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Functional Characterization of Alanine Racemase from *Schizosaccharomyces pombe*: a Eucaryotic Counterpart to Bacterial Alanine Racemase

TAKUMA UO,[1] TOHRU YOSHIMURA,[1] NAOTAKA TANAKA,[2] KAORU TAKEGAWA,[2] AND NOBUYOSHI ESAKI[1]*

Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011,[1] and Department of Life Sciences, Faculty of Agriculture, Kagawa University, Kagawa 761-0795,[2] Japan

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***Schizosaccharomyces pombe* has an open reading frame, which we named *alr1*$^+$, encoding a putative protein similar to bacterial alanine racemase. We cloned the *alr1*$^+$ gene in *Escherichia coli* and purified the gene product (Alr1p), with an M_r of 41,590, to homogeneity. Alr1p contains pyridoxal 5′-phosphate as a coenzyme and catalyzes the racemization of alanine with apparent K_m and V_{max} values as follows: for L-alanine, 5.0 mM and 670 μmol/min/mg, respectively, and for D-alanine, 2.4 mM and 350 μmol/min/mg, respectively. The enzyme is almost specific to alanine, but L-serine and L-2-aminobutyrate are racemized slowly at rates 3.7 and 0.37% of that of L-alanine, respectively. *S. pombe* uses D-alanine as a sole nitrogen source, but deletion of the *alr1*$^+$ gene resulted in retarded growth on the same medium. This indicates that *S. pombe* has catabolic pathways for both enantiomers of alanine and that the pathway for L-alanine coupled with racemization plays a major role in the catabolism of D-alanine. *Saccharomyces cerevisiae* differs markedly from *S. pombe*: *S. cerevisiae* uses L-alanine but not D-alanine as a sole nitrogen source. Moreover, D-alanine is toxic to *S. cerevisiae*. However, heterologous expression of the *alr1*$^+$ gene enabled *S. cerevisiae* to grow efficiently on D-alanine as a sole nitrogen source. The recombinant yeast was relieved from the toxicity of D-alanine.**

D-Alanine is an essential component of bacterial peptidoglycans and is produced by alanine racemase (EC 5.1.1.1), a pyridoxal 5′-phosphate (PLP)-dependent enzyme (22, 29). However, D-alanine also occurs in various natural compounds produced by other organisms. For example, cyclosporin A contains D-alanine as a component and is produced by a fungus, *Tolypocladium niveum* (10). Alanine racemase was shown to be involved in the biosynthesis of D-alanine in this fungus and was later purified and characterized. Even though it differs markedly in primary structure from bacterial alanine racemase, it rather resembles yeast threonine aldolase (4, 10).

D-Serine occurs in mammalian brains and serves as an endogenous ligand of the glycine site of the *N*-methyl-D-aspartate receptor (14). Serine racemase, a PLP-dependent enzyme, was purified from rat brain to homogeneity (35), and a cDNA clone for mouse brain enzyme was obtained (36); this enzyme is distinct from both bacterial and fungal alanine racemases but similar to bacterial threonine dehydratase in primary structure (36). Serine racemase occurs also in vancomycin-resistant *Enterococcus gallinarum* (1). However, it resembles bacterial alanine racemases but not brain serine racemase. Therefore, alanine racemases and serine racemases are classified into three groups: bacterial alanine racemases plus serine racemase from *E. gallinarum*, fungal alanine racemase, and serine racemase of mammalian brain. Similar structural divergence has been found between other types of PLP enzymes, as exemplified by ornithine decarboxylases. Bacterial and eucaryotic ornithine decarboxylases differ markedly from each other, and the eucaryotic one shares the same protein fold with bacterial alanine racemase (9).

Contrary to a long-standing belief, various D-amino acids, including D-alanine and D-serine, have been found in yeasts in a peptide-bound form (15). Although no evidence has been obtained for the occurrence of a free form of D-amino acids, they are most probably produced in yeast cells upon proteolytic cleavage of the peptides. Therefore, it is reasonable to assume that amino acid racemases occur in yeasts. In fact, we have found that fission yeast, *Schizosaccharomyces pombe*, has a gene encoding a putative amino acid racemase similar to bacterial alanine racemases and the serine racemase of *E. gallinarum*. We have named the gene *alr1*$^+$ because it shows alanine racemase activity upon cloning and expression in *E. coli*.

We report here the gene cloning, purification, and characterization of the gene product, alanine racemase ALR1p. Furthermore, we show that the Alr1p protein is involved in the catabolism of D-alanine in *S. pombe*, which we have confirmed through construction of a deletion mutation of the gene in *S. pombe* and heterologous expression of the gene in *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Materials. Oligonucleotides were provided by Espec Oligo Service, Tsukuba, Japan; restriction endonucleases were from Takara Shuzo, Kyoto, Japan, or New England Biolabs, Beverley, Mass.; D-amino acid oxidase (EC 1.4.3.3) of hog kidney and L-lactate dehydrogenase (EC 1.1.1.27) of pig heart were from Boehringer GmbH, Mannheim, Germany; and L-alanine dehydrogenase of *Bacillus stearothermophilus* (EC 1.4.1.1) was from Unitika, Osaka, Japan. All other chemicals were of analytical grade.

