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Molecular Cloning of an Extremely Thermostable Alanine Racemase from *Aquifex pyrophilus* and Enzymatic Characterization of the Expressed Protein

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A homologous gene to alanine racemase was cloned from a hyperthermophilic bacterium, *Aquifex pyrophilus*. The cloned gene encodes a protein of 341 amino acids, which has a significant homology to alanine racemase of *Bacillus stearothermophilus*, *Lactobacillus brevis*, and *E. coli*. When the gene was expressed in *Escherichia coli*, it produced a 40 kDa protein. The purified protein contains one mole pyridoxal 5-phosphate per one mole of protein, which is essential for catalytic activity of alanine racemase. The purified protein catalyzed racemization of L-alanine to D-alanine, or vice versa, indicating that the cloned gene encoded alanine racemase. It also showed significant racemization activity against L-serine and α-aminobutylic acid. The *A. pyrophilus* alanine racemase showed strong thermostability, and it maintained catalytic activity in the presence of organic solvents.

Keywords: Alanine racemase, *Aquifex pyrophilus*, Cloning, Hyperthermophile, Racemization.

Introduction

Alanine racemase catalyzes the racemization of L-alanine to D-alanine, which is used for the construction of a peptidoglycan layer of bacterial cell walls. The enzyme was found in bacterial species, including mesophilic bacteria such as *Bacillus subtilis* (Ferrari *et al.*,1985), *Pseudomonas putida* (Julius *et al.*, 1970), *E. coli* (Wild *et al.*, 1985), and *Salmonella typhimurium* (Wasserman *et al.*, 1984; Esaki and Walsh, 1986), thermophilic bacteria such as *Bacillus stearothermophilus* (Inagaki *et al.*, 1986; Toyama *et al.*, 1991), or psychrophilic bacteria, such as *Pseudomonas fluoresceus* TM5-2 (Yokoigawa *et al.*, 1993). It was also found in a fungus in which the enzyme was used for the synthesis of cyclosporin biosynthesis (Hoffmann *et al.*, 1994). Alanine racemase required pyridoxal 5-phosphate as a co-factor for its catalytic reaction. The pyridoxsal 5-phosphate is covalently linked via an aldimine linkage toa conserved lysine residue (Shaw *et al.*, 1997).

Due to an ubiquitous location in bacteria, and its pivotal role in the biosynthesis of the cell wall, the enzyme was recognized as a target for antibacterial drugs, such as D-cycloserine, β-chloro-D-alanine, and 1-aminoethyl-phosphonate (Hoffmann *et al.*, 1994). On the other hand, alanine racemase was used for the production of D-form amino acids, along with L-alanine dehydrogenase and D-amino acid aminotransferase (Esaki 1995). For the enzymatic production of D-amino acids, thermostable enzymes are required.

In this report, we cloned a gene coding alanine racemase from *Aquifex pyrophilus*, a hyperthermophile that grows optimally at 85°C. In particular, this organism was known as one of the most heat-resistant bacteria, and enzymes from this organism showed a higher stability than other mesophilic, or thermophilic bacteria (Lim *et al.*, 1997; Choi *et al.*, 1999; Kim *et al.*, 1999). The protein from the cloned gene was over-expressed in *E. coli* and its stability characterized.

Materials and Methods

Materials Restriction enzymes, and T4 DNA ligase, were purchased from Promega (Madison, USA). *Pfu* DNA polymerase for PCR amplification was obtained from New England Biolabs (Beverly, USA). Oligonucleotides for PCR primer and DNA sequencing were acquired from Biosynthesis (Lewisville, USA). DNA labeling, and the ECL detection system, were from Amersham (Piscataway, USA). Ni-NTA resin was purchased from Quagen (Hilden, Germany). L-Alanine dehydrogenase was obtained from Sigma (St. Louis, USA). Amino acids, nicotine amide deoxyadenosine (NAD^+), isopropyl-D-thiogalactoside (IPTG), and all other chemicals used in this study were of reagent grade.

