Extremophiles (1999) 3:175–183

ORIGINAL PAPER

Sang Suk Kim · In-Geol Choi · Sung-Hou Kim
Yeon Gyu Yu

Molecular cloning, expression, and characterization of a thermostable glutamate racemase from a hyperthermophilic bacterium, *Aquifex pyrophilus*

Received: September 11, 1998 / Accepted: January 12, 1999

Abstract A gene encoding glutamate racemase has been cloned from *Aquifex pyrophilus*, a hyperthermophilic bacterium, and expressed in *Escherichia coli*. The *A. pyrophilus* glutamate racemase is composed of 254 amino acids and shows high homology with glutamate racemase from *Escherichia coli*, *Bacillus subtilis*, or *Lactobacillus brevis*. This racemase converts L- or D-glutamate to D- or L-glutamate, respectively, but not other amino acids such as alanine, aspartate, and glutamine. The cloned gene was expressed and the protein was purified to homogeneity. The *A. pyrophilus* racemase is present as a dimer but it oligomerizes as the concentration of salt is increased. The K_m and k_{cat} values of the overexpressed *A. pyrophilus* glutamate racemase for the racemization of L-glutamate to the D-form and the conversion of D-glutamate to the L-form were measured as 1.8 ± 0.4 mM and $0.79 \pm 0.06\,s^{-1}$ or 0.50 ± 0.07 mM and $0.25 \pm 0.01\,s^{-1}$, respectively. Complete inactivation of the racemase activity by treatment with cysteine-modifying reagents suggests that cysteine residues may be important for activity. The protein shows strong thermostability in the presence of phosphate ion, and it retains more than 50% of its activity after incubation at 85°C for 90 min.

Key words *Aquifex pyrophilus* · Molecular cloning · Glutamate racemase · Overexpression · Thermostability

Communicated by G. Antranikian

S.S. Kim · I.-G. Choi · S.-H. Kim · Y.G. Yu (✉)
Structural Biology Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul, Korea
Tel. +82-2-958-5936; Fax +82-2-958-5939
e-mail: ygy@kistmail.kist.re.kr

S.-H. Kim
Department of Chemistry and Lawrence Berkeley National Laboratory, University of California, Berkeley, CA, USA

Introduction

Bacterial cell walls contain D-glutamate and D-alanine as peptidoglycan components (Dominique et al. 1982), and these D-amino acid residues are assumed to protect the cell wall from proteolysis. D-Alanine is produced directly from L-alanine by alanine racemase (Walsh 1989). D-Glutamate is produced either from the racemization of L-glutamate by glutamate racemase, as observed in *Lactobacillus* (Gallo and Knowles 1993; Yagasaki et al. 1995) and *Pediococcus* (Nakajima et al. 1986), or from the transamination between α-ketoglutarate and D-alanine by D-amino acid aminotransferase, as in *Bacillus* (Yonaha et al. 1975). The reaction mechanism and characteristics of the amino acid racemases have been of interest because the spontaneous racemization of amino acids without enzymes is a remarkably slow reaction, and some racemases can be used for the industrial production of D-amino acids that are used as precursors for pharmaceuticals. In addition, the amino acid racemases are target proteins for the development of antibacterial agents because they are exclusively found in bacteria and are critical for cell wall biosynthesis.

Amino acid racemases are grouped into two classes depending on their cofactors. Alanine and arginine racemases require pyridoxal 5′-phosphate (PLP) (Walsh 1989; Yorifugi et al. 1971), which forms a Schiff base with the amino acid substrate. In contrast, aspartate racemase (Yamauchi et al. 1992; Yohda et al. 1991, 1996) or the glutamate racemase from *E. coli* (Doublet et al. 1993) or *Lactobacillus* (Gallo and Knowles 1993; Yagasaki et al. 1995) are cofactor-independent enzymes. Extensive study of glutamate racemase from *Lactobacillus* shows that the reaction is catalyzed by a two-base mechanism in which one enzyme base eliminates the hydrogen from the α-carbon of glutamate and the conjugate acid of the second base transfers a proton to the α-carbon (Gallo et al. 1993). Two specific cysteines (cysteine-73 and cysteine-184) of the *Lactobacillus* enzyme are shown to function as the two bases, and they are shown to be responsible for abstracting the C-α proton from either form of glutamate (Gallo et al. 1993). Sequence comparison among

the glutamate racemases from various organisms shows that the sequences around the two catalytic cysteine residues are highly conserved (Yagasaki et al. 1995). The conserved cysteines are also observed in the aspartate racemase (Yamauchi et al. 1992). Hence, the two racemases are assumed to have a similar reaction mechanism although they cannot exchange their substrates.

Hyperthermophiles are organisms that grow optimally at 80°C or higher temperatures. They have been found near geothermal vents, and the majority of them belong to the Archaea. The cell wall components of hyperthermophilic Archaea are distinct from those of eubacteria, and the presence of D-glutamate or D-alanine has not yet been reported. Recently, the entire genomic sequences of several species of hyperthermophilic Archaea have been determined (Bult et al. 1996; Klenk et al. 1997). None of them have any genes homologous to the glutamate or the alanine racemase reported previously, except an aspartate racemase gene from *Archaeoglobus fulgidus* (Klenk et al. 1997). Besides Archaea, there are two genera of Eubacteria that are hyperthermophiles: *Aquifex* and *Thermotogales*. Among them, *Aquifex pyrophilus* is a gram-negative bacterium and its cell wall contains D-alanine and D-glutamate (Huber et al. 1992). Random analysis of the genomic DNA sequence of *Aquifex pyrophilus* reveals that there are genes homologous to glutamate racemase and alanine racemase (Choi et al. 1997). The two amino acid racemases have been also identified in *Aquifex aerolicus* (Deckert et al. 1998), a hyperthermophilic bacterium closely related to *A. pyrophilus.*

Enzymatic characteristics of glutamate racemases from several mesophiles have been studied but the enzyme from hyperthermophiles has not yet been reported. Here, we present the cloning and DNA sequence of glutamate racemase from *A. pyrophilus*, the expression of the cloned glutamate racemase in *E. coli*, and the characterization of the purified glutamate racemase.

Materials and methods

Materials

Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI, USA). *Pfu* DNA polymerase for PCR amplification was obtained from New England Biolabs (Beverly, MA, USA). Oligonucleotides were acquired from Biosynthesis (Lewisville, TX, USA). DNA labeling and the ECL detection system were from Amersham Pharmacia Biotech (Uppsala, Sweden). Glutamate dehydrogenase was obtained from Boehringer Mannheim (Mannheim, Germany). Amino acids, nicotine amide deoxyadenosine (NAD^+), isopropyl β-D-thiogalactoside (IPTG), iodoacetamide, and all other chemicals used in this study were of reagent grade.