Strains and culture conditions. All *S. pombe* strains described here were derived from the strain SP1 (h^+ *leu1-32 ura4-D18*) (25). *S. cerevisiae* ATCC 42752 (*MAT*α *ura3*) was used for the heterologous expression of the *alr1*$^+$ gene

* Corresponding author. Mailing address: Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan. Phone: 81-774-38-3240. Fax: 81-774-38-3248. E-mail: esaki@scl.kyoto-u.ac.jp.

from *S. pombe*. *S. pombe* and *S. cerevisiae* were routinely grown in a YPD medium (1% yeast extract, 2% peptone, 2% glucose) or an SD medium (0.67% Bacto yeast nitrogen base, 2% glucose). The minimal medium for *S. pombe* contained 2% glucose, 0.05% KH_2PO_4, 0.036% CH_3COOK, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.01% $CaCl_2 \cdot 2H_2O$, 0.1% (vol/vol) trace element solution (0.05% H_3BO_4, 0.004% $CuSO_4 \cdot 5H_2O$, 0.01% KI, 0.02% $FeCl_3 \cdot 6H_2O$, 0.04% $MnSO_4 \cdot H_2O$, 0.02% $Na_2MoO_4 \cdot 2H_2O$, and 0.04% $ZnSO_4 \cdot 7H_2O$), 1% (vol/vol) biotin (10 mg/ml), 1% (vol/vol) vitamin solution (0.1% pantothenic acid calcium salt, 1% nicotinic acid, and 1% myoinositol), and 10 mM ammonium sulfate. Cells were cultivated at 32°C with reciprocal shaking. The utilization of D- or L-alanine by *S. pombe* was examined as follows. *S. pombe* cells were harvested at a logarithmic growth phase, washed three times with minimal medium (described above) from which ammonium sulfate was omitted, and then cultured in the minimal medium or the alanine minimal medium, in which ammonium sulfate of the minimal medium described above was replaced by D- or L-alanine as a sole nitrogen source. Each strain was cultivated at 32°C with aeration. *Escherichia coli* cells were cultured at 37°C in Luria-Bertani broth supplemented with ampicillin (100 μg/ml), if necessary.

Cloning of the alanine racemase gene *alr1*$^+$. The *alr1*$^+$ gene of *S. pombe* was amplified by PCR with the genomic DNA of *S. pombe* as a template. The sense primer used for PCR was 5′-CAT TTA AAT TCA TAT GAG AGG TGC AAA GTC CCg-3′ (*Nde*I site underlined), and the antisense primer was 5′-GGT AGC ATA CCA ATG AAG CTT TGC TC-3′ (*Hin*dIII site underlined). The amplified product was digested with *Nde*I and *Hin*dIII, and the resulting fragment was ligated into pET21-a previously digested with the same enzymes to yield the plasmid pETALR1. *E. coli* BL21(DE3) was used as a host. In order to express the *alr1*$^+$ gene under the control of the *GAL1* promoter in *S. cerevisae*, another construct of the gene was prepared; *Bam*HI and *Hin*dIII sites were introduced at the 5′ and 3′ ends of the *alr1*$^+$ gene with a sense primer of 5′-GAT TCA TTT AAA GGA TCC ATG AGA GGT GCA AAG-3′ (*Bam*HI site underlined) and an antisense primer of 5′-GGT AGC ATA CCA ATG AAG CTT TGC TC-3′ (*Hin*dIII site underlined). The amplified product digested with *Bam*HI and *Hin*dIII was introduced between the *Bam*HI and *Hin*dIII sites of the p426Gal1 plasmid. *S. cerevisiae* ATCC 42752 (*MAT*α *ura3*) was transformed with the resulting plasmid, p426Gal1ALR1. Positive clones were selected on a minimal medium plate lacking uracil.

Assays. Alanine racemase was assayed in either direction of interconversion between L-alanine and D-alanine. The activity in the direction from D to L was determined with a mixture (1 ml) containing 100 mM CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid] (pH 9.0), 0.15 U of L-alanine dehydrogenase, 50 mM D-alanine, 2.5 mM NAD^+, and enzyme. The increase in absorbance at 340 nm due to NADH formation was monitored at 37°C. The other direction was followed at 37°C in a mixture (1 ml) containing 100 mM CHES (pH 9.0), 0.75 U of D-amino acid oxidase, 5.5 U of L-lactate dehydrogenase, 0.16 mM NADH, 50 mM L-alanine, and enzyme. Decrease in absorbance at 340 nm was monitored. The effect of pH on alanine racemase activity was examined with Bis-Tris propane (pH 6.5 to 9.0), CHES (pH 9.5 to 10.0), or CAPS [3-(cyclohexylamino)-1-propane sulfonic acid] (pH 9.5 to 11.0) replacing CHES (pH 9.0) in the mixtures described above. Activities toward other substrates were determined in a mixture (0.05 ml) containing 100 mM CHES buffer (pH 9.0), 50 mM L-amino acid, and enzyme. The reaction was carried out at 37°C for 30 min. The amount of the corresponding D-amino acid formed was determined by high-performance liquid chromatography after derivatization as described previously (27). Protein was assayed according to the method described by Bradford (2a) with bovine serum albumin as a standard.

