplied by K. Tanaka) were used to construct the fragment for *ppr1*$^+$ disruption.

IPTG-inducible vectors pQE30 and pQE31 (Qiagen) with a sequence coding six consecutive histidine residues at the 5′ end of cloning sites were used for expression of *MPR1* and *ppr1*$^+$ in *E. coli* strain JM109 [*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44*/(*F′ traD36 proAB*$^+$ *lacI*^{q} *Z*Δ*M15*)], respectively.

Culture Media—The media used for the growth of yeast cells were SD [2% glucose, 0.67% Bacto yeast nitrogen base without amino acids (Difco Laboratories)] and YPD (2% glucose, 1% Bacto yeast extract, 2% Bacto peptone). SD medium contains ammonium sulfate (0.1%) as the nitrogen source. When appropriate, required amino acids were added to the media for auxotrophic strains. For *S. pombe* transformation, 1/2 YEL medium (1.5% glucose, 0.25% Bacto yeast extract) containing adenine (30 μg/ml) was used. The *E. coli* recombinant cells were grown in Luria-Bertani (LB) (*15*) or M9 medium (*15*) containing 2% casamino acids (M9CA) with ampicillin (50 μg/ml). If necessary, 2% agar was added to solidify the medium.

DNA Manipulation and Transformation—One-step gene replacement by homologous recombination in *S. pombe* was performed according to the method of Rothstein (*16*).

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

Strain	Genotype	Background and/or source
S. pombe		
L972	*h*$^-$ wild-type *ppr1*$^+$	P. Nurse
ARC039	*h*$^-$ *leu1-32 ura4-294 ppr1*$^+$	K. Takegawa
ARC-Dppr	*h*$^-$ *leu1-32 ura4-294 ppr1::ura4*$^+$	This study
S. cerevisiae		
S288C	α wild-type *mal1 gal2*	C. Kaiser
Σ1278b	α wild-type *MPR1 MPR2*	M. Brandriss
CKY263	a *leu2-3, 112 ura3-52 GAL*	S288C, C. Kaiser

Conventional techniques were used for other manipulations such as *S. pombe* genomic DNA preparation and transformation (*17*).

Disruption of the ppr1$^+$ *Gene in S. pombe*—A DNA fragment of *ppr1*$^+$ was prepared by polymerase chain reaction (PCR) with genomic DNA from *S. pombe* L972 and oligonucleotide primers based on the available nucleotide sequence using a GeneAmp PCR system 2400 (PE Biosystems). The primers were SP-*Kpn*I (5′-GGG GTA CCC GGA TTC TTC ATT CG-3′) and SP-*Sph*I (5′-ACA TGC ATG CCT ACG GAT CAT TCT C-3′) (the underlined sequences are the positions of *Kpn*I and *Sph*I sites, respectively). The unique amplified band corresponding to 1,520-bp for *ppr1*$^+$ was digested with *Kpn*I and *Sph*I, and then ligated to the *Kpn*I and *Sph*I sites of pUC18. The plasmid harboring *ppr1*$^+$ was designated as pUC18-SP. Plasmid pUC-SP-URA4 was then constructed by deleting the 550-bp *Nco*I–*Sal*I fragment in *ppr1*$^+$ from pUC18-SP and inserting the 1.8-kbp *Hin*dIII fragment containing *ura4*$^+$ isolated from pBSUra4 by blunt-end ligation. The 2.7-kbp *Kpn*I–*Sac*I fragment containing *ppr1::ura4*$^+$ was integrated into the *ppr1*$^+$ locus in strain ARC039 by transformation. The resultant *ppr1* disruptant, ARC-Dppr, was selected from among several Ura$^+$ transformants, and the correct disruption of *ppr1*$^+$ was verified by chromosomal PCR analysis using primer SP-*Kpn*I or SP-*Sph*I in combination with primer URA4– (5′-CTG TTC CAA CAC CAA TG-3′) or URA4+ (5′-TAC AAA TCC CAC TGG CT-3′), which lie in *ura4*$^+$.

Construction of Expression Plasmids—For the expression of *ppr1*$^+$ in *S. pombe*, a DNA fragment of *ppr1*$^+$ containing the putative promoter and terminator regions was prepared by PCR with genomic DNA from *S. pombe* L972, and oligonucleotide primers 5′-CCC CTC GAG CGC AAA CCG AAA ATA CAC AG-3′ and 5′-CCG CTC GAG ATT TAT CGG CGT CTT CTA CG-3′ (the underlined sequences are the positions of *Xho*I sites). The unique amplified band corresponding to 1,430-bp was digested with *Xho*I, and then ligated to the *Xho*I site of *ARS1*-based plasmid pAL-SK to construct pAL-ppr. The nucleotide sequence of *ppr1*$^+$ was confirmed by DNA sequencing with a Model 377 DNA sequencer from PE Biosystems using the dideoxy chain termination method.

