

α -Aminoadipate aminotransferase from an extremely thermophilic bacterium, *Thermus thermophilus*

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The extremely thermophilic bacterium *Thermus thermophilus* HB27 synthesizes lysine through α -aminoadipate (AAA). In this study, a *T. thermophilus* gene encoding the enzyme that catalyses transamination of AAA was cloned as a mammalian kynurenine/AAA aminotransferase (*Kat2*) gene homologue. A *T. thermophilus* mutant with disruption of the *Kat2* homologue required a longer lag phase for growth and showed slower growth in minimal medium. Furthermore, addition of AAA or lysine shortened the lag phase and improved the growth rate. The *Kat2* homologue was therefore termed *lysN*. *LysN* recognizes not only 2-oxoadipate, an intermediate of lysine biosynthesis, but also 2-oxoisocaproate, 2-oxoisovalerate and 2-oxo-3-methylvalerate, intermediates of leucine, valine and isoleucine biosyntheses, respectively, along with oxaloacetate, a compound in the TCA cycle, as an amino acceptor. These results suggest multiple roles of *LysN* in several cellular metabolic pathways including lysine and branched-chain amino acid biosyntheses.

INTRODUCTION

Lysine is one of the essential amino acids for higher animals including mammals. However, there are organisms that can synthesize lysine via either of two different lysine biosynthetic pathways: through diaminopimelate as found in plants and bacteria, or through α -aminoadipate (AAA) as found in fungi and yeast (Broquist, 1971; Rico *et al.*, 1995; Vogel, 1964). Our previous studies revealed that the extremely thermophilic bacterium *Thermus thermophilus* HB27 synthesizes lysine through AAA, following a pathway similar to that in lower eukaryotes up to the step for AAA synthesis (Kobashi *et al.*, 1999; Miyazaki *et al.*, 2001, 2002, 2003; Nishida *et al.*, 1999). However, the latter part of the newly found pathway is different from the pathway of fungi and yeast, which contains saccharopine as a biosynthetic intermediate (Cunin *et al.*, 1986), but similar to the conversion of glutamate to ornithine in arginine biosynthesis (Nishida *et al.*, 1999). We have previously cloned most of the genes for lysine biosynthesis from *T. thermophilus* HB27 and have provided further support for the idea that lysine biosynthesis through AAA has an

evolutionary relationship with other amino acid biosyntheses and the TCA cycle (Miyazaki *et al.*, 2001, 2002; Nishida *et al.*, 1999); this has already been shown in part by Irvin & Bhattacharjee (1998).

In lower eukaryotes, the enzyme α -aminoadipate aminotransferase (AAAAT; EC 2.6.1.39), which catalyses the transamination reaction using 2-oxoadipate (2-OA) as an amino acceptor to yield AAA, is suggested to be involved in lysine biosynthesis. Matsuda & Ogur (1969) isolated and characterized glutamate:2-OA aminotransferases 1 and 2 from *Saccharomyces cerevisiae* and reported, based on the catalytic properties, that enzymes 2 and 1 were biosynthetic and catabolic enzymes, respectively, in lysine metabolism. However, to date, the corresponding genes have not been identified in *S. cerevisiae* or other organisms. To elucidate the whole pathway of lysine biosynthesis in *T. thermophilus* and the evolutionary relationships between lysine biosynthesis and other metabolic pathways, including leucine biosynthesis, arginine biosynthesis and the TCA cycle, we attempted to clone the gene from the micro-organism and analyse its function.

In this paper, we describe the cloning of a gene (*lysN*) encoding a mammalian kynurenine/AAAAT homologue, which is presumably involved in lysine biosynthesis in *T. thermophilus* HB27. We also report the kinetic properties of *LysN*, which shows AAAAT activity and branched-chain amino acid aminotransferase activity.

Abbreviations: AAA, α -aminoadipate; AAAAT, α -aminoadipate aminotransferase; BcAT, branched-chain amino acid aminotransferase; 2-OA, 2-oxoadipate; 2-OIC, 2-oxoisocaproate; 2-OIV, 2-oxoisovalerate; 2-OMV, 2-oxo-3-methylvalerate.