Purification of the Alr1p protein. All procedures were carried out at 4°C. The standard buffer used throughout the purification was a 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 10 μM PLP. *E. coli* BL21(DE3) cells harboring pETALR1 (about 50 g, wet weight) were suspended in 200 ml of the standard buffer and sonicated. The lysate was centrifuged at 18,000 × *g* for 20 min, and protamine sulfate was added to the supernatant solution to a final concentration of 0.4%, followed by centrifugation. After dialysis, the supernatant solution was loaded onto a DEAE-TOYOPEARL column (200 ml) equilibrated with the standard buffer. The column was washed with the standard buffer, and the enzyme was eluted with a linear gradient of 0 to 0.4 M KCl in the standard buffer. The active fractions were combined and brought to 30% saturation with ammonium sulfate. After centrifugation, the supernatant solution was loaded onto a butyl-TOYOPEARL column (50 ml) equilibrated with the standard buffer supplemented with ammonium sulfate (30% saturation). The column was washed with the standard buffer containing a 10% saturation of ammonium sulfate, and then the enzyme was eluted with a linear gradient of 10 to 0% saturation with ammonium sulfate in the standard buffer. The active fractions were combined and dialyzed against the standard buffer. After centrifugation, the supernatant solution was applied to a Q-Sepharose HP column (50 ml) equilibrated with the standard buffer. The column was washed with 0.1 M KCl, and the enzyme was eluted with a linear gradient of 0.1 to 0.3 M KCl in the standard buffer. The pooled active fractions were brought to 30% saturation with ammonium sulfate, and the supernatant solution was loaded onto a butyl-TOYOPEARL column (50 ml) equilibrated with the standard buffer supplemented with 30% saturation of ammonium sulfate. The column was washed with the standard buffer containing 10% saturated ammonium sulfate, and the enzyme was eluted with the standard buffer. The active fractions were combined and dialyzed against a 2 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 10 μM PLP. After centrifugation, the supernatant was loaded onto a Gigapite column (30 ml) equilibrated with a 2 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 10 μM PLP. The enzyme was eluted with the same buffer, and the active fractions were pooled and dialyzed against the standard buffer. The purity of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting. The extracts of *S. pombe* cells were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes. Western blot analysis was performed with the anti-Alr1p antiserum (raised by standard procedures), and the proteins were detected by chemiluminescence using CDP-Star (Roche Diagnostics, Basel, Switzerland).

Disruption of the *alr1*$^+$ gene in *S. pombe*. For the construction of the *alr1::LEU2* yeast strain, a *LEU2* deletion cassette for *alr1*$^+$ was prepared by PCR as follows. Two DNA fragments, one (0.6 kbp) containing the upstream region and the other (0.8 kbp) containing the downstream region of *alr1*$^+$, were obtained by PCR with the genomic DNA of *S. pombe* as a template. The primers used were as follows: for the upstream region, 5′-CAG TTT CAT TTC AAA ACG AAT GTT ATA GAA CAT C-3′ (sense primer) and 5′-CGA ATT CCT GCA GCC CGG GGG ATC CAA TTG CTA AAA CAT GAC CGC TTG G-3′ (antisense primer); and for the downstream region, 5′-GCT TAT CGA TAC CGT CGA CCG GAG AGT TCC TTT ACA GTA CAC-3′ (sense primer) and 5′-CAG ACT ACA TAG ATT TAC ACA TCC TTC TTT ACC-3′ (antisense primer). The 3′ end of the upstream region and the 5′ end of the downstream region are complementary to the 5′ and 3′ ends of the *LEU2* gene, respectively. The second PCR was carried out with the DNA fragment (2.2 kbp) containing the *LEU2* gene as a template and the products of the first PCR as primers. The resulting PCR product (3.6 kbp) containing the *LEU2* gene flanked by the upstream and downstream regions of *alr1*$^+$ was used for the transformation of the haploid yeast strain. Positive clones were selected on a minimal medium not supplemented with L-leucine, and disruption of *alr1*$^+$ was verified by Southern blotting and PCR analysis. However, the resulting *alr1*Δ strain (h^+ *leu1-32 ura4-D18 alr1::LEU2*) and the SP1 strain are not suitable for examination of nitrogen sources because even low concentrations of uracil and leucine, which must be added to supplement the defects in mutated *ura4* and *leu1* genes, respectively, can support the growth without addition of nitrogen sources. Therefore, nitrogen sources for the deletion mutant were examined by introduction of the *ura4*$^+$ gene on a plasmid, pART2, which we prepared by exchanging the *LEU2* marker in the pART1 plasmid with the *ura4*$^+$ marker derived from the pBSUra4 plasmid at the *Hin*dIII and *Sal*I sites. The SP1 strain was transformed with plasmids pART1 and pART2 and then used for examination of the utilization of nitrogen sources.

RESULTS

Cloning of the alanine racemase gene from *S. pombe*. We found by a BLAST search of protein databases that *S. pombe* contains a gene in its chromosome III (SPCC965.08c) that is homologous to those of bacterial alanine racemases and the serine racemase of *E. gallinarum* (1) (Fig. 1). Similarities in the amino acid sequence of the putative gene product were 39% to Alr alanine racemase from *E. coli* and 28% to serine racemase of *E. gallinarum*. We confirmed by cloning and expression of the gene whether it codes for alanine racemase or serine racemase. We constructed a plasmid, pETALR1, by ligation of the gene amplified by PCR into an expression vector, pET21-a. An *E. coli* BL21(DE3) clone harboring pETALR1 showed 18 U of alanine racemase activity/mg, which was about 220-fold higher than that of the control clone carrying the empty vector