To express *ppr1*$^+$ under control of the *ADH1* promoter in *S. cerevisiae*, a DNA fragment of the open reading frame was prepared by PCR with genomic DNA of strain

L972, and oligonucleotide primers 5′-CCC AAG CTT CCA CAT GGC TAT AAA ATC CC-3′ and 5′-CCC GAG CTC ATC GAA AGT GTG GCA TCC AA-3′ (the underlined sequences are the positions of *Hin*dIII and *Sac*I sites, respectively). The unique amplified band corresponding to 750-bp was digested with *Hin*dIII and *Sac*I, and then ligated to the *Hin*dIII and *Sac*I sites of a 2μ-based plasmid, pAD4, to construct pAD-ppr. The nucleotide sequence of *ppr1*$^+$ was confirmed by DNA sequencing. In comparison with *ppr1*$^+$, the 950-bp *Hin*dIII–*Bal*I fragment from pMH1 (*7*), which contains *MPR1*, was also ligated to the large fragment of pAD4 digested with *Hin*dIII and *Sma*I to construct pAD-MPR.

Another plasmid was further constructed to express *ppr1*$^+$ in *E. coli*. The 690-bp *Bam*HI–*Sac*I fragment isolated from pAD-ppr was ligated into the *Bam*HI and *Sac*I sites of pQE31. The plasmid in which *ppr1*$^+$ was placed under the T5 promoter/*lac* operator was designated as pDE-ppr. pQE-ppr carries *ppr1*$^+$ with a sequence coding six consecutive histidine residues at the amino terminal threonine residue, which substituted for a native lysine. For expression of *MPR1* in *E. coli*, a pQE30-based plasmid, pQE-MPR, was used as described previously (*8*).

Expression and Purification of the Mpr1p and Ppr1p in E. coli—An *E. coli* strain, JM109, was transformed with pQE-MPR and pQE-ppr, and then the transformed cells were grown at 37°C in 200 ml of M9CA medium containing ampicillin. When the absorbance at 600 nm reached 0.5, IPTG was added to the culture medium to a final concentration of 0.1 mM to induce gene expression. After cultivation for 4 h at 37°C, harvested cells were suspended in 20 ml of ice-cold sonication buffer [50 mM NaH_2PO_4, 50 mM Na_2HPO_4 (pH 7.8) and 300 mM NaCl], and cell-free extracts were prepared by sonic oscillation (200 W) under cooling. The sonicated cells were centrifuged at 10,000 ×g for 20 min.

The soluble fraction of the supernatant was then purified using Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen) according to the procedure recommended by the supplier. The His-tagged fusion proteins were bound to the Ni-NTA resin. The resin was then collected on a column, washed with 50 mM Na-phosphate (pH 6.0), 300 mM NaCl, and 10% (v/v) glycerol, and eluted with 300 mM imidazole in the same buffer. The eluted fractions were dialyzed exhaustively against 100 volumes of 50 mM Tris-HCl buffer (pH 8.3).

Relative Molecular Mass and Subunit Structure—The purity of the proteins and the subunit molecular mass were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of the protein was estimated by gel-permeation liquid chromatography at 4°C using a calibrated column of Superdex 200 PC3.2/30 (Amersham Pharmacia Biotech) connected to a SMART™ system (Amersham Pharmacia Biotech), with a flow rate of 40 μl/min. A calibration curve was made from the elution pattern of yeast glutamate dehydrogenase (290 kDa), pig heart lactate dehydrogenase (142 kDa), yeast enolase (67 kDa), yeast myokinase (32 kDa), and horse heart cytochrome *c* (12.4 kDa).