The GenBank accession number for the sequence reported in this paper is AB097117.

METHODS

Strains, media and chemicals. The extreme thermophile *T. thermophilus* HB27 was cultivated as described previously (Kobashi *et al.*, 1999; Koyoma *et al.*, 1986; Tanaka *et al.*, 1981). *Escherichia coli* DH5 α was used for DNA manipulation, and *E. coli* BL21-Codon-Plus(DE3)-RIL [F⁻ *ompT hadS* (r_B m_B) *dcm Tet*^r *gal* λ (DE3) *endA Hte* (*argU ileY leuW Cam*^r)] (Stratagene) was used as the host for gene expression. The medium 2 \times YT (Sambrook *et al.*, 2001), was generally used for cultivation of *E. coli* cells.

All the chemicals were purchased from Sigma or from Kanto Chemicals. NAD⁺-dependent glutamate dehydrogenase was purchased from Toyobo. Enzymes for DNA manipulation were purchased from Takara Shuzo. Oligonucleotide primers used in this study are listed in Table 1. DNA manipulation was performed according to the methods of Sambrook & Russell (2001).

Molecular cloning and sequencing. Based on amino acid sequence alignments among DR1588 (NP_295311), ST1411 (NP_377369), APE0169 (NP_147018) and PH0207 (NP_142204), all of which possess significant identity in amino acid sequence to Kat2, oligonucleotides AAA1 and AAA2 were designed and used as degenerate primers for PCR to clone a DNA fragment containing a *Kat2* homologue of *T. thermophilus* HB27. An amplified 666 bp fragment was blunted, phosphorylated and ligated into the *EcoRV* site of pBluescript II KS (+) (Stratagene). The resulting plasmid was named pKSAAA101. Based on the information on the partial nucleotide sequence of the cloned fragment, oligonucleotides AT-N3 and AT-C3 were designed and used as primers for inverse PCR (Sambrook *et al.*, 2001). For the inverse PCR, a *BglII*-digested genome fragment of approximately 4.5 kb, which was positive in Southern hybridization using the 666 bp fragment as the probe, was purified by agarose gel electrophoresis, self-ligated and used as the template. The amplified DNA fragment of approximately 4.1 kb was then sequenced to assign both translational start and stop codons for the *Kat2* homologue, *lysN*. Based on 5' and 3' flanking sequences of *lysN*, oligonucleotides lysN-N and lysN-C, containing *NdeI* and *EcoRI* sites, respectively, were designed and used as primers for PCR to amplify the full-length *lysN* gene. The amplified 1194 bp *lysN* fragment was then cloned into pET26b(+) (Novagen, EMD Biosciences). After sequencing four clones to avoid PCR errors, the

resulting plasmid carrying the correct sequence was named pETLysN7 and used for expression of the *lysN* gene. The nucleotide sequence was determined by the method of Sanger *et al.* (1977).

Disruption of *lysN* in *T. thermophilus* HB27. To introduce an *XbaI* site in the *lysN* gene, PCRs were performed using two pairs of oligonucleotides as primers, A3AT-1/A3AT-2 and A3AT-3/A3AT-4. After appropriate treatment with restriction endonucleases, the two amplified fragments were inserted into pUC18 to generate pUC18/A3AT. The 1.1 kb DNA fragment encoding the thermostable kanamycin resistance gene (Hoseki *et al.*, 1999) was amplified as the *XbaI* fragment by PCR, using the oligonucleotides HTK-1 and HTK-2 as primers, and inserted into pUC18/A3AT at the *XbaI* site to yield pUCATKmR. Disruption of the chromosomal copy of *lysN* was performed by using pUCATKmR as described previously (Hidaka *et al.*, 1994; Koyoma *et al.*, 1986), and confirmed by PCRs using the oligonucleotides A3AT-1/AAA3 and A3AT-1/A3AT-5 as primers.