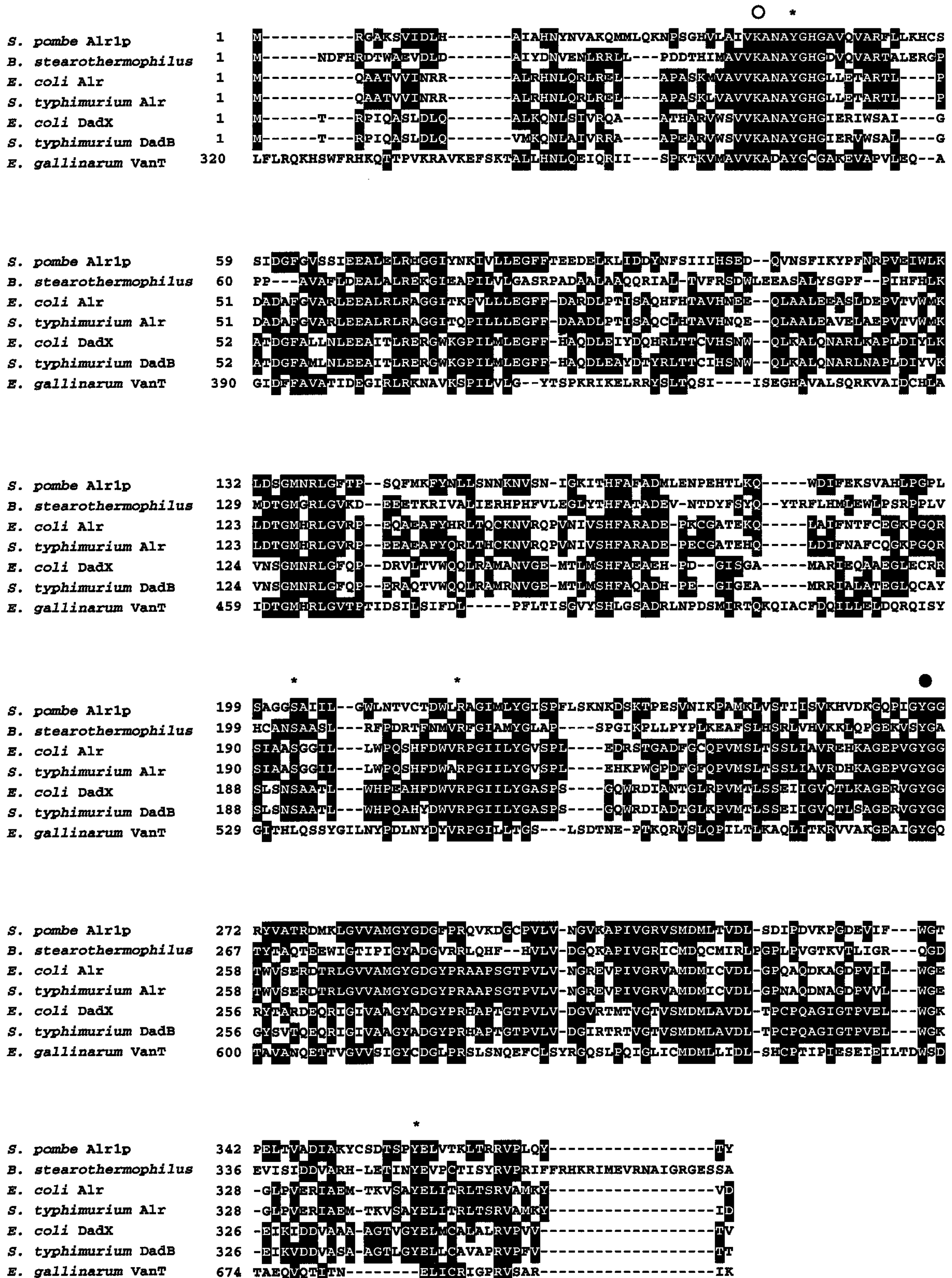

FIG. 1. Comparison of the amino acid sequences of *S. pombe* Alr1p (GenBank accession no. AL023590) with the bacterial alanine racemases (plus GenBank accession numbers) of *B. stearothermophilus* (M19142), *E. coli* Alr (AE00478), *S. enterica* serovar Typhimurium Alr (M12847), *E. coli* DadX (LO2948), *S. enterica* serovar Typhimurium DadB (KO2119), and *E. gallinarum* VanT (AF162694). The amino acid sequences were aligned with the MegAlign program of the DNASTAR package (5). Conserved residues are boxed. The open circle indicates the Lys residue that binds PLP and is proposed to abstract α-proton from D-alanine. The closed circle indicates the Tyr residue that is proposed to abstract α-hydrogen from L-alanine. Asterisks show the residues involved in binding with PLP.