Bacterial strains and plasmids

Lambda DASH II from Stratagene (La Jolla, CA, USA) was used for construction of the genomic DNA library. Plasmids pUC19 and pET21a (Novagene, Milwaukee, WI, USA) were used for DNA cloning and as an expression vector, respectively. *A. pyrophilus* (stock number 6858) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM, Braunschweig, Germany). *E. coli* strains XL1-blue (MRA-P2), DH5α, and BL21 (DE3) were used as the hosts for the lambda library, plasmid DNA amplification, and protein expression, respectively.

Cell culture and construction of genomic DNA library

Aquifex pyrophilus was grown in a modified SME medium with gentle shaking at 85° for 12–14 h as described by Huber et al. (1992). The cultured cells were collected by centrifugation and lysed with lysozyme and proteinase K. The genomic DNA was isolated from the cell lysate as previously described by Choi et al. (1997). After partial digestion with *Hin*dIII, the fragments of the genomic DNA were separated on a 0.7% agarose gel. DNA fragments with an average size of 15–20 kb were recovered from the gel and ligated with the prerestricted and dephosphorylated λ-DASH II DNA (Sambrook et al. 1989). The recombinant λ-DNAs were packaged into the phage assembly and transfected into *E. coli* strain XL1-blue (MRA-P2). The transfected cells were plated and the lambda phages from about 4000 primary plaques were pooled. The resulting phage library was used for screening of a specific gene.

Cloning and sequencing

The lambda library was screened using a DNA tag containing a partial sequence homologous to the glutamate racemase gene of *Lactobacillus fermenti* 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). Sequence comparison and analysis were performed using the Blast program (Altschul et al. 1990).

Phylogenic analysis

The phylogenic tree was constructed using an analysis program package, PHYLIP version 3.5 (Felsenstein 1993). The amino acid sequences of the glutamate racemases and the aspartate racemases were aligned with the CLUSTAL W program for the generation of an original data set. This result was used to produce 1000 bootstrap replicates using the SEQBOOT program. The PROTPARSS, NEIGHBOR, and CONSENSE programs in the same package were used sequentially to produce a branch point in the maximum "parsimony tree," which infers an unrooted phylogeny by the "protein sequence parsimony method." The tree was drawn using the DRAWTREE program.

Construction of glutamate racemase expression vector

The open reading frame (ORF) of the cloned *A. pyrophilus* glutamate racemase gene was amplified from the genomic DNA of *A. pyrophilus* by polymerase chain reaction (PCR) with an N-terminus primer containing the first nine codons and flanking *Nde*I site (5′-GGCCCGGCCCATATGAAG-ATAGGTATCTTTGACCGTGGT-3′) and a C-terminus primer containing the last eight codons and flanking *Xho*I site (5′-GGGCCCGCTCGAGTTAATGTGTAAAAAC-CCCCTCCGCAAG-3′). The amplified DNA was cloned into pET21a plasmid. In-frame ligation of the amplified DNA into the PET21a vector was confirmed by DNA sequencing. The resulting plasmid (pGR1) contains the whole coding region of the cloned gene downstream of the T7 promoter and the ribosomal binding site.

Expression and purification of the glutamate racemase

Plasmid pGR1 was transformed into the *E. coli* strain BL21 (DE3), and the cells were grown at 37°C in Luria-Bertani (LB) broth with ampicillin (100 μg/ml). When the optical density of the culture at 600 nm reached 0.6, the expression of the protein was induced by adding IPTG at a final concentration of 1.0 mM. After 3 h of induction, the cells were harvested by centrifugation at 5000 × g for 15 min. About 10 g of cell pellet (wet weight) was resuspended in 50 ml of lysis buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.8, 1 mM dithiothreitol and phenylmethylsulfonyl fluoride) and passed through a French press twice under 12 000 psi. The cell debris was removed by centrifugation at 10 000 × *g* for 20 min, and the cell lysate was incubated for 40 min at 80°C. Denatured *E. coli* proteins were removed by centrifugation at 15 000 × *g* for 20 min, and the heat-treated sample was loaded onto a CM-sepharose column (1.5 × 20 cm; Pharmacia LKB, Uppsala, Sweden) equilibrated with buffer A (50 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol, pH 7.5). Proteins were eluted with 400 ml of a linear gradient of 0.05–0.8 M NaCl in buffer A.

Size determination

The native size of the purified *A. pyrophilus* glutamate racemase was determined using size-exclusion chromatography. The protein (1.0 mg/ml) was eluted from a OHpac SB-803 HQ column (Shodex, Tokyo, Japan), and the retention time was compared with the values of lysozyme (14 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), bovine serum albumin (67 kDa), and alcohol dehydrogenase (150 kDa). The size of the glutamate racemase was also obtained by measuring the scattered light from the protein solution (1.0 mg/ml) using a DynaPro (Protein Solution, Charlottesville, VA, USA), and the apparent molecular weight was calculated.

Assays

The protein concentration was determined using the absorption coefficient of the protein at 280 nm (Gill and von Hippel 1989). The racemase activity was measured using a coupled enzyme assay method as described by Gallo et al. (1993). The reaction was initiated by adding the purified racemase (80–100 μg) to 0.1 ml of reaction buffer (50 mM Tris-HCl, pH 8.5, 2 mM dithiothreitol) containing 10 mM D-glutamate. After incubation at 80°C for 60 min, the reaction was terminated by adding 0.3 vol of 7% perchloric acid. The pH of the reaction mixture was neutralized by adding 0.2 vol of 1 N NaOH, and the produced L-glutamate was reduced to α-ketoglutarate by adding 25 units of L-glutamate dehydrogenase, 10 mM hydrazine hydrate, and 5 mM NAD^+ in 0.1 vol of 100 mM of KPO_4 pH 7.0. The amount of NADH produced from the reduction of NAD^+ by L-glutamate dehydrogenase and L-glutamate was calculated from the absorption value at 340 nm using a Shimadzu UV-1601PC spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of optimum pH and temperature

The optimal pH for the activity of the glutamate racemase was determined by comparing the specific activity of the enzyme in reaction mixtures of different pH values. A reaction buffer containing 50 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 10, 11), Tris-HCl (pH 7–9), or 2-[*N*-morpholino]ethansulfonic acid (MES) (pH 6–7) was used for the enzyme assay. The temperature optimum of the racemase reaction was determined by using various incubation temperatures in the pH 8.5 buffer. For determination of thermostability, the racemase was incubated at 85°C for various time periods and the residual activity was measured.

