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Lysine racemase from a lactic acid bacterium, *Oenococcus oeni*: structural basis of substrate specificity

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***Oenococcus oeni*, a lactic acid bacterium, possesses a lysine racemase, which has a specific activity towards basic amino acids. A comparison of amino acid residues around the active site suggested that Ile222 and Tyr354 of the *Geobacillus stearothermophilus* alanine racemase, which shares 60% sequence similarity with lysine racemase, were replaced by Thr224 and Trp355 in the *O. oeni* lysine racemase. T224I/W355Y double mutations significantly decreased the activity of lysine racemase, whereas I222T/Y354W double mutations endowed alanine racemase with lysine racemization activity. These results suggest that the two residues play an important role in lysine racemization.**

Keywords: alanine racemase/lactic acid bacteria/lysine racemase/pyridoxal 5′-phosphate/substrate specificity.

Abbreviations: DAAT, D-amino acid transaminase; D-Ala, D-alanine; D-Glu, D-glutamate; D-Lys, D-lysine; IPTG, isopropyl β-D-thiogalactopyranoside; KPB, potassium phosphate buffer; PCR, polymerase chain reaction; PLP, pyridoxal-5′-phosphate.

Because of the physiological significance of D-amino acids (*1*), those occurring in foods need to be investigated. Recently, we analysed fermenting wine samples and detected D-alanine (D-Ala), D-glutamate (D-Glu) and D-lysine (D-Lys) (*2*). Because D-Lys is not frequently found in foods, unlike D-Ala and D-Glu, we attempted to clarify the origin of D-Lys in the fermenting wine. A microfloral analysis of the fermenting wine suggested that the D-amino acids were produced by the lactic acid bacterium *Oenococcus oeni*, which is responsible for malolactic fermentation (*2*). Generally, D-amino acids are made from their corresponding L-amino acids by the action of an amino acid racemase. Bacterial amino acid racemases are divided into two groups: pyridoxal 5′-phosphate (PLP)-independent enzymes, such as aspartate racemase and glutamate racemase, and PLP-dependent enzymes, including alanine racemase (*3*), amino acid racemase of broad substrate specificity (*4*), arginine racemase (*5*) and others. These bacterial PLP-dependent racemases share sequence homology, and amino acid racemase of broad substrate specificity and arginine racemase have activity towards various amino acids, including lysine. Thus, we searched for gene(s) homologous to the PLP-dependent amino acid racemase gene in the metagenome of fermenting wine to find the racemase gene responsible for D-Lys synthesis.

Oenococcus oeni PSU-1, whose genome has been elucidated, contains two alanine racemase gene homologs, *oeoe_0162* and *oeoe_1641*. On the basis of the sequences of these genes, we prepared primers and amplified DNA fragments by polymerase chain reaction (PCR) using a metagenomic DNA mixture extracted from a white wine sample (*2*) as a template. The DNA fragment obtained with the primers designed based on the sequence of *oeoe_1641* was revealed to encode alanine racemase (data not shown). The PCR product obtained with the primers 0162FW and 0162RV designed according to the sequence of *oeoe_0162* (Supplementary Table S1) was ligated into the *Xho*I-*Bam*HI sites of the plasmid pCold I (Takara Shuzo, Kyoto, Japan). The amplified DNA fragment of the resultant plasmid, pC1-0162m, was sequenced and compared with *oeoe_0162*. One difference was detected: A132 of *oeoe_0162* from the PSU-1 strain was replaced by a C in the sequence of the amplified DNA fragment. However, the mutation is silent, and the deduced amino acid sequences from the two genes were identical (data not shown).

Escherichia coli BL21 cells harbouring pC1-0162m were then cultivated in Luria Bertani medium containing ampicillin (100 μg·ml^{-1}). Expression of the putative racemase was induced by cold shock treatment at 15°C in the presence of 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After further cultivation for 24 h at 15°C, the cells were harvested. The putative racemase (which we have called OEOE0162 hereafter, as the putative racemase is identical to OEOE0162 at the amino acid level) was purified using a His Bind™ Resin column (Novagen, Tokyo, Japan) according to the supplier's recommended protocol. The purified protein was dialyzed against 50 mM potassium phosphate buffer (KPB) (pH 8.0) containing 20 μM PLP and subjected to the following analyses.