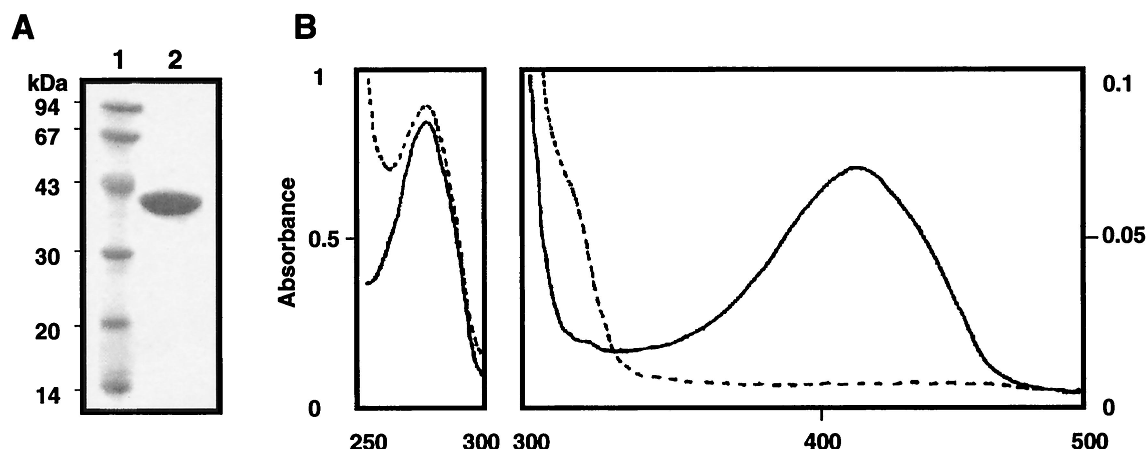

FIG. 2. SDS-PAGE gel (A) and absorption spectra (B) of purified *S. pombe* Alr1p. (A) Lane 1, marker proteins (the molecular masses are shown in kilodaltons); lane 2, purified *S. pombe* Alr1p (10 μg). Protein was stained with Coomassie brilliant blue R-250. (B) The spectrum of the enzyme solution (1.0 mg/ml) in a 20 mM potassium phosphate buffer (pH 7.8) containing 0.1% 2-mercaptoethanol is shown as a solid line, while that of the enzyme dialyzed against 500 volumes of a 20 mM potassium phosphate buffer (pH 7.8) containing 0.1% 2-mercaptoethanol and 10 mM sodium borohydride is shown as a dashed line.

pET21-a. The *E. coli* BL21/pETALR1 clone also showed racemization activity toward serine, but the activity was much lower than with alanine. Therefore, we named the alanine racemase gene *alr1*$^{+}$.

The alanine racemase of *B. stearothermophilus* has been studied extensively by X-ray crystallography and site-directed mutagenesis, and Lys39 and Tyr265 most probably function as catalytic bases abstracting the α-hydrogen of D-alanine and L-alanine, respectively (20, 31, 32). X-ray crystallography of the enzyme also showed that Tyr43, Arg136, Ser204, and Arg219 interact with PLP (20). All these residues are conserved in alanine racemase of *S. pombe* (Fig. 1).

Purification and characterization of alanine racemase from *S. pombe*. Alanine racemase of *S. pombe* (Alr1p) was purified to homogeneity through six steps from the cell extract of *E. coli* BL21(DE3) cells harboring pETALR1 (Fig. 2A). A summary of the purification is shown in Table 1.

The purified enzyme absorbed at around 420 nm (Fig. 2B). When the enzyme was dialyzed against sodium borohydride, the 420-nm peak disappeared and a new band appeared at around 330 nm (Fig. 2B). The enzyme was completely inactivated by the treatment. The behavior is characteristic of PLP-dependent enzymes, indicating that Alr1p contains PLP as a coenzyme. The sequence around Lys38 is conserved among all bacterial alanine racemases, and X-ray crystallography of the *B. stearothermophilus* enzyme shows that the corresponding lysine residue (Lys39) of the enzyme forms an aldimine linkage with PLP (20). Therefore, Lys38 of Alr1p is most probably bound to PLP and functions as a catalytic base abstracting α-hydrogen from D-alanine according to the mechanism proposed for the *B. stearothermophilus* enzyme (31).

X-ray crystallography of the *B. stearothermophilus* enzyme has shown that it is a dimer of identical subunits (20). Tyr265 of the enzyme functions as the base functioning pairwise with Lys39, as described above, but it comes from another subunit. Tyr265 is conserved (as Tyr269 in Alr1p) as well as many residues functioning as a kind of glue (i.e., residues forming ion pairs, hydrophobic interactions, and hydrogen bonds) at the subunit interface, such as Glu69 (Glu70 in Alr1p), Arg136 (Arg138 in Alr1p), and His166 (His167 in Alr1p) (20). This strongly suggests that Alr1p also occurs as a dimer. However, Alr1p showed an unexpected molecular weight of about 44,000 upon gel filtration through a Superose12 column, suggesting a monomeric structure. Both alanine racemases of *Salmonella enterica* serovar Typhimurium, DadB and Alr, were thought to be monomers based on the results of gel filtration before X-ray crystallography of the *B. stearothermophilus* enzyme (6). If all these enzymes, including Alr1p, are dimers, then their unusual behaviors on gel filtration are due to a characteristic nature common to the enzymes, such as sticky surfaces of the protein molecules.

Alr1p is virtually specific toward alanine. Apparent K_m and V_{max} values were determined: L-alanine, 5.0 mM and 670 μmol/min/mg, respectively; and D-alanine, 2.4 mM and 350 μmol/min/mg, respectively. V_{max}/K_m values for the conversions from L- to D-alanine and D- to L-alanine were similar to each other: 134 and 146 μmol/min/mg/mM, respectively. The ratio of the values is theoretically unity (3), and the experimental value (0.92) is consistent with the fact that the enzyme catalyzes racemization (3). L-Serine and L-2-aminobutyrate are poor substrates of Alr1p: their rates are 3.7 and 0.37% of that

TABLE 1. Purification of ALR1p from *E. coli* BL21(DE3) cells harboring pETALR1

Step of purification	Total protein (mg)	Total activity (U)	Sp act (U/mg)[a]
Crude extracts	3,200	21,000	5.5
Protamine sulfate	1,800	19,000	11
DEAE-TOYOPEARL	340	14,000	42
First Butyl-TOYOPEARL100	100	12,000	120
Q-Sepharose HP	39	8,200	210
Second Butyl-TOYOPEARL100	25	7,500	300
Gigapite	20	6,800	340

[a] Alanine racemase activity was determined as described in Materials and Methods with D-alanine as a substrate at 37°C.

of L-alanine, respectively. The opposite situation can be seen for the VanT serine racemase of *E. gallinarum*: L-alanine is racemized at a rate that is 14% of that of L-serine (2). No activity of Alr1p was found toward L-valine, L-methionine, L-tryptophan, L-phenylalanine, L-histidine, L-tyrosine, L-threonine, L-asparagine, L-glutamine, L-aspartate, L-glutamate, L-arginine, L-lysine, L-norvaline, L-norleucine, L-homoserine, or L-ornithine.