Cloning and sequencing The genomic DNA lambda library of *A. pyrophilus* constructed by Kim *et al.* (1999) was screened

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using a DNA tag (pAQpU219) containing a partial sequence homologous to the alanine gene of *B. stearothermophilus* as a probe (Choi *et al.*, 1997). Plaque and southern hybridization were carried out using the ECL kit, according to the manufacturer's instructions. Conventional procedures for plasmid manipulation were followed, as described by Sambrook *et al.* (1989). The nucleotide sequence of the cloned gene was determined by the dideoxy chain termination method using a Dye Primer Taq sequencing kit (Applied Biosystems, Foster City, USA). Sequence comparison and analysis were performed using the Blast program (Altschul *et al.*, 1990).

Expression and purification of alanine racemase The open reading frame (ORF) of the cloned alanine racemase gene was amplified from the genomic DNA of *A. pyrophilus* by PCR with an N-terminus primer, containing the first nine codons and flanking *Nde*I site (5'GGCCCGGCCATATGAGAAGGGAGGTTCTTGAGATACT GGA3') and a C-terminus primer containing the last eight codons and flanking *Xho*I site (5'GCCGGGCCCTCGAGCCTTAGTTCTCCTGTTCACCTTTC3'). The amplified DNA was digested with *Nde*I and *Xho*I and cloned into the *Nde*I and *Xho*I sites of plasmid pET28a. In-frame ligation of the amplified DNA into the pET28a vector was confirmed by DNA sequencing. The resulting plasmid (pAR1) contains the whole coding region of the cloned gene downstream of the T7 promoter and the ribosomal binding site. Plasmid pAR1 was transformed into the *E. coli* strain BL21 (DE3) and the cells were grown at 37°C in Luria-Bertani's (LB) broth containing 100 μg/ml of ampicillin. When the optical density of the culture at 600 nm reached 1.0, the expression of the protein was induced by adding IPTG in the growth medium to a final concentration of 1.0 mM. After 4 h of induction, the cells were harvested by centrifugation at 4,000 rpm for 15 min. About 10 g of cell pellet (wet weight) was resuspended in 50 ml of lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM dithiothreitol, and 100 μM pyridoxal 5-phosphate) and passed through a French press twice under 12,000 psi. The cell debris was removed by centrifugation at 16,000 rpm for 20 min, and the cell lysate was incubated for 1 h at 85°C. The denatured *E. coli* proteins were removed by centrifugation at 16,000 rpm for 20 min and the expressed protein, in the supernatant fraction, was purified using Ni-NTA agarose column by the procedure in the instruction manual (Quiagen, Hilden, Germany).

Size determination The native size of the purified *A. pyrophilus* alanine racemase is determined using size exclusion chromatography. The protein is eluted from a Superdex 200 column (Pharmacia, Uppsala, Sweden) and the retention time was compared with the values of carbonic anhydrase (29 kDa), ovalbumin (44 kDa), bovine serum albumin (67 kDa) and Alcohol dehydrogenase (150 kDa).

Enzyme assays The racemase activity was measured using a coupled enzyme assay method, as described by Badet *et al.* (1984). The reaction was initiated by adding the purified racemase (10 μg) to 0.1 ml of the reaction buffer [50 mM Tris-HCl, pH 9.5, 5 μM pyridoxal 5-phosphate (PLP), 2 mM dithiothreitol] containing 10 mM D-alanine. After incubation at 85°C for 60 min, adding a 0.3 volume of 7% perchloric acid terminated the reaction. The pH of the reaction mixture was neutralized by adding 0.2 volume of 1 N NaOH, and the produced L-alanine was reduced to pyruvate by adding 25 units of L-alanine dehydrogenase and 5 mM NAD^+ in 0.1 volume of 0.5 M of Tris-HCl, pH 9.5. The amount of NADH produced from the reduction of NAD^+ by L-alanine dehydrogenase and L-alanine was calculated from the absorption value at 340 nm, using a Shimadzu UV-1601PC spectrophotometer (Shimadzu, Tokyo, Japan). A unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of a L-alanine per min. The protein was assayed using a dye binding method (Bradford, 1976).