Assaying of Acetyltransferase Activity—To determine acetyltransferase activity, whole-cell extracts of yeast strains expressing *MPR1* or *ppr1*$^+$ were prepared by vortexing the cells with glass beads followed by sonic oscillation under cooling, respectively, and then used as enzyme sources. Also, purified proteins from the transformed *E. coli* cells were used to further determine the enzymatic properties. Acetyltransferase activity was basically assayed at 30°C by monitoring the increase in 5-thio-2-nitrobenzoic acid (TNB) because of the reaction of acetyl-CoA with DTNB described previously with some modifications (*18*). The initial rate of the increase in absorbance at 412 nm of the reaction mixture (final volume, 1 ml) comprising 50 mM Tris-HCl buffer (pH 8.3), 1 mM L-proline, HP, AZC, DHP, TAC, or PA, 1 mM DTNB, 0.1 mM acetyl-CoA, and enzyme solution was measured, and then that obtained for a solution containing all the materials except L-proline, HP, AZC, DHP, TAC, or PA (blank) was subtracted. The reaction rate was calculated using an extinction coefficient for TNB of 15,570 M^{-1} cm^{-1}. One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol TNB/min at 30°C. Protein concentrations were determined with a Bio-Rad protein assay kit. Bovine serum albumin was used as the standard protein.

Kinetic Studies—Steady-state kinetic studies were performed with 50 mM Tris-HCl buffer (pH 8.3). The kinetic parameters, k_{cat} and K_m, were obtained from initial rate measurements by monitoring the increase in absorbance at 412 nm with a DU-640 spectrophotometer (Beckman). When the apparent K_m value for AZC was determined, the AZC concentration was varied from 0.25 to 4 mM in the presence of a fixed concentration of acetyl-CoA (0.1 mM). When the apparent K_m value for acetyl-CoA was determined, the acetyl-CoA concentration was varied from 0.025 to 0.4 mM in the presence of a fixed concentration of AZC (1 or 2 mM).

Data Deposition—The GenBank™ accession numbers for *MPR1* and *ppr1*$^+$ are AB031349 and AB083128, respectively.

RESULTS

Identification of the MPR1 Homologue in S. pombe—On a BLAST search (*19*) of protein databases, we found that *S. pombe* has a hypothetical 23.8 kDa protein encoded by the SPAC21E11.04 gene on its chromosome I (accession No. Z67999) that is homologous to that in Mpr1p. Figure 2 shows that, within the overall region of 209 amino acids, 32% of the amino acids are identical and 46% are considered to be similar. No introns were found in the open reading frame. It should be additionally noted that *S. pombe* has one copy of the gene on chromosome I, whereas *S. cerevisiae* Σ1278b has two copies of the gene (*7*). We therefore took the putative AZC acetyltransferase gene *ppr1*$^+$ (*pombe MPR1*) to be the *S. pombe MPR1* homologue.

Also, the sequence of the *ppr1*$^+$-encoded protein (Ppr1p) was homologous to the amino-terminal sequence of the *S. cerevisiae SPT10*-encoded protein (Spt10p) of 640 amino acids, which is a negative transcriptional regulator (*20–22*). On sequence comparison, these three proteins, Mpr1p, Ppr1p, and Spt10p, were found to belong to the NAT superfamily. The NAT superfamily, which comprises over 50 members, has four conserved regional motifs, A–D (*9*). In particular, the most highly conserved motif (A) would be involved in the binding of acetyl-CoA

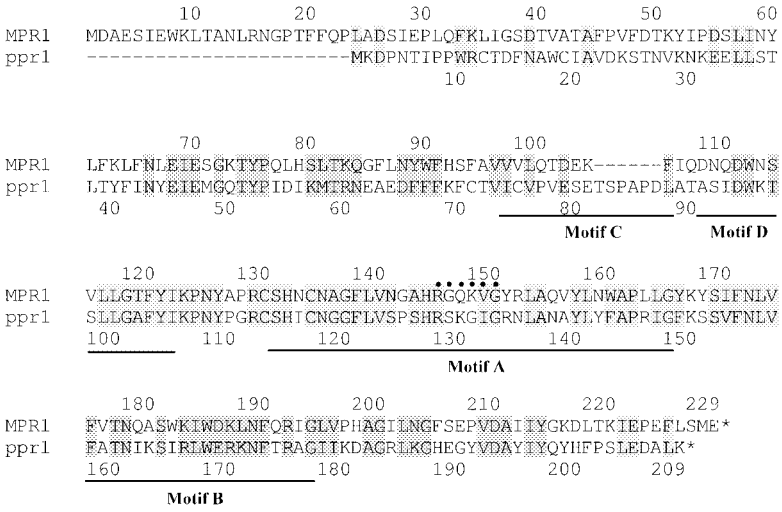

Fig. 2. **Comparison of the amino acid sequence of *S. cerevisiae* Mpr1p (MPR1) with that of *S. pombe* Ppr1p (ppr1).** The numbering above the sequences refers to Mpr1p and that below the sequences to Ppr1p. Identical or similar amino acids are shown in shaded boxes. Dashes indicate the absence of the corresponding amino acid residues at those positions. Motifs A–D, which correspond to the motifs described by Neuwald and Landsman (*9*), belong to the *N*-acetyltransferase superfamily. Highly conserved residues in motif A, (Q/R)*XX*G*X*(G/A), in common within members of the superfamily are indicated by dots. The GenBank™ accession numbers for Mpr1p and Ppr1p are AB031349 and AB083128, respectively.