Alr1p showed the maximum activity toward D- and L-alanine at pHs 9.0 and 9.5, respectively. The alkalophilic nature is common to most bacterial alanine racemases.

Role of D- and L-alanine as a sole nitrogen source in the growth of *S. pombe*. We found that *S. pombe* grows on either enantiomer of alanine as a sole nitrogen source (Fig. 3A). Since *S. pombe* has Alr1p, which catalyzes the interconversion between the enantiomers, it is reasonable to assume that the yeast survives on either enantiomer through racemization with only a catabolic pathway for L- or D-alanine. Alternatively, it may have a separate pathway for each enantiomer. Therefore, we constructed a deletion mutant of *S. pombe* lacking the *alr1*$^+$ gene (Fig. 3B). The *alrl*Δ strain grew as well as the original strain on a complete medium such as YPD, indicating that the *alr1*$^+$ gene is not essential for *S. pombe*. We found, however, that the deletion mutant grows about 50% less slowly than the original strain on a medium containing D-alanine as a sole nitrogen source (Fig. 3B). The response to the antipode was different: both strains grew equally well on L-alanine as a sole nitrogen source. Moreover, the growth rate on L-alanine was twice that on D-alanine. These results indicate that *S. pombe* has catabolic pathways for each enantiomer of alanine and that the pathway for L-alanine plays a major role in the catabolism of D-alanine by coupling with racemization. L-Alanine is utilized in *Candida maltosa* by L-alanine aminotransferase, which is inducibly formed by L-alanine (26). *S. pombe* also has two putative homologs of this enzyme (GenBank accession no. CAB46671.1 and CAA93786.1). D-Alanine is utilized by D-amino acid oxidase in *Rhodotorula gracilis* and *Candida boidinii* (8, 17). We found that *S. pombe* grown on D-alanine as a sole nitrogen source also shows D-amino acid oxidase activity toward D-alanine as a substrate (data not shown). Thus, these results suggest that alanine racemase participates in the catabolism of D-alanine by providing L-alanine aminotransferase with its substrate L-alanine and thereby supports the growth of the yeast, which would otherwise not be efficient with D-amino acid oxidase alone. We found by Western blotting that Alr1p is produced in a minimal medium containing D-alanine as a sole nitrogen source (Fig. 4). However, no positive bands reactive with the anti-Alr1p antibody were observed either when ammonium sulfate or L-alanine replaced D-alanine or in a complete medium, such as YPD (Fig. 4).

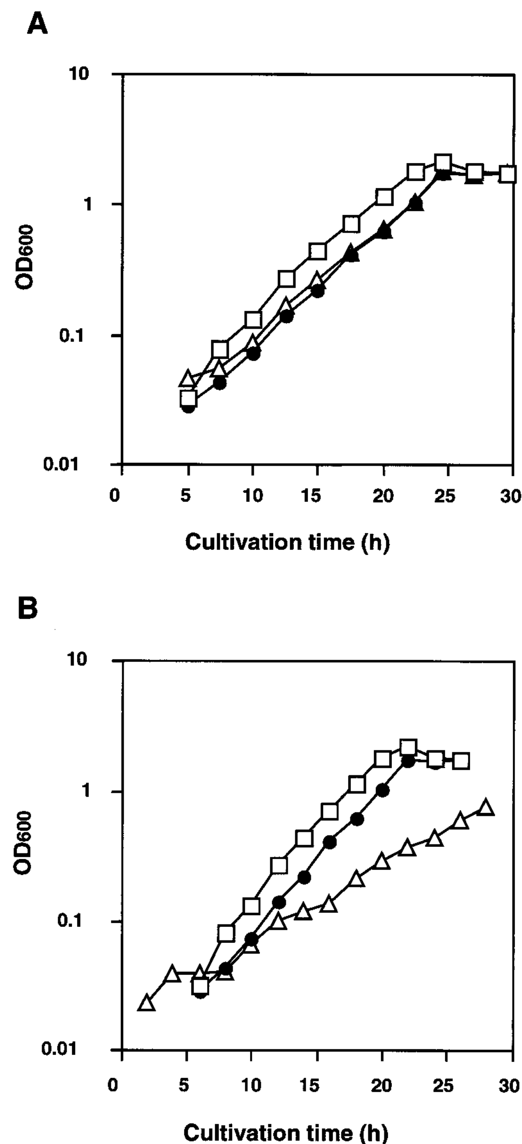

FIG. 3. Growth characteristics when D- or L-alanine was a sole nitrogen source of wild-type *S. pombe* (A) and a deletion mutant (*alr1*Δ) of *S. pombe* (B). (A) *S. pombe* SP1 (Leu$^-$ Ura$^-$) was made Leu$^+$ Ura$^+$ by introduction of both pART1 and pART2 (see Materials and Methods). (B) *alr1*::*LEU2* strain (Alr1Δ Leu$^+$ Ura$^-$) derived from *S. pombe* SP1 was transformed with pART2 to become Alr1Δ Leu$^+$ An Ura$^+$. Each recombinant was precultured in the minimal medium described in Materials and Methods and transferred to a culture containing, as a sole nitrogen source, 5 mM ammonium sulfate (squares), 10 mM D-alanine (triangles), or L-alanine (circles). The culture was grown at 32°C with reciprocal shaking.