Results

Cloning and sequencing of glutamate racemase gene from *A. pyrophilus*

The gene coding for glutamate racemase was cloned from a genomic DNA library of *A. pyrophilus*. For screening for the glutamate racemase gene, a sequence tag from the *A. pyrophilus* genomic DNA (AQpU181) was used as a probe. The probe DNA contains 733 nucleotides of *A. pyrophilus* DNA that show high sequence similarity to glutamate racemases (Choi et al. 1997). Two lambda clones were identified after screening the genomic DNA lambda library. The insert DNAs of the two lambda phages hybridized with the probe DNA but they failed to hybridized with each other. Analysis of the boundary sequences near the cloning sites of the lambda DNAs shows that the 5′-half of the probe DNA is located at the end of one lambda DNA, pλGR1, whereas the 3′-half is found at the end of the other lambda DNA, pλGR2. These results indicate that the *Hin*dIII site in the middle of the probe sequence was digested during the construction of the genomic DNA library and the two parts are presented in two separate lambda clones. The nucleotide sequence of the 1.3-kb region that contains the probe and flanking sequences was determined on both strands and is

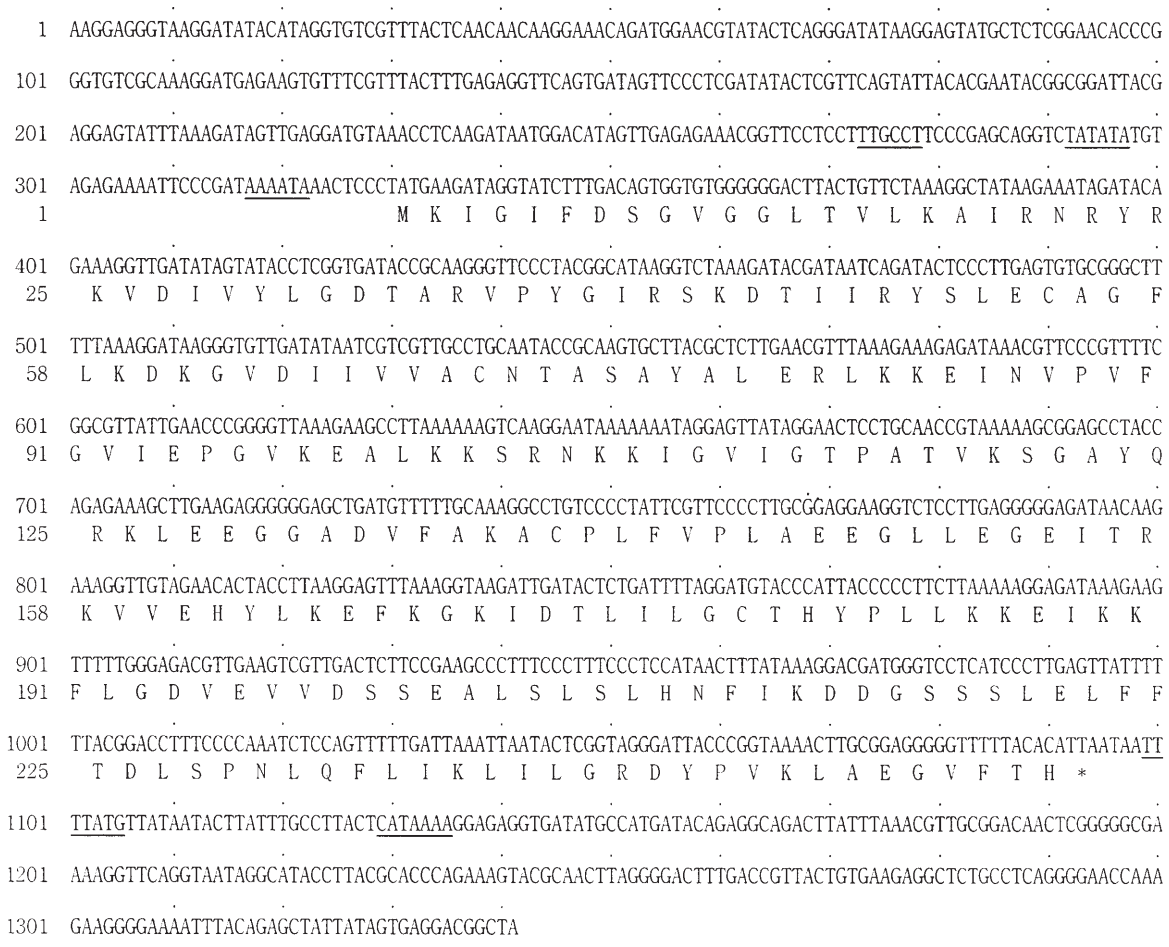

Fig. 1. Nucleotide and deduced amino acid sequence of the cloned glutamate racemase from *Aquifex pyrophilus*. The putative promoters (−35 and −10 regions) and *underlined* at the 5′-flanking region. The stop codon is marked by an *asterisk* and the transcription termination sequence is a few nucleotides downstream of the termination codon (*underlined*)

presented in Fig. 1. In this region, there is a unique ORF that starts with ATG at nucleotide position 331 and terminates at TAA at position 1093. The nucleotide sequence of the probe matches exactly the sequence from nucleotide 307 to 1039. The ORF codes for a single polypeptide of 254 amino acids whose sequence is indicated under the nucleotide sequence.

Putative promoter sites at the 5′-end of the coding region have been observed. The TATATA sequence located 33 nucleotides upstream of the first methionine codon is homologous to the canonical −10 (TATAAT) promoter sequence, and the TTGCCT sequence located 19 bases upstream from the TATATA sequence is similar to the −35 (TTGACA) promoter (Pribnow et al. 1975). A transcription termination signal characterized as a palindrome sequence is found downstream of the termination codon. The traditional ribosome binding site (AGGAGG) has not been observed around the first Met codon. Instead, a high purine-rich sequence (AAAATA) is observed 8 bases upstream from the Met codon. This sequence may be used as a ribosome-binding site. There is only one ATG codon downstream of the putative promoter site, and an alternative start codon, GTG, also has not been found in that region. For this reason, we have tentatively assigned the first Met at nucleotide 331 as the start site of translation.