The ultraviolet-Vis absorption spectrum of OEOE0162 exhibited an absorption peak at ~420 nm (data not shown), suggesting that the protein contained PLP. The substrate specificity of OEOE0162 was examined by an end-point assay, described here. First, OEOE0162 was added to a reaction mixture (final volume of 100 μl) consisting of 50 mM KPB (pH 8.0), 20 μM PLP and 40 mM each of L- or D-amino acid (because of its insolubility, tyrosine was

used at 1 mM). This mixture was incubated for 10–30 min at 30°C, boiled for 10 min and then centrifuged at 20,000 × *g* for 15 min at 4°C. Amino acid content of the resulting supernatant was analysed by high-performance liquid chromatography, as described previously (*2*). OEOE0162 exhibited racemase activity towards lysine, arginine and ornithine (Supplementary Table S2). All other standard amino acids, including alanine, were inert (data not shown). Because of OEOE0162's greater active towards lysine than both arginine and ornithine, we propose *oeoe_0162* to be named as '*lysine racemase*'.

The properties of lysine racemase including thermal stability, pH profile and kinetics were determined by the following rate assay methods. The rate of L- to D-Lys reaction was determined using a reaction mixture (final volume of 1 ml) consisting of 50 mM KPB (pH 8.0), 20 μM PLP, 50 mM L-Lys, 10 mM α-ketoglutarate, 2 mM *o*-amino-benzaldehyde, 100 μg D-amino acid transaminase (DAAT) (*6*) and lysine racemase. The reaction was started by the addition of lysine racemase and monitored at 30°C by following the increase in absorbance at 465 nm derived from the formation of adduct from *o*-amino-benzaldehyde and Δ^1-piperidine-2-carboxylic acid. The latter compound is formed from α-keto-ε-aminocaproic acid—a DAAT reaction product from D-Lys, which is produced from L-Lys by lysine racemase. The D- to L-Lys reaction was assayed using the same method except for a substitution of D-Lys and L-Lys transaminase (LysAT) (*7*) for L-Lys and DAAT, respectively. In this reaction, lysine racemase converts D-Lys to L-Lys, which is then converted to α-aminoadipate-δ-semialdehyde on transamination by LysAT. α-Aminoadipate-δ-semialdehyde is spontaneously dehydrated to form Δ^1-piperidine-6-carboxylic acid, which reacts with *o*-amino-benzaldehyde to produce an adduct absorbing at 465 nm. These rate assay methods using D- and L-Lys can be also used for assays with D- and L-ornithine, respectively.

The thermal stability of lysine racemase was examined by measuring residual activity using L-Lys as a substrate after incubation of the enzyme at various temperatures for 60 min. The enzyme was stable on heat treatment at 50°C, and the residual activity gradually decreased as the treating temperature increased (Supplementary Fig. S1A). The optimum pH of the enzyme was found to be 9.0 (Supplementary Fig. S1B). The enzyme exhibited maximum activity under alkaline conditions, similar to the well-characterized *Geobacillus stearothermophilus* alanine racemase (*8*).

The kinetic parameters of the lysine racemase are summarized in Table I. The agreement between the $k_{cat} \cdot K_m^{-1}$ values for L-Lys ($19\,s^{-1} \cdot mM^{-1}$) with that for D-Lys ($17\,s^{-1} \cdot mM^{-1}$) confirms that the enzyme is a racemase (*9*). Although lysine racemase shares ~60% amino acid sequence similarity with the *G. stearothermophilus* alanine racemase, the k_{cat} values of lysine racemase for L- and D-Lys are only 0.23 and 0.28% of those for L- and D-Ala of the alanine racemase, respectively (Table I) (*3*). This marked difference in the reaction rate was also reported between two homologous alanine racemases of *Salmonella typhimurium*, DadB and Alr. Although *dadB* and *alr* genes exhibit 40% sequence homology, the V_{max} value of DadB is 60 times higher than that of Alr (*10*). The structural basis for this V_{max} difference is currently unclear. DadB and Alr possess different physiological functions: DadB is inducible and required for cell growth on L-Ala, whereas Alr is constitutive and serves an anabolic function in peptidoglycan assembly (*10*). These differences in the physiological roles between DadB and Alr are suggestive when we speculate on the role of lysine racemase.

Not only the amino acid sequence but also the crystal structure of lysine racemase, which has previously been solved (PDB ID: 3CO8, the enzyme is annotated as alanine racemase in PDB), resembles that of the *G. stearothermophilus* alanine racemase (PDB ID: 1SFT) (*11*) as shown in Fig. 1A and B. The alanine racemase reaction proceeds through a two-base mechanism with the PLP-binding lysyl residue and the tyrosyl residue that faces the lysyl residue across the PLP (*12*). These lysyl and tyrosyl residues are conserved in the lysine racemase as Lys39 and Tyr266, respectively (Fig. 1A). We mutated Lys39 and Tyr266 of lysine racemase to alanyl residues by quick-change mutagenesis by using pC1-0162m as a template and the following mutagenic primer pairs: K39Af-K39Ar and Y266Af-Y266Ar (Supplementary Table S1) for the construction of the Lys39→Ala39 mutant (K39A) and Tyr266→Ala266 mutant (Y266A) genes, respectively. Both mutant enzymes were purified as described previously for the purification of the wild-type enzyme. We found that K39A lost the spectral features of the PLP enzyme (data not shown) and that both K39A and Y266A lost racemase activity. These results suggest that lysine racemase reaction proceeds through the similar two-base mechanism to that of the alanine racemase.