Effect of heterologous expression of the *S. pombe alr1*$^+$ gene on *S. cerevisiae*. *S. cerevisiae* contains no genes for homologs of bacterial alanine racemases in its chromosome. Gly1p for threonine aldolase is similar to fungal alanine racemase in primary structure but shows no alanine racemase activity (12). Contrary to *S. pombe*, D-alanine is toxic to *S. cerevisiae* (19, 23). When the *alr1*$^+$ gene of *S. pombe* was cloned into *S. cerevisiae* with an expression vector, p426Gal1, controlled by the *GAL1* promoter, the recombinant *S. cerevisiae* carrying the resulting plasmid, p426Gal1ALR1, grew well on a medium containing glucose as a carbon source and either ammonium sulfate or L-alanine as a nitrogen source in the same manner as the control recombinant carrying the empty p426Gal1 vector. However, neither of the two recombinant yeasts grew on D-alanine as a sole nitrogen source (Fig. 5). When the *GAL1* promoter was turned on by addition of galactose, only the recombinant carrying the *alr1*$^+$ gene grew on a medium containing D-alanine as a sole nitrogen source and raffinose as a carbon source (Fig. 5). This indicates that D-alanine is converted to its antipode by Alr1p and is then utilized as a nitro-

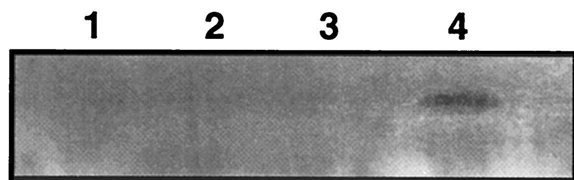

FIG. 4. Western blot analysis of Alr1p. *S. pombe* SP1 cells exponentially growing on YPD medium (lane 1) or a minimum medium containing, as a sole nitrogen source, ammonium sulfate (lane 2), L-alanine (lane 3), or D-alanine (lane 4) were harvested. Cell extracts were subjected to SDS–13.0% PAGE, blotted onto a polyvinylidene difluoride membrane, and analyzed with polyclonal anti-Alr1p antibody. Each lane was loaded with 10 μg of protein.

gen source by the recombinant carrying *alr1*$^+$. Moreover, the growth rate of the recombinant on the D-alanine medium is similar to that on the glucose-ammonium sulfate medium (data not shown). Therefore, D-alanine is not toxic to the recombinant yeast expressing Alr1p. These results indicate that the heterologous Alr1p not only renders *S. cerevisiae* capable of utilization of D-alanine as a nitrogen source but also relieves the yeast from the toxicity of D-alanine.

DISCUSSION

PLP enzymes have been classified into five groups with different fold types (9). Bacterial alanine racemases belong to fold type III (7, 9, 11), while fungal alanine racemase belongs to fold type I (4, 12). This is probably a case of convergent evolution. The enzyme of fold type I has possibly evolved from an ancestor protein common to that of threonine aldolase in the fungus.

The BLAST search in GenBank and EMBL databases showed that, in addition to occurring in eubacteria, the alanine racemase of fold type III occurs only in *S. pombe*; no homologs could be found in the database of expressed sequence tags or in the genomes of archaea and eucaryotes, including *S. cerevisiae, Caenorhabditis elegans*, and *Drosophila melanogaster*. The phylogenetic analysis of alanine racemases indicates that they are classified into three groups (Fig. 6). It is interesting that Alr1p of *S. pombe* shows high similarity only to alanine racemases of γ-proteobacteria (phylum of gram-negative bacteria) (Fig. 6, group A). Lower similarity was found when Alr1p was compared to other groups: group B, containing mainly high-G+C-content gram-positive bacteria, and group C, containing mainly low-G+C-content gram-positive bacteria. The *alr1*$^+$ gene of *S. pombe* was possibly acquired from γ-proteobacteria through some events of horizontal gene transfer, such as conjugation: *S. pombe* is known to be a recipient of genes from *E. coli* through direct conjugation (21). Whatever the mechanism of acquisition of the enzyme gene was in the past, it has been kept by *S. pombe* throughout evolution. The enzyme has probably been required by this yeast in its habitat due to, for example, the availability of D-alanine as a sole nitrogen source.

E. coli and *S. enterica* serovar Typhimurium contain two isoenzymes of alanine racemases with distinct physiological functions: one is biosynthetic, and the other is catabolic (18). Alr alanine racemases of *S. enterica* serovar Typhimurium and *E. coli* are biosynthetic and constitutive, while DadB (*S. enterica* serovar Typhimurium) and DadX (*E. coli*) isozymes are catabolic and inducibly formed by L-alanine (13, 30, 34). Alr provides D-alanine for the biosynthesis of peptidoglycans. Both *S. enterica* serovar Typhimurium and *E. coli* grow on D-alanine as the sole carbon and energy source because they have D-amino acid dehydrogenase decomposing D-alanine into pyruvate and ammonia. However, they have no direct catabolic pathways for L-alanine and get it racemized with the DadB and DadX racemases (16, 33). *S. pombe* differs from these bacteria in that it has direct catabolic pathways for each enantiomer of alanine. A methylotrophic yeast, *C. boidinii*, also grows on D-alanine as a sole nitrogen or carbon source. The utilization of D-alanine is attributed to D-amino acid oxidase (8, 17), but deletion of the enzyme gene showed no significant effect on the growth on D-alanine as a sole nitrogen source (37). This suggests that another degradation pathway for D-alanine occurs in this methylotrophic yeast. Alternatively, it may have alanine racemase and a degradation pathway for L-alanine in the same manner as *S. pombe*. However, *S. cerevisiae* is quite different from these yeasts because it cannot utilize D-alanine. Similar examples are known in nitrogen metabolic pathways: a pathway common to *S. pombe* and *Candida* is missing in *S. cerevisiae*, as exemplified by purine degradation through uric acid and allantoin (17, 28). *S. cerevisiae* lacks uricase and cannot utilize purine as a nitrogen source.