Results

Cloning and sequencing of alanine racemase gene from *A. pyrophilus* Previously, we identified genomic DNA fragments of *A. pyrophilus* that had a high sequence similarity to the alanine racemases from *Bacillus* (Choi *et al.*, 1997). With this DNA tag as a probe, a lambda clone, which hybridized with the probe, was identified after screening the *A. pyrophilus* genomic DNA lambda library. A 1.4 kb fragment, which contained an open reading frame harboring the probe sequence, was further identified from the cloned lambda DNA and the nucleotide sequence of the 1.4 kb fragment determined. The restriction map, and sequencing strategy for the 1.4 kb fragment, is shown in Fig. 1. The nucleotide, and amino acid sequence of the open reading frame, is shown in Fig. 2. The open reading frame consisted of 1,023 base-pairs starting at the ATG (position 285) and terminating at the TAA (position 1308). It encoded a protein of 341 amino acid residues.

Sequence comparison When the amino acid sequence of the ORF was compared with those in the Genbank and the PIR databases, it showed a strong sequence similarity to the alanine racemases from various organisms. Sequence identity values between the cloned *A. pyrophilus* gene and the alanine racemase from *B. stearothermophilus*, *E. coli*, and *Lactobacillus brevis* were 38.4%, 29.3%, and 33.4%, respectively. The linear alignment of the amino acids from these genes is shown in Fig. 3. Multiple amino acid sequence alignments showed highly conserved domains. Specially, the

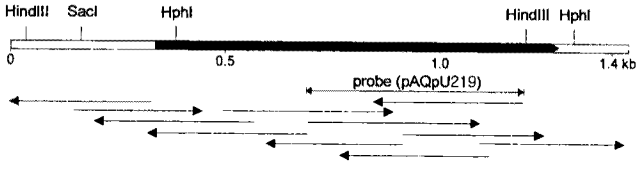

Fig. 1. Restriction map of the 1.4 kb *A. pyrophilus* DNA fragment containing a homologue of the alanine racemase. The black arrow represents an open reading frame. The arrows under the restriction map indicate the direction and length of the sequenced DNAs. The location of the probe sequence (pAQpU219) is indicated as an arrow.