Amino acid racemase of broad substrate specificity (*4*) and arginine racemase (*5*) acting on basic amino acids also catalyze the alanine racemization. In contrast, lysine racemase does not act on alanine despite the structural similarity with alanine racemase. We attempted to find out the determinant of the substrate specificity of lysine racemase. The distribution of active site residues in lysine racemase and the *G. stearothermophilus* alanine racemase was quite similar, but Ile222 and Tyr354 of alanine racemase were substituted to Thr224 and Trp355, respectively, in lysine racemase (Fig. 1C). Thus, we simultaneously mutated these specific residues of both enzymes and examined the activity of the mutant enzymes. Plasmids for the lysine racemase mutants T224I and W355Y and the double mutant, T224I/W355Y, were constructed, as described for the K39A mutant by using the following sets of mutagenic primers: T224If-T224Ir and W355Yf-W355Yr (Supplementary Table S1). The expression and purification of the mutants were performed, as described previously for the wild-type enzyme. Plasmids for the alanine racemase mutants I222T, Y354W and I222T/Y354W were also constructed by quick-change mutagenesis using the pMDAlr3 plasmid expressing the wild-type alanine racemase (*13*) as a template and the following sets of mutagenic primers: I222Tf-I222Tr and Y354Wf-Y354Fr (Supplementary

Table I. Kinetic parameters of racemases.

Enzyme		Substrate	Direction	k_{cat} (min^{-1})	K_m (mM)	$k_{cat} \cdot K_m^{-1}$ ($min^{-1} \cdot mM^{-1}$)
Lysine racemase	Wild-type	Lys	L → D	$2.1 \pm 0.1 \times 10^2$	11 ± 1	19
			D → L	$1.7 \pm 0.1 \times 10^2$	9.8 ± 1.5	17
		Orn	L → D	$1.1 \pm 0.3 \times 10^2$	27 ± 4	4.1
			D → L	$1.1 \pm 0.1 \times 10^2$	22 ± 2	5.0
	T224I	Lys	L → D	19 ± 3	15 ± 3	1.5
			D → L	17 ± 2	12 ± 2	1.4
	W355Y	Lys	L → D	62 ± 7	23 ± 3	2.7
			D → L	53 ± 6	21 ± 4	2.5
	T224I/W355Y	Lys	L → D	9.7 ± 1.4	27 ± 3	0.36
			D → L	8.9 ± 1.2	31 ± 5	0.29
Alanine racemase	Wild-type	Ala	L → D	$9.2 \pm 0.3 \times 10^4$	5.1 ± 0.63	1.8×10^4
			D → L	$6.1 \pm 0.2 \times 10^4$	2.8 ± 0.41	2.2×10^4
	I222T	Ala	L → D	$8.5 \pm 0.1 \times 10^4$	4.7 ± 0.29	1.8×10^4
			D → L	$5.9 \pm 0.1 \times 10^4$	2.6 ± 0.27	2.3×10^4
	Y354W	Ala	L → D	$6.8 \pm 0.2 \times 10^4$	4.5 ± 0.41	1.5×10^4
			D → L	$3.1 \pm 0.2 \times 10^4$	2.3 ± 0.29	1.3×10^4
	I222T/Y354W	Ala	L → D	$6.8 \pm 0.24 \times 10^4$	8.3 ± 0.89	8.2×10^3
			D → L	$3.8 \pm 0.29 \times 10^4$	4.0 ± 0.51	9.5×10^3
		Lys	L → D	$2.5 \pm 0.26 \times 10^2$	32 ± 2.12	7.8
			D → L	$1.5 \pm 0.21 \times 10^2$	18 ± 2.01	8.3