(*23–25*), and this motif has a short consensus sequence, (Q/R)*XX*G*X*(G/A), in common with other members of the superfamily.

S. pombe Wild-Type Strain Shows AZC Resistance and AZC Acetyltransferase Activity—As a first step of analysis of *ppr1*$^+$, we examined the growth of yeast strains on SD agar plates containing toxic AZC (Fig. 3). *S. pombe* wild-type strain L972, as well as *S. cerevisiae* strain Σ1278b expressing *MPR1* and *MPR2*, showed greater AZC resistance than *S. cerevisiae* S288C, which is sensitive to AZC. Acetyltransferase activity toward AZC was clearly detected in total cell extracts of strains Σ1278b and L972 (Fig. 3). These results suggest that *ppr1*$^+$ is involved in the resistance of *S. pombe* to AZC.

The ppr1$^+$ *Gene Also Encodes an Acetyltransferase That Detoxifies AZC*—To further examine the function(s) of *ppr1*$^+$ in *S. pombe*, we disrupted *ppr1*$^+$ by one-step gene replacement as described under "MATERIALS AND METHODS." *ppr1*-disruptant ARC-Dppr grew as well as the wild-type parent strain (ARC039) on SD medium,

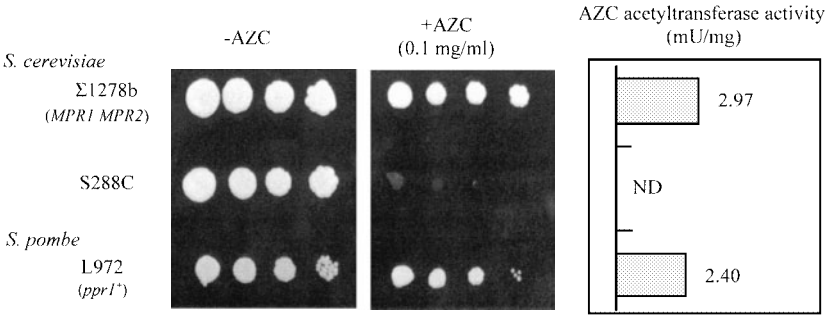

Fig. 3. ***S. pombe* wild-type strain shows AZC resistance and AZC acetyltransferase activity.** The growth phenotypes on AZC-containing medium and AZC acetyltransferase activities of various yeast strains were examined. Approximately 10^6 cells of each strain and serial dilutions of 10^{-1} to 10^{-3} (from left to right) were spotted onto SD plates containing L-leucine and uracil in the absence (–AZC) and presence of 100 μg/ml AZC (+AZC). The plates were incubated at 30°C for 3 days. AZC acetyltransferase activity was assayed using the enzyme solution prepared from each strain grown in SD medium at 30°C. The data shown are the means for three independent experiments. The variations in the values were less than 5%. ND, not detected.

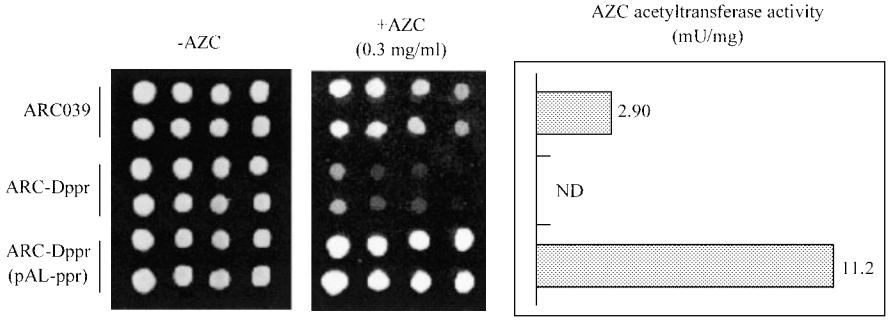

Fig. 4. **The *ppr1*$^+$ gene encodes an AZC acetyltransferase.** The growth phenotypes on AZC-containing medium and AZC acetyltransferase activities of *S. pombe* strains ARC039 (wild-type), ARC-Dppr (*ppr1*-disruptant), and ARC-Dppr harboring pAL-ppr (high-copy *ppr1*$^+$) were examined. Approximately 10^6 cells of each strain and serial dilutions of 10^{-1} to 10^{-3} (from left to right) were spotted onto SD plates containing L-leucine and uracil in the absence (–AZC) and presence of 300 μg/ml AZC (+AZC). The plates were incubated at 30°C for 3 days. AZC acetyltransferase activity was assayed using the enzyme solution prepared from each strain grown in SD medium at 30°C. The data shown are the means for three independent experiments. The variations in the values were less than 5%. ND, not detected.