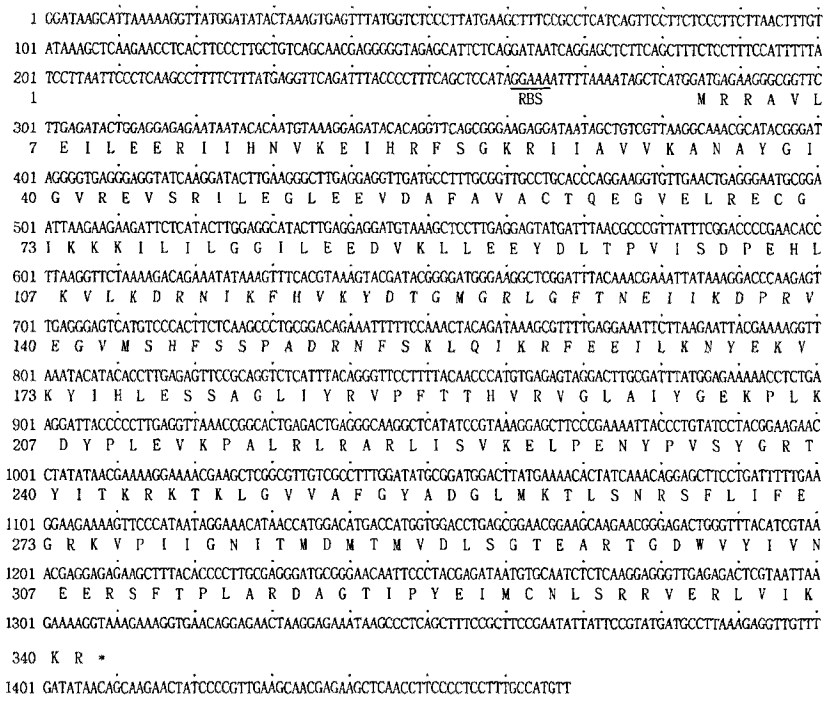

Fig. 2. Nucleotide and deduced amino acid sequence of the cloned alanine racemase from *A. pyrophilus*. The putative ribosome binding sequence (RBS) is underlined at the 5'-flanking region. The stop codon is marked by an asterisk.

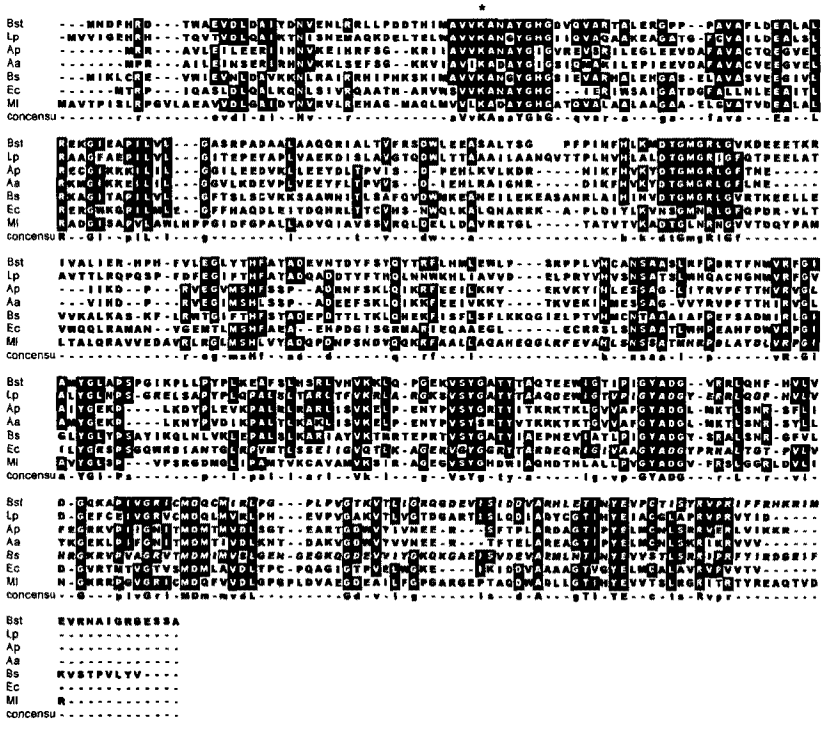

Fig. 3. Linear alignment of amino acid sequence of the cloned gene and alanine racemases. The identical amino acid residues are indicated as black box. The lysine residue at the active site is marked with asterisks. The amino acid sequences of alanine racemase are obtained from SWISS-PROT. Bst (*B. stearothermophilus*; P10724), Lp (*L. plantarum*; O08445), Ap (*A. pyrophilus*; this study), Aa (*A. aerolicus*; O67687), Bs (*B. subtillus*; P94494), Ec (*E. coli*; P29012), Ml (*M. leprae*; P38056).