In *S. cerevisiae*, various D-amino acids are incorporated by the general amino acid permease, which is characterized by its low affinity and high capacity (19, 24). However, some of them, such as D-alanine, D-serine, D-leucine, and D-tyrosine, are toxic to the yeast (23). The toxicity of D-leucine and D-tyrosine is explained by their unfavorable reactions with the corresponding tRNAs by leucyl- and tyrosyl-tRNA synthetases, respectively. D-Tyrosyl-tRNA^{Tyr} deacylase (*DTD1*) of *S. cerevisiae*

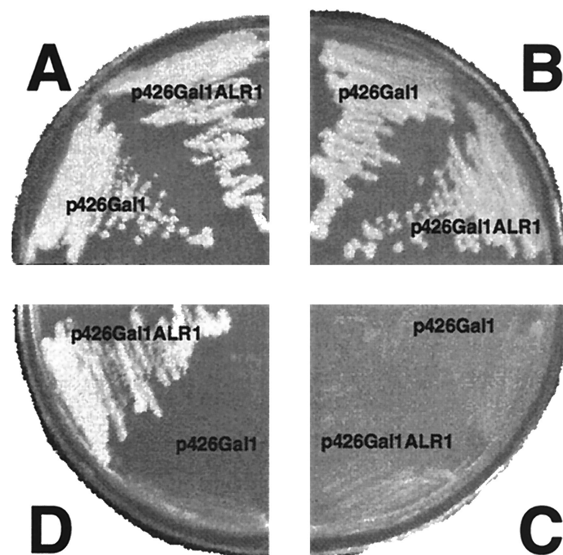

FIG. 5. Heterologous expression of *alr1*$^+$ gene in *S. cerevisiae S. cervisiae* cells harboring p426Gal1 or p426Gal1ALR1 were streaked on agar plates containing the minimal medium supplemented with 5 mM ammonium sulfate (see Materials and Methods) (A), 10 mM L-alanine (B), or 10 mM D-alanine (C and D). (D) Glucose in the medium was replaced by a mixture of raffinose (2%) and galactose (0.2%). After 3 days of incubation at 30°C, each plate was photographed.

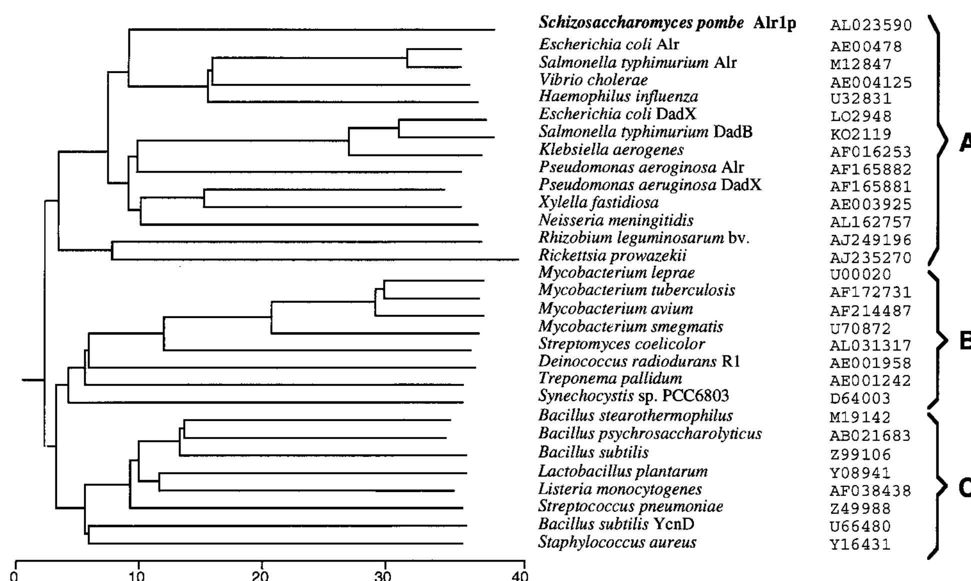

FIG. 6. Phylogenetic tree for alanine racemases. Sequences obtained from GenBank were aligned, and a phylogenetic tree was constructed with the MegAlign program by using the CLUSTAL method (5). The scale indicates the percentages of sequence divergence. The accession numbers of alanine racemase genes from various sources are shown.

hydrolyzes resultant D-leucyl-tRNA^{Leu} and D-tyrosyl-tRNA^{Tyr} and thereby reduces their toxicity (23). A similar defense system against the toxicity of D-leucine and D-tyrosine probably occurs in *S. pombe* because it also has a gene for the *DTDI* homolog (GenBank accession no. CAB16293.1) although it has not yet been proven biochemically. However, little is known about the mechanism of toxicity of D-alanine and D-serine toward *S. cerevisiae*. Whatever the mechanism of toxicity, *S. cerevisiae* recombinant cells expressing alanine racemase are relieved from the toxicity of D-alanine. This may be another role of alanine racemase in *S. pombe*. A deletion mutant of *S. pombe* lacking both *alr1*$^+$ and the D-amino acid oxidase gene is being prepared in order to show the effect of D-alanine and D-serine on this yeast.

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