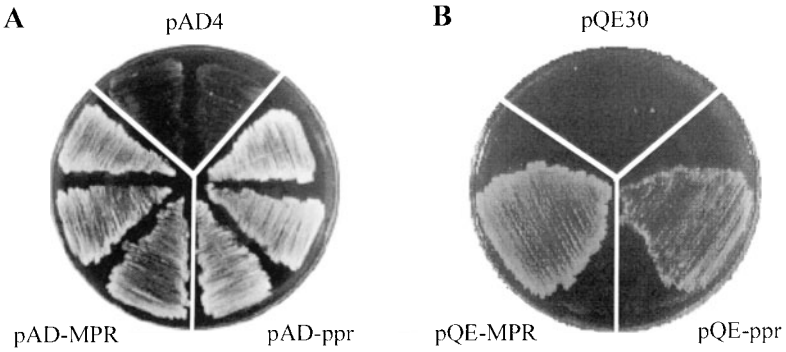

Fig. 5. **The *ppr1*⁺ gene also confers AZC resistance to *S. cerevisiae* and *E. coli*.** The expression of *ppr1*⁺ in *S. cerevisiae* CKY263 (A) and *E. coli* JM109 (B) was examined. (A) The yeast transformants were cultivated on SD plates containing uracil and 100 μg/ml AZC. The plates were incubated at 30°C for 3 days. (B) The *E. coli* transformants were cultivated on M9 plates containing 100 μg/ml AZC in the presence of 0.1 mM IPTG. The plates were incubated at 37°C for 1 day.

indicating that *ppr1*⁺ is not essential for *S. pombe* (Fig. 4). However, strain ARC-Dppr failed to grow on AZC-containing agar plates (Fig. 4), which simultaneously resulted in the complete loss of AZC acetyltransferase activity in the cell extract (Fig. 4).

When high-copy-number plasmid pAL-ppr harboring *ppr1*⁺ was introduced into strain ARC-Dppr lacking *ppr1*⁺, all of the Leu⁺ transformants showed restoration of the AZC-resistant phenotype (Fig. 4). The cells containing pAL-ppr showed a prominent 4-fold increase in acetyltransferase activity as compared to that of wild-type strain ARC039 (Fig. 4). Thus, *ppr1*⁺ was found to have a dosage effect on both AZC resistance and acetyltransferase activity, comparable to that of *MPR1* (*8*). These results demonstrate that *ppr1*⁺ encodes an acetyltransferase required for AZC detoxification in *S. pombe*.

The ppr1⁺ *Gene Also Confers AZC Resistance to S. cerevisiae and E. coli*—We constructed expression plasmids for *ppr1*⁺ in *S. cerevisiae*. As shown in Fig. 5A, when *ppr1*⁺ was introduced into *S. cerevisiae* CKY263 with a S288C background and was controlled by the *ADH1* promoter in pAD-ppr, the transformed cells grew as well as the control cells containing *MPR1* in pAD-MPR on SD medium containing AZC. Moreover, AZC acetyltransferase activity in the cells expressing *ppr1*⁺ was significantly high, and was virtually the same as that in the case of *MPR1* (20.1 mU/mg for *ppr1*⁺ *versus* 18.1 mU/mg for *MPR1*).

Similar results were obtained for the transformed *E. coli* cells. We introduced expression plasmids pQE-ppr and pQE-MPR for *ppr1*⁺ and *MPR1*, respectively, under the IPTG-inducible promoter into *E. coli* JM109. The cells expressing *ppr1*⁺ or *MPR1* in the presence of IPTG acquired the AZC-resistant phenotype, whereas the vector-harboring cells failed to grow on AZC-containing plates (Fig. 5B). These results indicate that the two yeast genes encoding AZC acetyltransferases are expressed in other microorganisms, where they play global roles in AZC detoxification.