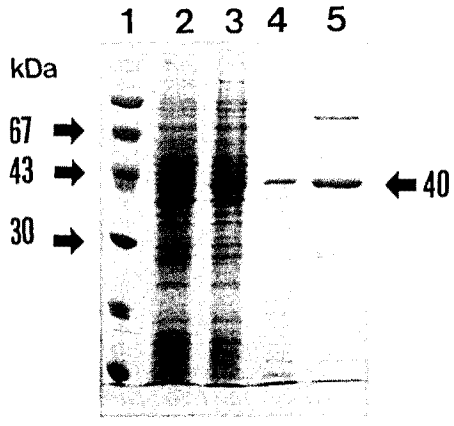

Fig. 4. SDS-PAGE of *A. pyrophilus* alanine racemase at different stages of purification. The proteins were analyzed on 15% SDS-PAGE. Lane 1, molecular weight markers (14.4, 21.5, 30, 43, 66 and 97 kDa); lane 2, crude extract of *E. coli* BL21(DE3) before induction; lane 3, crude extract after induction with 1 mM IPTG for 3 h; lane 4, soluble fraction of the crude extract after heat treatment for 60 min at 85°C; lane 5, purified alanine racemase after Ni-NTA affinity column. The arrow indicats the 40 kDa alanine racemase.

amino acid, sequence around Lys33 of the cloned gene, is well conserved. The ε-amino group of the active site lysine residue that is involved in the formation of an internal Schiff base between the formyl group of PLP (which is involved in the deprotonation of L-alanine) and the residue was conserved among various alanine racemases.

Expression and purification The *A. pyrophilus* gene was expressed in *E. coli* using the pET expression system. Since the open reading frame was inserted at the *Nde*I site of pET28 plasmid DNA, the resulting protein contained His-tag sequence at the N-terminus of *A. pyrophilus* alanine racemase. After a 4 h induction of the BL21(DE3) strain of *E. coil* harboring pAR1 with 1 mM IPTG, a 40 kDa protein was expressed to a level less than 3% of the total cellular protein (Fig. 4, lane 3). The molecular weight of the expressed protein is well matched with the calculated value from the amino acid sequence. The expressed protein was not denatured by heat treatment for 1 h at 85°C, indicating that the His-tag sequence at the N-terminus did not affect its thermostability. However, most of the *E. coli* proteins were denatured and precipitated (Fig. 4, lane 4). After the heat treatments, the protein was further purified to about 90% homogeneity, using Ni-NTA affinity column. The apparent molecular weight of the purified protein was determined by a gel-filtration chromatography with a Superdex 200 resin. The protein was eluted at the position of 80 kDa, indicating that the protein was a dimer.

Alanine racemase activity To examine the purified protein for alanine racemase activity, the presence of PLP as a co-factor in the purified protein, and the racemase activity against

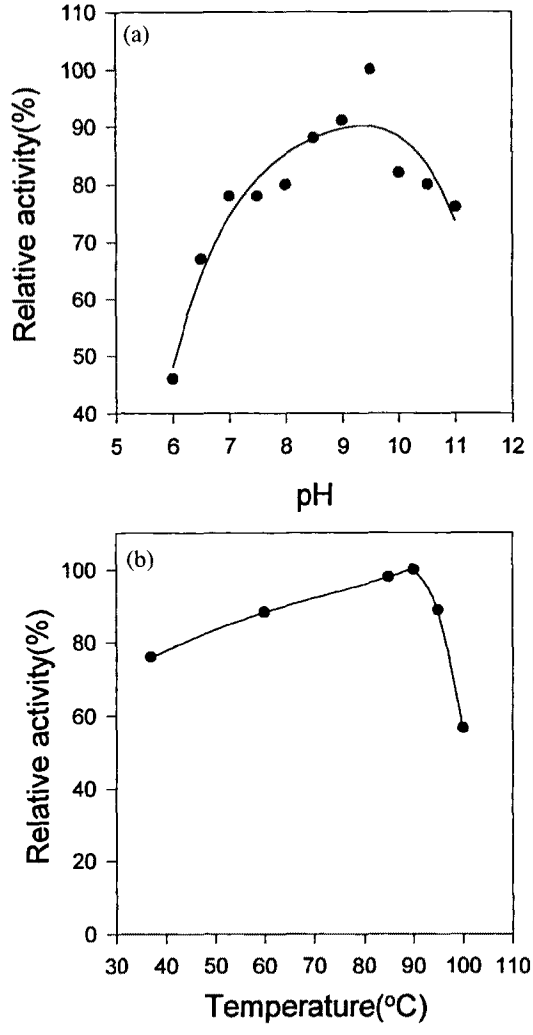

Fig. 5. Effect of pH and temperature on the activity of *A. pyrophilus* alanine racemase. (a) Alanine racemase activity of the purified protein was measured in 50mM sodium phosphate (6, 6.5), Tris-HCl (7-9.5), CAPS (10-11). (b) The protein was assayed at various temperatures in 50 mM Tris-HCl buffer (pH 9.5).