Sequence comparison and phylogenetic analysis

When the amino acid sequence of the cloned gene was compared with those in the Genebank and the PIR databases, it showed strong sequence similarity to the glutamate racemases from various organisms and weak similarity to the aspartate racemases. Sequence identity values between the cloned *A. pyrophilus* gene and the glutamate racemase from *A. aerolicus*, *Bacillus subtilis*, *E. coli*, and *Lactobacillus brevis* were 72%, 36.1%, 26.6%, and 35.5%, respectively. The linear alignment of the glutamate racemases and aspartate racemase from various organisms shows several homologous regions (Fig. 2a). Among them, the sequences in three regions (DSGVGG, ACNT, and LGCTH) are highly conserved. It is noticeable that the cysteines at ACNT and LGCTH motives are known to serve as bases during the deprotonation reaction.

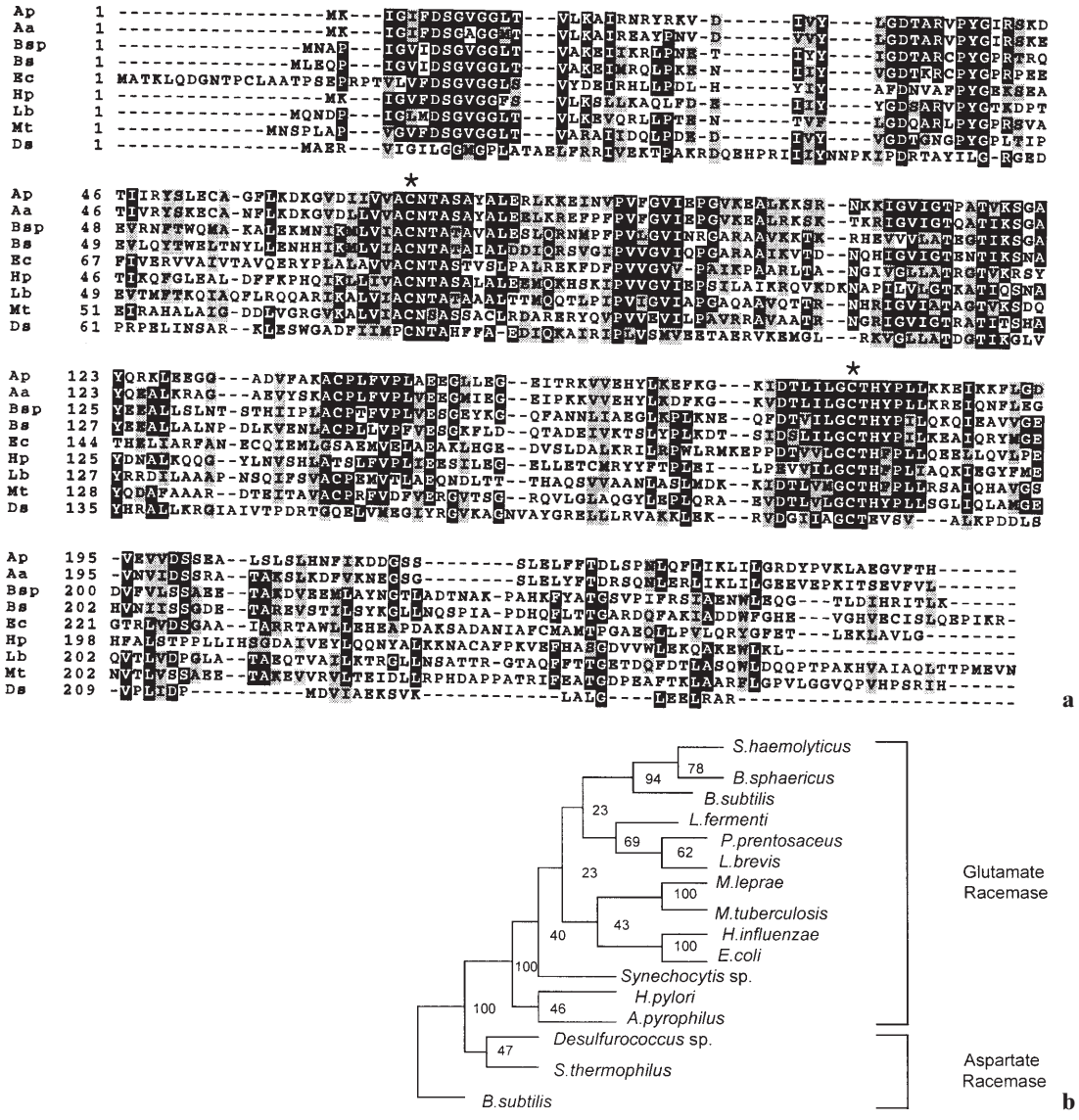

Fig. 2a,b. Comparison of primary structure of glutamate racemases. **a** Linear alignment of amino acid sequence of glutamate racemases and an aspartate racemase. The identical and homologous amino acid residues are indicated as *black* and *shaded backgrounds*, respectively. Two cysteine residues involved in the catalytic reaction are marked with *asterisks*. Glutamate racemases: Ap, *A. pyrophilus*; Aa, *A. aerolicus*; Bsp, *Bacillus sphaericus*; Bs, *B. subtilis*; Lb, *Lactobacillus brevis*; Mt, *Mycobacterium tuberculosis*; Hp, *Haemophilus pylori*; Ec, *E. coli*. Asparate racemase: De, *Desulfurococcus* species. **b** Phenogram of consensus maximum parsimony tree of glutamate and aspartate racemases using the PHYLIP package. The *number* in the phenogram indicates the frequency (%) of the occurrence of the node in 1000 bootstrap replicates. The amino acid sequences were obtained from GeneBank (*gb*), PIR (*pir*), or SWISS-PROT (*sp*). Glutamate racemases: *A. aerolicus* (gb:AE000657), *Streptococcus haemolyticus* (gb:U12405), *B. sphaericus* (gb:U26733), *B. subtilis* (sp:P94556), *Lactobacillus fermenti* (gb:L02916), *Pediococcus pentosarceus* (gb:L22448), *L. brevis* (gb:D29627), *M. leprae* (sp:P46705), *M. tuberculosis* (sp:Q10626), *H. influenzae* (sp:P52973), *E. coli* (sp:P22634), *H. pylori* (sp:P56068). Aspartate racemase: *Desulfurococcus* sp. (gb:D84067), *S. thermophilus* (pir:S16175), *B. subtilis* (pir:B36908)

A consensus maximum parsimony tree was established from the amino acid sequences of the glutamate racemase and the aspartate racemase from several microorganisms (Fig. 2b). The glutamate racemase of *A. pyrophilus* appears farthest from the other bacterial glutamate racemase genes. This low value of homology is comparable to the location of *A. pyrophilus* in the deepest branch of the bacteria (Olsen et al. 1994).