Purification and Relative Molecular Masses of the Recombinant Mpr1p and Ppr1p—Given that both enzymes remained active in *E. coli* cells, we purified the recombinant enzymes from *E. coli* cells and characterized their enzymatic properties. The recombinant His-tagged fusion Mpr1p and Ppr1p (rMpr1p and rPpr1p) were each purified to give a single band, corresponding to relative molecular masses of approximately 28.3 and 24.9 kDa, respectively, on an SDS-PAGE gel (Fig. 6A). The molecular masses of native rMpr1p and rPpr1p were estimated to be approximately 51 and 43 kDa, respectively, by gel-permeation liquid chromatography (Fig. 6B). These results suggest that rMpr1p and rPpr1p are homodimeric proteins.

Substrate Specificity and Optimum pH—Table 2 shows the acetyltransferase activities of rMpr1p and rPpr1p toward various L-proline–related compounds. DHP and TAC are five-membered ring analogues of L-proline. HP is a natural constituent of animal proteins like collagen and elastin. PA, an L-homoproline, is a six-membered ring L-lysine metabolite. Among the substrates tested, only AZC was acetylated by rMpr1p and rPpr1p. These results suggest that these enzymes do not acetylate five-

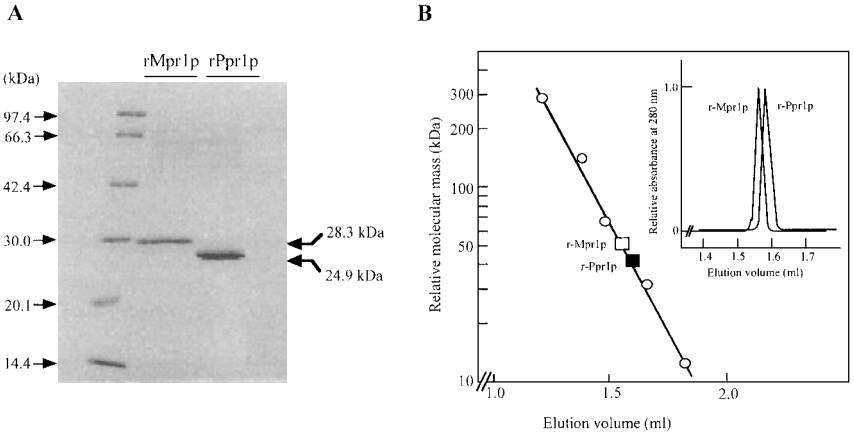

Fig. 6. **Molecular masses of purified rMpr1p and rPpr1p.** (A) SDS-PAGE of the enzymes. The molecular mass standards, sizes in kilodaltons, are in the left lane. The gel was stained for protein with Coomassie Brilliant Blue R-250. (B) Gel permeation liquid chromatography of the enzymes on Superdex 200 PC3.2/30. Determination of the relative molecular masses of the enzymes is shown. Open circles represent the protein standards. The inset shows the elution profiles of the enzymes. The ultraviolet absorption at 280 nm is expressed as the relative absorbance. The absorbance is relative to the full-scale deflection (1.0) on the recorder.

Table 2. **Acetyltransferase activities of rMpr1p and rPpr1p toward various L-proline-related compounds.**

Enzyme	Specific activity (mU/mg) for					
	Pro	AZC	DHP	TAC	HP	PA
rMpr1p	<0.01	6.77 ± 1.91	0.05 ± 0.03	0.12 ± 0.01	<0.01	<0.01
rPpr1p	<0.01	2.10 ± 0.51	0.07 ± 0.02	0.08 ± 0.04	<0.01	<0.01

Assays were performed in 50 mM Tris-HCl (pH 8.3) at 30°C using 2 μg of purified enzyme. The data shown are means ± SD for three independent experiments.

Table 3. **Kinetic analysis of acetyltransferase activities of rMpr1p and rPpr1p.**

Temperature	rMpr1p		rPpr1p	
	AZC	Acetyl-CoA	AZC	Acetyl-CoA
20°C	1.00	140	0.59	3.32
	(1.15, 1.15)	(1.98, 0.014)	(0.48, 0.86)	(0.46, 0.14)
30°C	1.25	134	0.4	3.63
	(2.10, 1.71)	(2.82, 0.021)	(0.61, 1.52)	(0.52, 0.15)
40°C	1.66	187	0.31	1.64
	(3.47, 2.10)	(3.95, 0.021)	(1.05, 3.46)	(0.53, 0.31)

Assays were performed in 50 mM Tris-HCl (pH 8.3) at 20, 30, and 40°C. Values represent k_{cat}/K_m (s^{-1}/mM); values in parentheses represent k_{cat} (s^{-1}) and K_m (mM^{-1}), respectively. The data shown are means for three independent experiments. Variations in the values were below 5%.

or six-membered ring compounds. The optimal pH of rMpr1p and rPpr1p for AZC was about 8.5 to 9.0, at which level the activities were measured with Na-acetate, 2-morpholinoethanesulfonic acid, Tris-HCl, and Gly-NaOH buffers (data not shown). When various cations were added to the reaction mixture, it was found that the activities were greatly inhibited by divalent cations Co^{2+}, Ni^{2+}, and Mn^{2+} (data not shown).