various amino acids, were examined. PLP was known to have a absorption maximum at 420 nm, and the content of PLP in the protein sample was determined by spectroscopic method-based on the extinction coefficient at 420 nm of 8,450 (Inagaki *et al.*, 1986). The concentration of PLP was calculated to be 0.85 fold of the protein concentration, indicating that a single molecule of PLP was present at each monomer of the purified protein. The substrate specificity of the purified protein was examined by measuring its racemase activity against 19 natural L-form amino acids and L-α-aminobutylic acid by monitoring the decrease of the CD signal at 204 nm. The *A. pyrophilus* enzyme catalyzed racemization of L-alanine, indicating that the cloned gene represented alanine racemase. In addition, it showed substantial racemase activity against L-form of serine (50%) and α-aminobutylic acid (18%) compared to L-alanine.

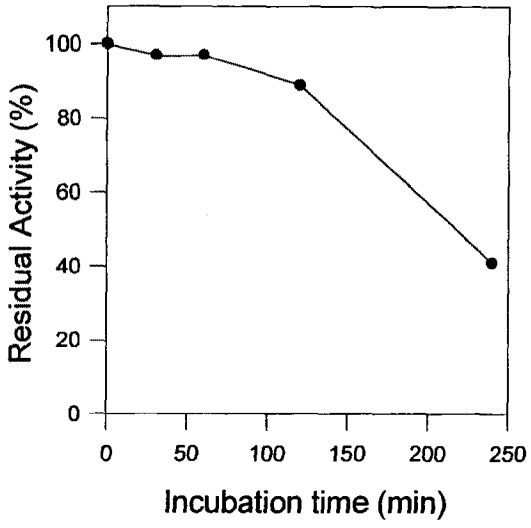

Fig. 6. Thermostability of *A. pyrophilus* alanine racemase. The enzyme was incubated at 95°C for 0-4 h, and then the residual activity was assayed. The relative activity was plotted against the incubation time.

However, it failed to racemize the rest of the tested amino acids.

The characteristics of the *A. pyrophilus* alanine racemase, such as optimum pH and temperature, were determined. The enzyme showed maximum reactivity at pH 9.5 (Fig. 5a) when its racemase activity was examined in 50 mM sodium phosphate (pH 6-6.5), Tris-HCl (pH 7-9.5), CAPS (pH 10-11). When the enzyme was assayed at various temperatures in the standard buffer (pH 9.5), it remained active over a broad range of temperatures and showed the maximum activity at 90°C (Fig. 5b). The K_m and k_{cat} values of the enzyme, for the conversion of D- to L-alanine in pH 9.5 and 90, was measured as 1.8 mM and 24.8 sec^{-1}, respectively.

Stability of *A. pyrophilus* alanine racemase The thermostability of the *A. pyrophilus* alanine racemase was examined by measuring the residual activity of the enzyme after incubation at 95°C in 50 mM of Tris-HCl, 0.5 M sodium phosphate, pH 8.0. The protein showed a strong heat resistance and maintained more than 90% of activity for 2 h (Fig. 6). The *A. pyrophilus* alanine racemase also showed a strong resistance against denaturants, or organic solvents (Table 1). There was more than 60% activity in the presence of 4 M guanidine-HCl, or 2M urea. When 50% of acetonitrile, or isopropyl alcohol, was present in the reaction mixture, the enzyme maintained its activity more than 90% or 30%, respectively. The stability of *A. pyrophilus* alanine racemase against heat, denaturants, or organic solvents may be valuable for the enzyme-catalyzed chemical synthesis of D-form amino acids.