Expression and purification

The coding region of the cloned gene was expressed in *E. coli* using a pET expression system. After a 3-h induction of the BL21 (DE3) strain of *E. coil* harboring pGR1 with 1 mM IPTG, a 28-kDa protein was expressed in a soluble form. The expression level was about 15% of the total cellular protein (Fig. 3, lane 3). The molecular weight of the

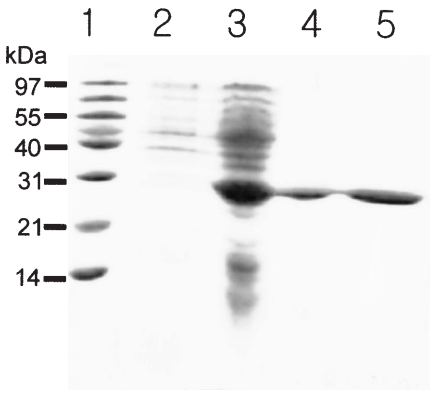

Fig. 3. SDS-PAGE of *A. pyrophilus* glutamate racemase at different stages of purification. The proteins were analyzed on 15% SDS-PAGE. *Lane 1*, molecular weight markers (14.4, 21.5, 31, 40, 42.7, 55, 66, and 97 kDa); *lane 2*, crude extract of *E. coli* BL21 (DE3) before induction; *lane 3*, crude extract after induction with 1 mM isoprogyl β-D-thiogalactoside (IPTG) for 3 h; *lane 4*, soluble fraction of the crude extract after heat treatment for 20 min at 80°C; *lane 5*, purified glutamate racemase after CM-sepharose column chromatography

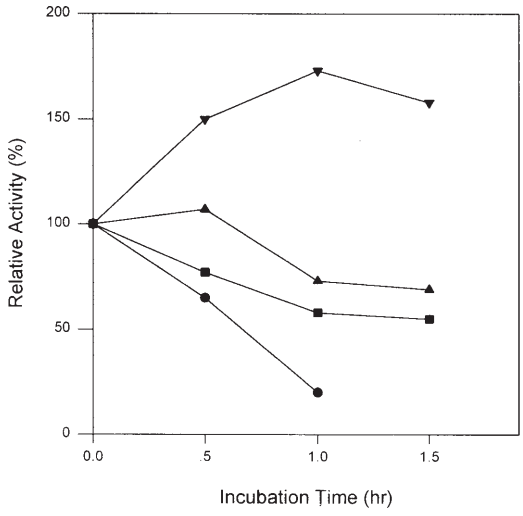

Fig. 4. Effect of heat treatment in the presence of salt on the activity and stability of *A. pyrophilus* glutamate racemase. The enzyme was incubated at 85°C for indicated times in 50 mM Tris-HCl, pH 7.5 containing 0 (*circles*), 0.2 (*squares*), 0.5 (*triangles*), or 1.0 M (*inverted triangles*) $NaPO_4$. After incubation, the activity was measured as described in Materials and methods

expressed protein matched the calculated value (27 993 Da) from the amino acid sequence deduced from the gene sequence. The *A. pyrophilus* glutamate racemase remained in the soluble fraction after incubation at 80°C for 40 min, whereas more than 80% of the *E. coli* proteins were denatured and removed by centrifugation (Fig. 3, lane 4). The expressed protein from *E. coli* is assumed to have the same conformation as the native form in *A. pyrophilus* because it shows strong heat stability, which is a common characteristic of the proteins from hyperthermophiles. The protein was purified to more than 95% purity using CM-sepharose ion-exchange chromatography (lane 5) and used for further characterization.

Confirmation of the glutamate racemase activity and size determination

To confirm that the cloned gene is a glutamate racemase gene, the racemase activity of the expressed and purified protein on L-form glutamate, glutamine, aspartate, asparagine, alanine, proline, lysine, and D-glutamate was examined. When the change of molar ellipticity of the amino acids at 204 nm was monitored after exposure to the purified enzyme, only the ellipticity of L- or D-glutamate was changed (data not shown). These results confirm that the cloned gene is a glutamate racemase gene and that the enzyme only uses D- or L-glutamate among the tested amino acids as substrates.

The size of the purified glutamate racemase has been examined by gel filtration chromatography. In low salt concentration buffer (50 mM Tris-HCl, pH 7.5), the protein eluted at 26 kDa. However, in high-salt buffer (50 mM Tris-HCl, 0.5 M $NaPO_4$, pH 7.5) it eluted as a 61-kDa protein. The apparent size of the protein in these buffers measured much higher by dynamic light scattering; however, it measured as 43 kDa and 96 kDa in low- or high-salt buffer, respectively (Table 1). These results suggested that the protein may present as the dimeric form in low salt condition and become oligomerized as the salt concentration is increased. The dimeric nature of the protein in low-salt condition was further confirmed by X-ray crystallographyc analysis. When the protein was crystallized and its atomic structure solved, it was revealed that the protein formed a dimer (Hwang et al. 1999).

Table 1. Size of native *Aquifex pyrophilus* glutamate racemase (kDa)

	Size exclusion chromatography	Dynamic light scattering
50 mM Tris-HCl, pH 7.5	26	43
50 mM Tris-HCl, pH 7.5 /500 mM $NaPO_4$	61	96

Activation of the enzymatic activity by heat treatment in the presence of high salt concentration

Aquifex pyrophilus glutamate racemase exists as a dimer or tetramer depending on salt concentration. To examine the relative stability of these forms, the residual activity of the enzyme after incubation at 85°C in different salt concentrations has been examined. When the protein was incubated without salt, its activity quickly decreased (Fig. 4). In contrast, the activity declined more slowly in the presence of 0.2 or 0.5 M of $NaPO_4$. Interestingly, the activity of the enzyme increased up to 80% after incubation for 1 h in the presence of 1.0 M $NaPO_4$, but decreased on further incubation. When NaCl or KPO_4 were tested for their effect on activation of *A. pyrophilus* glutamate racemase, 1.0 M of KPO_4 activated the enzyme to a similar level of $NaPO_4$ but not NaCl. These