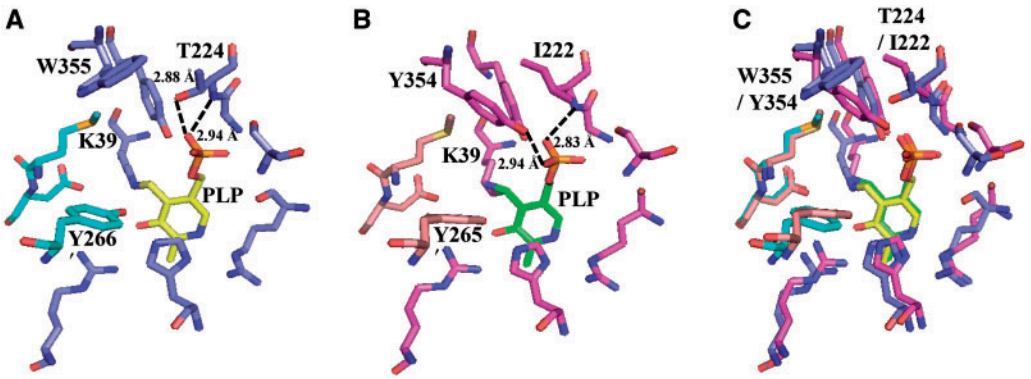

Fig. 1 Structure of the catalytic site of lysine racemase (A) and alanine racemase (B) and their superimposition (C). These figures were created using PyMOL software version 0.99 based on their published crystal structures (PDB ID: 3CO8 and 1SFT for lysine racemase from *O. oeni* and alanine racemase from *G. stearothermophilus*, respectively).

Table S1). Expression of alanine racemases was induced by the addition of 0.1 mM IPTG. The protein purification was carried out as described by Inagaki *et al.* (*3*) with some modifications: Butyl-Toyopearl 650 M (Tosoh, Tokyo, Japan) column chromatography was carried out before DEAE-Toyoperal column chromatography and the gel filtration was omitted.

Kinetic parameters towards alanine using the alanine and lysine racemases were analysed as follows. The reaction mixture (final volume of 1 ml) contained 50 mM KPB (pH 8.0), 20 μM PLP, 7.4 mM 4-aminoantipyrine, 0.03% *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, 10 units of horseradish peroxidase, various concentrations of L- or D-Ala and 50 μg of D-amino acid oxidase from *Schizosaccharomyces pombe* (*14*) or 1 mg of L-amino acid oxidase from *Crotalus atrox* (Sigma). The reaction was initiated by the addition of lysine racemase or alanine racemase and carried out at 30 or 37°C, respectively. The reaction was monitored by following the increase in the absorbance at 550 nm; this increase was due to the formation of the formazan pigment.

In the case of lysine racemase, the introduction of T224I and W355Y mutations decreased the enzyme activity. The T224I, W355Y and T224I/W355Y mutant lysine racemases exhibited 17.6, 25.0 and 3.4% L-Lys racemization activity of the wild-type enzyme, respectively (Supplementary Table S2). These mutations decreased the k_{cat} values and increased the K_m values (Table I). The three lysine racemase mutants showed a slight activity towards D- and L-Ala, but the activities were so weak that we could not determine the kinetic parameters. These results indicate that Thr224 and Trp355 provide the structure that accommodates lysine as the substrate and that the conversion of these residues to Ile and Tyr, respectively, caused no significant enhancement of alanine racemization activity.

The corresponding mutations in alanine racemase (I222T and Y354W) significantly affected the substrate specificity of the enzyme. Although wild-type alanine racemase catalyzes the racemization of alanine and serine specifically, the two single mutants, I222T and Y354W, showed activity towards glutamine, asparagine, methionine and leucine in addition to alanine

and serine but did not display any lysine racemization activity (Supplementary Table S3). Each mutation had no significant effects on the kinetic parameters towards alanine (Table I). The double mutant, I222T/Y354W, reacted with almost all the standard amino acids except for tryptophan (Supplementary Table S3). Lysine racemization activity was also enhanced, and the $k_{cat}{\cdot}K_m^{-1}$ value towards lysine of the I222T/Y354W alanine racemase mutant was comparable with that of the wild-type lysine racemase (Table I). Considering that only the double mutant exhibited activity towards lysine, these mutations might synergistically affect substrate specificity and, in particular, the specificity for lysine. The double mutant still retained ~50% of the wild-type $k_{cat}{\cdot}K_m^{-1}$ values towards D- and L-alanine (Table I). The mutation studies of alanine racemase led us to the same conclusion as that obtained through the mutation studies of lysine racemase: Thr224 and Trp355 of lysine racemase provide the structure that is essential for accommodating lysine as the substrate, but Ile222 and Tyr354, the corresponding residues of alanine racemase, are not critical for alanine racemization. However, mutation of these residues altered the substrate specificity of the alanine racemase. In alanine racemase, the α-amino group of Ile222 and the hydroxyl group of Tyr354 have been suggested to interact with the phosphate group of PLP through a hydrogen bond (*12*). Mutation of these residues probably affects the hydrogen bond to alter the substrate specificity.

In this study, we demonstrated that one of the alanine racemase homologs of *O. oeni* (OEOE0162) is a lysine racemase, which is highly specific for basic amino acids. D-Lys acts as a catabolic nutrient in *Pseudomonas taetrolens* (*5*) and is a component of the cell wall peptidoglycan in *Thermotoga maritima* (*15*). However, the physiological importance of D-Lys in *O. oeni* is still unknown. Our future task is to elucidate the physiological functions of D-Lys and the lysine racemase OEOE0162 in *O. oeni* cells.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest
None declared.

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