Discussion

For the first time, alanine racemase from hyperthermophiles was cloned and characterized in this study. Until now, hyperthermophiles, which grow at higher than 80°C, belonged mostly to Archaea. Only a couple of species of bacteria, such as *Thermotoga* and *Aquifex*, were able to grow at temperatures higher than 85°C. Hyperthermophilic Archaeas did not possess any genes homologues to glutamate, or alanine racemese, which produce D-Glu and D-Ala for the cell wall synthesis (Bult *et al.*, 1996). However, *Aquifex* species, like other bacterial species, were shown to have glutamate and alanine racemase genes (Choi *et al.*, 1997; Deckert *et al.*, 1998). The glutamate racemase from *A. pyrophilus* was shown to be one of the most heat stable glutamate racemases (Kim *et al.*, 1999), and its atomic structure was determined (Hwang *et al.*, 1999). The dependency of PLP for the enzymatic activity, and the dimeric form of the *A. pyrophilus* alanine racemase, are consistent with the alanine racemases from other bacteria. However, it showed outstanding thermostability compared to alanine racemases from other bacteria, including *B. stearothermophilus* (Inagake *et al.*, 1986)

The expression level of the cloned gene in *E. coli* is much lower than other foreign proteins expressed in *E. coli* using the pET expression system (Choi *et al.*, 1998). *A. pyrophilus* and *E. coli* showed the biased codon frequency differently (Choi *et al.*, 1997). Specially, the frequency of AGA or AGG codons for arginine, and ATA codon for isoleucine, are less than 5% in *E. coli*. However, these codons were dominantly used in the *A. pyrophilus* alanine racemase gene (Fig. 2). Since tRNAs complementing to these codons were low in *E. coli*, the rate of translation of the gene might be severely retarded, resulting in a low level expression. For high-level expression of *A.*

Table 1. Stability of *A. pyrophilus* alanine racemase against denaturants and organic solvents.

Denaturants			
Urea (M)	Relative activity (%)*	Guanidine-HCl (M)	Relative activity (%)*
0	100	0	100
1	80	1	102
2	62	2	111
4	38	4	77
5	31	5	52
Organic solvents			
Isopropyl alcohol (%)	Relative activity (%)*	Acetonitrile (%)	Relative activity (%)*
0	100	0	100
5	110	5	103
10	107	10	107
20	70	20	107
40	33	40	103
50	33	50	92

*The 100% activity was obtained from the reaction of *A. pyrophilus* alanine racemase without any denaturant or organic solvent in the reaction mixture.

pyrophilus alanine racemase, a host organism other than *E. coli,* such as *Bacillus* or yeast, should be exploited.

The cloned gene showed general sequence similarity, in particular at the region around the catalytic Lys residue. Involvement of pyridoxal 5-phosphate also indicated that the *A. pyrophilus* enzyme might use the same catalytic mechanism as the enzymes from other bacteria. However, the *Aquifex* enzyme showed some differences. First, it showed broader substrate specificity. Alanine racemase from *B. stearothermophilus* used strictly alanine, and failed to use other amino acids (Inagaki *et al.*, 1986). However, *A. pyrophilus* alanine racemase catalyzed the racemization of D-serine significantly. It also showed a slight activity against valine, lysine, leucine, and α-aminobutylic acid (data not shown). Secondly, the *A. pyrophilus* enzyme showed a very strong thermo-stability against heat, such as other hyperthermophilic protein. Up to now, the *A. pyrophilus* alanine racemase is the most heat stable enzyme among the alanine racemases. It also showed a high resistance against organic solvents. These properties may be beneficial for industrial usage. It would be interesting to examine the nature of broad substrate specificity and thermostability of the *A. pyrophilus* alanine racemase by examining its atomic structure.

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