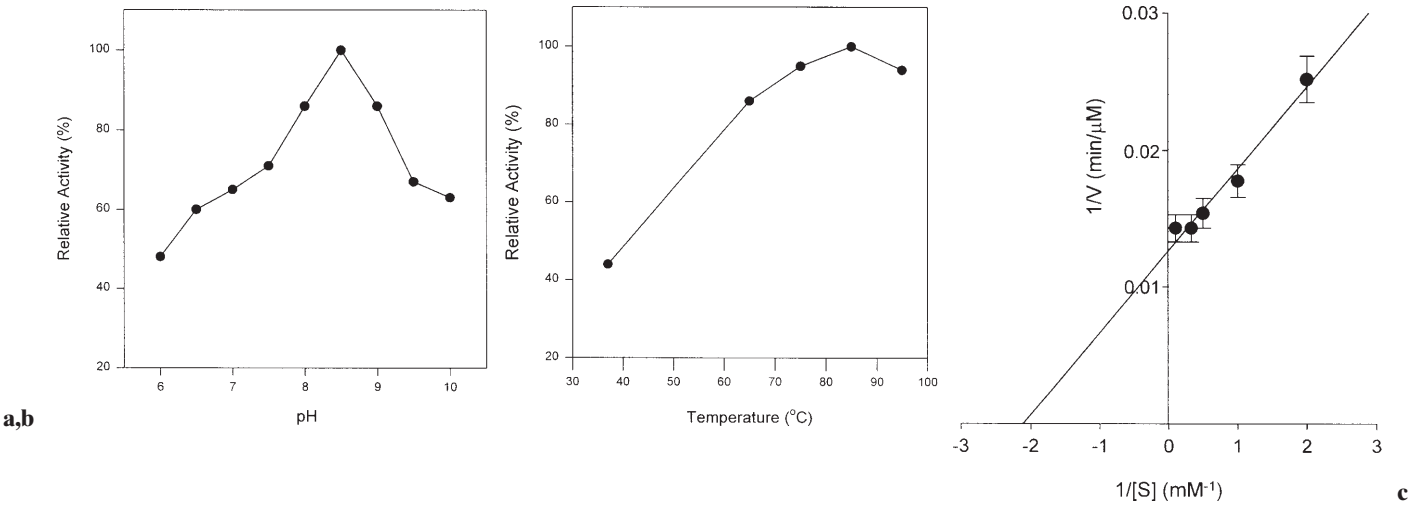

Fig. 5a–c. Effect of pH and temperature on the activity of *A. pyrophilus* glutamate racemase and its kinetic parameters. After incubation at 85°C for 1 h in the presence of 1.0 M $NaPO_4$, the activity of glutamate racemase was measured at 85°C for 20 min in buffers at different pHs (**a**) or temperatures (**b**). The activity of the enzyme was measured at 85°C for 20 min in 50 mM Tris-HCl, pH 8.5 in different concentration of D-glutamate, and the double reciprocal plot of glutamate racemase for the conversion of D- to L-glutamate was obtained (**c**)

results indicate that heat treatment of the enzyme in the presence of a high concentration of phosphate ion may change the protein into a more active and stable conformation.

Enzymatic characterization of *A. pyrophilus* glutamate racemase

The characteristics of the *A. pyrophilus* glutamate racemase have been determined after the enzyme was activated by incubation at 85°C for 1 h in the presence of 1.0 M $NaPO_4$. When the activity of the enzyme was measured at different pHs, it showed highest activity at pH 8.5 (Fig. 5a). The activity decreased by 40% when the pH was increased or decreased by 2 pH units. The enzyme remained active at high temperature. The enzyme showed highest activity at 85°C, the optimum growth temperature of *A. pyrophilus*. More than 85% of the activity was maintained between 65° and 95°C (Fig. 5b). At low temperature (37°C), however, the relative activity of the enzyme decreased to less than 50%. The oligomeric state of the protein differed depending on salt concentration. The relative activity of the enzyme decreased slightly to a level of 70% activity as the concentration of $NaPO_4$ in the reaction mixture was increased up to 1.0 M (data not shown). Hence, the optimum condition for glutamate racemase (50 mM Tris-HCl, pH 8.5, and incubation temperature of 85°C) was further used to measure the kinetic constant of the enzyme. Both the K_m and k_{cat} values for the conversion of D-glutamate to L-glutamate were determined as 0.50 ± 0.07 mM and 0.25 ± 0.01 s^{-1}, respectively, from the reciprocal plot of substrate concentration and reaction velocity (Fig. 5c). For the reverse reaction, the K_m and k_{cat} values were measured as 0.50 ± 0.07 mM and 0.25 ± 0.01 s^{-1}.

Previous studies on glutamate racemase from *Lactobacillus* showed that two cysteines are involved in the active site. The cloned *A. pyrophilus* glutamate racemase has four cysteines and two of them are in the conserved regions (Fig. 2a). To test whether the cysteine residues were critical for enzyme activity, the effect of a cysteine-modifying reagent on the enzyme activity was examined. When the enzyme was incubated in the presence of thiol group-specific modifying reagents (1 mM iodoacetamide or 2 mM of mercuribenzoate), the glutamate racemase activity was completely inhibited (data not shown). This result indicates that the cysteines are involved at the active site.

Thermostability of *A. pyrophilus* glutamate racemase

The thermostability of *A. pyrophilus* glutamate racemase has been examined by measuring the residual activity after incubation of the protein at high temperature in the presence or absence of salt. As shown in Fig. 6a, the enzyme maintained about half its activity after incubation at 85°C for 90 min in the presence of 1.0 M $NaPO_4$, but it lost more than 90% of its activity when it was incubated at 95°C for 20 min. In the absence of salt, the stability of the enzyme was significantly reduced. The half-life of the enzyme at 85°C was measured as less than 10 min, and the activity was completely inactivated after incubation at 95°C for 5 min (Fig. 6b). However, this enzyme was very stable at 60°C regardless of salt. The activity of the enzyme remained unchanged within error over 3 h, and more than 90% of the activity remained after incubation for 24 h at 60°C (data not shown). The CD spectrum of the glutamate racemase has a minimum at 220 nm (data not shown). When temperature-dependent denaturation was observed by monitoring the increase of ellipticity at 220 nm, the protein started to dena-