Ppr1p Is a Highly Thermostable Enzyme Relative to Mpr1p—The acetyltransferase activity toward AZC *versus* assay temperature is shown in Fig. 7A. The optimum temperature of rPpr1p was approximately 20°C, whereas that of rMpr1p was 35°C. To further analyze the heat stability of the purified enzymes, the rate of thermal inactivation was measured at 50°C (Fig. 7B). It is noteworthy that the half-life ($t_{1/2}$) of rPpr1p was approximately 20 times longer than that of rMpr1p, indicating that rPpr1p is highly thermostable relative to rMpr1p ($t_{1/2}$, 60 min for rPpr1p *versus* 3 min for rMpr1p).

Mpr1p Shows Rather High Acetyltransferase Activity toward AZC Relative to That of Prp1p—Kinetic constants k_{cat} and K_m were determined at 20, 30, and 40°C from the initial rate measurements of AZC and acetyl-CoA. As shown in Table 3, the acetyltransferase activity of rMpr1p gradually increased with an increase in the assay temperature, and showed 2- to 100-fold greater catalytic efficiency than that of rPpr1p at any temperature tested. In particular, rMpr1p exhibited a high k_{cat}/K_m value for acetyl-CoA, which was predominantly caused by a smaller K_m and indicated a high affinity for acetyl-CoA. The K_m values for AZC were virtually unchanged for either of the two enzymes. However, with increasing assay temperature, rPpr1p was inclined to show a decrease in catalytic efficiency, this finding being in agreement with the results as to the temperature profile (Fig. 7B). It was shown that at 40°C, the K_m value increases rapidly, with little increase in k_{cat}. These results suggest that at high temperature, the tertiary structure of Ppr1p becomes more rigid than that of Mpr1p.

DISCUSSION

We found that *ppr1*$^+$ is involved in the toxin AZC resistance of *S. pombe* and encodes an AZC acetyltransferase, like the *S. cerevisiae MPR1* gene (*7*, *8*). Prior to this study, no AZC acetyltransferase, nor any proteins exhibiting homology with Mpr1p, had been identified in any

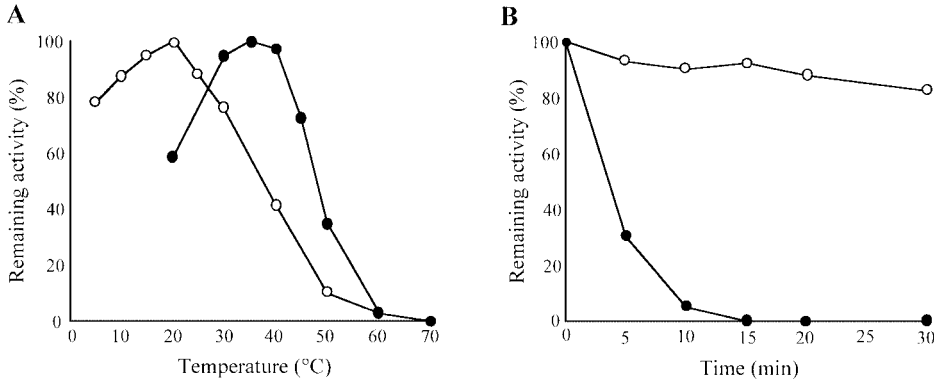

Fig. 7. **Ppr1p is a highly thermostable enzyme relative to Mpr1p.** (A) Temperature dependence of the relative AZC acetyltransferase activities of rMpr1p (filled circles) and rPpr1p (open circles). Relative activity is expressed as a percentage of the maximum activity. The variations in the values were less than 5%. (B) Thermostability of rMpr1p (filled circles) and rPpr1p (open circles). AZC acetyltransferase activity remaining after heating at 50°C was determined at 30°C and was expressed as a percentage of the original activity.

prokaryotic or eukaryotic organisms. It is known that *S. pombe* is as phylogenetically distant from *S. cerevisiae* as it is from humans (*26*). The *MPR* genes found in *S. cerevisiae* Σ1278b are missing in other *S. cerevisiae* laboratory strains, including genome project strain S288C and sake yeast strain K-9 (*7*). In standard laboratory yeast strains, chromosome length polymorphism is thought mainly to originate from movement of Ty elements in and out of chromosomes, and from Ty-associated duplications or deletions (*27*). However, no repeated sequence involving any Ty element was found at the end of *MPR1* or *ppr1*$^+$. Much more research on genome evolution is needed to elucidate the origin of these genes. We are currently searching for other *MPR1* homologue genes in the genomes of *S. cerevisiae* complex species. Some details of this search will be presented elsewhere (*28*).

Toxin AZC, which is distributed only in plants belonging to the Lilaceae family (*13*, *14*), is incorporated into newly synthesized proteins through competition with L-proline in the formation of L-prolyl-tRNA. After incorporation into proteins, AZC causes the polypeptide chain to turn at an angle 15° smaller than that characteristic of L-proline, leading to nonfunctional proteins and a deleterious overall effect (*3*). We now speculate that *N*-acetylation of AZC is catalyzed by Mpr1p or Ppr1p in the cytoplasm, and that the *N*-acetyl AZC accumulated may be transported into vacuoles by an unidentified transporter protein.

The question arises as to why the yeast strains possess this novel acetyltransferase. Our findings here suggest that this enzyme may have a physiologically conserved function additional to detoxification of unusual imino acid AZC. The natural substrates of these enzymes in yeast cells might be unknown four-membered ring compounds. In *S. cerevisiae* Σ1278b, when the wild-type and *mpr1 mpr2*-disrupted strains were cultured in medium containing various nitrogen sources, the growth phenotypes and total intracellular amino acid contents were virtually unchanged in all of the strains (data not shown). It is possible that Mpr1p and Ppr1p would acetylate a toxic intermediate accumulated in the cytoplasm *via* some amino acid metabolic pathway. To investigate this possibility, we will analyze the substrate specificity for intermediate(s) of the L-proline metabolic pathway, using the purified recombinant enzymes.

In comparison with Ppr1p, Mpr1p exhibited high activity, mainly due to a larger k_{cat}/K_m value for acetyl-CoA but not for AZC (Table 3). Recently, the crystal or solution structures of several proteins belonging to the superfamily were determined in a complex with acetyl-CoA (*23*, *25*, *29*). For instance, the X-ray structure of a GCN5-related *N*-acetyltransferase, *Serratia marcescens* aminoglycoside 3-*N*-acetyltransferase, bound to CoA showed that acetyl-CoA recognition is mediated by a β-α structure derived from motif A for hydrogen bonding with the cofactor. In addition, amino acid substitutions of any of these residues were shown to reduce the activities of NAT enzymes (*30*, *31*). We previously performed Ala-scan mutagenesis through a sequence of six amino acid residues (Arg145 to Gly150) in Mpr1p (*8*). The Ala substitutions of Arg145, Val149, and Gly150, led to a growth defect in AZC and simultaneously resulted in a complete loss of AZC acetyltransferase activity, suggesting that Ala substitutions at these positions may interfere with acetyl-CoA binding to Mpr1p. On the other hand, the Gly146Ala, Gln147Ala, and Lys148Ala mutants exhibiting AZC resistance in the growth assay also retained a significant level of AZC acetylation activity. These data demonstrated a clear correlation between residues within Mpr1p that are critical for AZC acetylation and residues that are absolutely required for AZC resistance.

In the case of the Ppr1p sequence, the corresponding region consists of Arg128-Ser129-Lys130-Gly131-Ile132-Gly133, showing a high degree of similarity to other family members. It is worth noting that positions 129, 130, and 132 in Ppr1p are occupied by amino acid residues having larger and more hydrophobic side-chains than these positions in Mpr1p (Gly146, Gln147, and Val149, respectively). It was suggested that such steric hindrance helps to weaken the ability of acetyl-CoA binding by Ppr1p, which leads to a larger K_m value than that of Mpr1p. In contrast, Prp1p was more thermostable than Mpr1p (Fig. 7B), although the catalytic efficiency of the former gradually decreased with increasing assay temperature. It is probable that Ppr1p, which forms a homodimer, undergoes many hydrophobic interactions involving the intersubunit, which is not the case for Mpr1p, thereby increasing the thermostability of the former. The conformation of a molecule with lower catalytic efficiency is likely to lose flexibility, which may explain the rigidity of Ppr1p. Based on that Mpr1p does not contain any carbohydrate (*8*), and that both rMpr1p and rPpr1p remain active in *E. coli* cells, crystallization of the recombinant proteins, rMpr1p and rPpr1p, overexpressed in *E. coli* is currently being attempted in order to determine their tertiary structures.

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