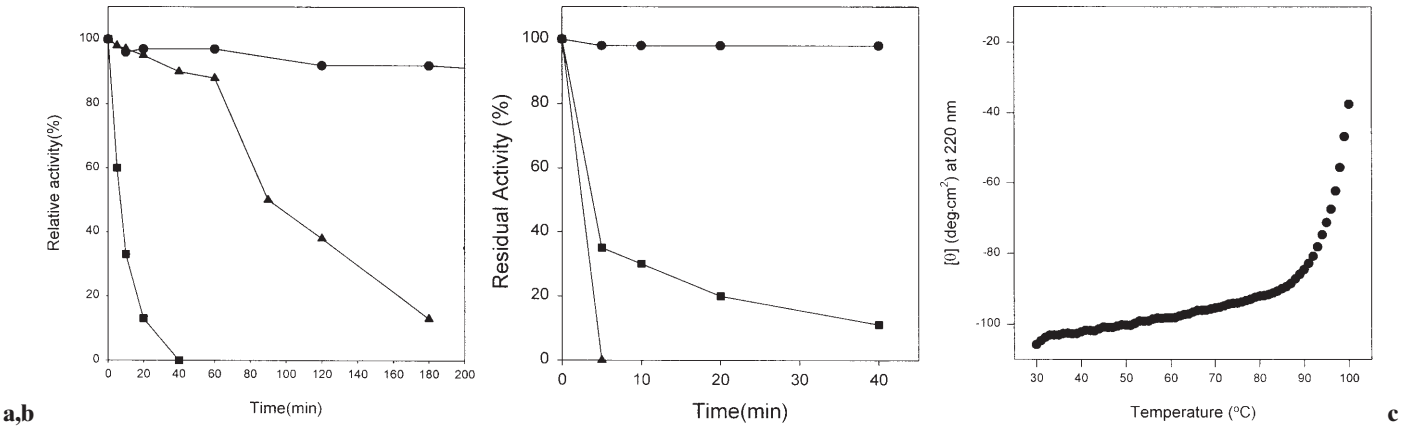

Fig. 6a–c. Thermostability of *A. pyrophilus* glutamate racemase. **a** The enzyme was incubated at 60°C (*circles*), 85°C (*triangles*), or 95°C (*squares*) for 0–3 h in the presence of 1.0 M $NaPO_4$ and the residual activity was assayed. The relative activity was plotted against the incubation time. **b** The activity of the enzyme was measured after incubation at 60°C (*circles*), 85°C (*triangles*), or 95°C (*squares*) in the absent of salt. (**c**) The ellipcity at 220 nm of *A. pyrophilus* glutamate racemase in 50 mM Tris-HCl, 1.0 M $NaPO_4$, pH 7.5 was measured at increments of temperature of 0.5°C/min

ture at temperatures higher than 90°C (Fig. 6c). These results indicate that the enzyme is a very stable protein and that the half-life of the enzyme at 85°C is comparable to the doubling time of *A. pyrophilus* at the same temperature (Huber et al. 1992).

Discussion

The cloned glutamate racemase gene has strong sequence homology to glutamate racemases as well as aspartate racemases from other microorganisms. The relationship between these racemases, analyzed by the construction of a phylogenetic tree using the PHYLIP program, is generally consistent with the 16S RNA-based phylogenetic tree according to the occurrence of branches representing related groups in the tree. The glutamate racemase of *A. pyrophilus* is remotely related to other bacterial racemase genes. This is consistent with the location of *A. pyrophilus* at the deepest branch in the phylogenetic tree of 16S rRNA (Olsen et al. 1994) in the bacterial kingdom. The glutamate racemase of *A. pyrophilus* is unexpectedly close to that of *Haemophilus pylori*. The reason for higher sequence similarity to the *H. pylori* protein is unknown. Because glutamate racemases and aspartate racemases have sequence similarity, and both racemases have active cysteines, it may be possible that they are of the same phylogenetic lineages.

Compared to the heat-sensitive glutamate racemases from mesophilic or thermophilic bacteria (personal communication, Dr. M.-H. Sung, Korea Research Institute of Bioscience and Biotechnology), the *A. pyrophilus* enzyme shows significantly higher thermostability but has a lower conversion rate than the *Lactobacillus* enzyme. This enzyme has relatively low specific activity. The conversion rate (k_{cat}) of the enzyme from L- to D-glutamate is 0.79 s^{-1}, a rate much slower than that of the gram-positive *Lactobacillus* enzyme (k_{cat} of 69 s^{-1}; Gallo and Knowles 1993) but comparable to the conversion rate (3.1 s^{-1}) of *E. coli* (Doublet et al. 1993). The low conversion rate of glutamate racemases from the gram-negative bacteria may be related to the relative low abundance of the peptidoglycan layer compare to that of the gram-positive bacteria.

Different oligomeric states have been reported for glutamate racemase. Glutamate racemases from *E. coli* (Doublet et al. 1994) or *Lactobacillus fermenti* (Gallo and Knowles 1993) are shown to be present as monomers using size-exclusion chromatography. However, the same enzyme from *E. coli* was measured as a dimer by size-exclusion chromatography (Yoshimura et al. 1993). The native size of *A. pyrophilus* glutamate racemase was measured as 26 kDa in size-exclusion chromatography. However, the apparent size becomes bigger as the salt concentration increases, and it measured as a dimer or tetramer by dynamic light scattering analysis, depending on the salt concentration. Because of the potential interaction between the protein and gel matrix in the size-exclusion column, the values determined from the light scattering measurement are believed to be more accurate. Furthermore, the protein presents as dimer when it is crystallized in a low concentration of salt. Oligomerization of protein from hyperthermophiles may contribute to their stability. Ftr (formylmethanofuran: tetrahydromethanopterin formyltransferase) protein from *Methanopyrus kandleri* is inactive and unstable as a monomer at low concentrations of KPO_4. However, it assembles as an active and thermostable tetramer in the presence of 10 M KPO_4 (Ermler et al. 1997). Similarly, the highly assembled form of *A. pyrophilus* glutamate racemase in high-salt condition is more active and stable than the dimer in low-salt conditions.

The crystal structures of several proteins from hyperthermophiles have been determined (Korndorfer et al. 1995; Lim et al. 1997; Yip et al. 1995). When these structures are compared to their counterparts from mesophilic or ther-

mophilic organisms, two characteristics are recognized: (1) a high level of inter- or intra-subunit ion-pair interaction, suggesting that this is a major factor for the thermostability of proteins, and (2) a higher percentage of charged amino acids involved in such interactions. When the content of the charged amino acids of the *Aquifex* enzyme was compared to those of the other glutamate racemases, the average percentage of charged amino acids increased 30% to 90% higher than those of the mesophiles. Especially noteworthy is that the percentage of lysines increased 1.5 to 3 fold. This increased number of charged amino acids probably participate in the formation of ion-pair interactions that stabilize the protein at high growth temperatures.

Acknowledgments This work was supported by the grants from the Ministry of Science and Technology, Korea and the Office of Biosciences and Environmental Research, Office of Energy Research, Department of Energy (DE-AC03-76SF0098).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb J-F, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NSM, Weidman JF, Fuhrman J, Nguyen D, Utterback TR, Kelley JM, Peterson JD, Sadow PW, Hanna MC, Cotton MD, Robert KM, Hurst MA, Kaine BP, Borodovsky M, Klenk HP, Fraser CM, Smith HO, Woese CR, Venter JC (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058–1073

Choi IG, Kim SS, Ryu JR, Han YS, Bang WG, Kim SH, Yu YG (1997) Random sequence analysis of genomic DNA of a hyperthermophile: *Aquifex pyrophilus*. Extremophiles 1:125–134

Dominique ML, Flourt B, van Heijenoort J (1982) Cytoplasmic steps of peptidoglycan synthesis in *Escherichia coli*. J Bacteriol 151:1109–1117

Doublet P, van Heijenoort J, Bohin JP, Dominique ML (1993) The *murI* gene of *Escherichia coli* is an essential gene that encodes a glutamate-racemase activity. J Bacteriol 175:2970–2979

Ermler U, Merckel MC, Thauer RK, Shima S (1997) Formymethanofuran: tetramethanopterin formyltransferase from *Methanopyrus kandler* – new insights into salt-dependence and thermostability. Structure 5:635–646

Felsenstein J (1993) PHILIP (Phylogeny Inference Package) version 3.4c. Distributed by author. Department of Genetics, University of Washington, Seattle

Gallo KA, Knowles JR (1993) Purification, cloning, and cofactor independence of glutamate racemase from *Lactobacillus.* Biochemistry 32:3981–3990

Gallo KA, Tanner ME, Knowles JR (1993) Mechanism of the reaction catalyzed by glutamate racemase. Biochemistry 32:3991–3997

Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem 182:319–326

Huber R, Williarm T, Huber D, Trincore A, Burggraf S, Konig H, Rachel R, Rockinger I, Fricke H, Stetter KO (1992) *Aquifex pyrophilus*, gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst Appl Microbiol 15:340–351

Hwang KY, Cho C-S, Kim SS, Sung HC, Yu YG, Cho Y (1999) Structure and mechanism of glutamate racemase from *Aquifex pyrophilus*. Nature Struct Biol 6:422–426

Klenk H-P, Clayton RA, Tomb J-F, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD, Richardson DL, Kerlavage AR, Graham DE, Kyrpides NC, Fleischmann RD, Quackenbush J, Lee NH, Sutton GG, Gill S, Kirkness EF, Dougherty BA, McKenney K, Adams MD, Loftus B, Peterson S, Reich CI, McNeil LK, Badger JH, Glodek A, Zhou L, Overbeek R, Gocayne JD, Weidman JF, McDonald L, Utterback T, Cotton MD, Spriggs T, Artiach P, Kaine BK, Sykes SM, Sadow PW, D'Andrea KP, Bowman C, Fujii C, Garland SA, Mason TM, Olsen GJ, Fraser CM, Smith HO, Woese CR, Venter JC (1997) The complete genome sequence of the hyperthermophilic, sulfate-reducing archaeon *Archaeoglobus fulgidus*. Nature (Lond) 390:364–370

Korndorfer I, Steipe B, Huber R, Tomschy A, Jaenicke R (1995) The crystal structure of holo-glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima* at 2.5 Å resolution. J Mol Biol 246:511–521

Lim J-H, Yu YG, Han YS, Cho S-J, Ahn B-Y, Kim S-H, Cho Y (1997) The crystal structure of an Fe-superoxide dismutase from hyperthermophile *Aquifex pyrophilus* at 1.9 Å resolution: structural basis for thermostability. J Mol Biol 270:259–274

Nakajima N, Tanizawa K, Tanaka H, Soda K (1986) Cloning and expression in *Escherichia coli* of the glutamate racemase gene from *Pediococcus pentosaceus*. Agric Biol Chem 50:2823–2830

Olsen GJ, Woese CR, Overbeek R (1994) The wind of (evolutionary) change: breathing new life into microbiology. J Bacteriol 176:1–6

Pribnow D (1975) Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. Proc Natl Acad Sci USA 72:184–188

Sambrook J, Fritch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Walsh CT (1989) Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J Biol Chem 264:2393–2396

Yagasaki M, Iwata K, Ishino S, Azuma M, Ozaki A (1995) Cloning, purification, and properties of a cofactor-independent glutamate racemase from *Lactobacillus brevis* ATCC 8287. Biosci Biotechnol Biochem 59:610–614

Yamauchi T, Choi S-Y, Okada H, Yohda M, Kumaga H, Esaki N, Soda K (1992) Properties of aspartate racemase, a pyridoxal 5′-phosphate-independent amino acid racemase. J Biol Chem 267: 18361–18364

Yip KSP, Stillman TJ, Britton KL, Artymiuk PJ, Baker PJ, Sedelnikova SE, Engel PC, Pasquo A (1995) The structure of *Pyrococcus furiosus* glutamate dehydrogenase reveals a key role for ion-pair networks in maintaining enzyme stability at extreme temperatures. Structure 3:1147–1158

Yohda M, Okada H, Kumagai H (1991) Molecular cloning and nucleotide sequencing of the aspartate racemase gene from lactic acid bacteria *Streptococccus thermophilus*. Biochim Biophys Acta 1089:234–240

Yohda M, Endo I, Abe Y, Ohta T, Iida T, Maruyama T, Kagawa Y (1996) Gene for aspartate racemase from the sulfur-dependent hyperthermophilic archaeum, *Desulfurococcus* strain SY. J Biol Chem 271:22017–22021

Yonaha K, Misono H, Yamamoto T, Soda K (1975) D-Amino acid aminotransferase of *Bacillus sphaericus*. J Biol Chem 250:6983–6989

Yorifugi T, Misono H, Soda K (1971) Arginine racemase of *Pseudomonas grareolens*. I. Purification, crystallization, and properties. J Biol Chem 246:5085–5092

Yoshimura T, Ashiuchi M, Esaki N, Kobatake C, Choi S-Y, Soda K (1993) Expression of *glr* (*murI*,*dga*) gene encoding glutamate racemase in *Escherichia coli*. J Biol Chem 268:24242–24246