Phenotypic analysis of *lysN* mutant. The *lysN* knockout mutant of *T. thermophilus* was cultured in 1 ml TM medium overnight. TM medium contains 0.8% polypeptone, 0.4% Bacto Yeast Extract, 0.2% NaCl, 0.4 mM CaCl₂, and 0.4 mM MgCl₂. After centrifugation of the culture at 20 000 g for 1 min, the precipitate was washed with minimal medium (MP medium; Kobashi *et al.*, 1999; Tanaka *et al.*, 1981) four times and resuspended in 1 ml MP medium. A 10 μ l volume of the resuspended culture was added to 5 ml MP medium and incubated at 70 °C. Growth of the cells was analysed by monitoring the OD₆₀₀ of the cell culture at appropriate intervals.

Purification of recombinant LysN from *E. coli*. *E. coli* BL21-Codon-Plus (DE3)-RIL cells harbouring pETLysN7 were cultured in 800 ml of 2 \times YT medium containing 50 μ g kanamycin ml⁻¹ and 30 μ g chloramphenicol ml⁻¹. When the culture had reached an OD₆₀₀ of 0.5, IPTG (final concentration, 0.1 mM) was added. The culture was continued at 25 °C for an additional 12 h after the induction. *E. coli*(pETLysN7) cells (wet weight, 4.5 g) collected from the 800 ml culture were suspended in 27 ml buffer I (20 mM potassium phosphate buffer, pH 6.5, 0.5 mM EDTA) and disrupted by sonication. The supernatant prepared by centrifugation at 40 000 g for 20 min was heated at 80 °C for 20 min, and denatured proteins from *E. coli* cells were removed by centrifugation at 40 000 g for

Table 1. Primers

Name	Sequence
AAA1	5'-AAGGT(G/C)TCCT(G/C)GACGAG-3'
AAA2	5'-CTCCATCCA(G/C)ACGAACAT(G/C)CC-3'
AAA3	5'-CTCCATCCAGACGAACATGCC-3'
AT-N3	5'-CGCCAGGGTTTTCCAGTCACGACCCCGGGCCACCGTGAGGAAGCG-3'
AT-C3	5'-GAGCGGATAACAATTTACACAGGATGCTCAACCAGATGCTGGTCCACGAG-3'
A3AT-1	5'-CCCCGAATTC AAGGTCTTCTGGACGAGGG-3'
A3AT-2	5'-GAGGCTCGGGAGTCTAGACTCCCCGAAGTA-3'
A3AT-3	5'-TACTTCGGGGAGTCTAGACTCCCCGAGCCTC-3'
A3AT-4	5'-CCAGACGAGCATGCCGCCCTTGGGCCTTGT-3'
A3AT-5	5'-GCATGCCGCCCTTGGGCCTTGTGTAGCGCA-3'
lysN-N	5'-GGGGTCTAGACATATGAAACCCTGTAAGCTG-3'
lysN-C	5'-GGGGGAATTCCTTAGACCAAGGCCAAAAGCC-3'
HTK1	5'-CCCCTCTAGACGTTGACGCGGATATGGTA-3'
HTK2	5'-AAAATCTAGACGTAACCAACATGATTAACA-3'

20 min. Supernatant fractions were applied to an anion-exchange column (DE-52; Whatman), pre-equilibrated with buffer I and eluted with buffer I containing 0.1 M NaCl. After the addition of ammonium sulfate to a final concentration of 65% saturation to active fractions, the resultant precipitate was collected by centrifugation at 40 000 g for 30 min. The precipitated proteins were solubilized with buffer I and applied onto a Hi-load 26/60 Superdex 200 prep-grade column (Amersham-Pharmacia Biotech) equilibrated with buffer I containing 0.2 M NaCl to yield the purified preparation. Protein concentration was determined by the method of Bradford (1976) using a protein assay kit (Nippon Bio-Rad).

Sedimentation equilibrium analysis. Sedimentation equilibrium analysis of purified LysN was performed with a Beckman Optima XL-A analytical ultracentrifuge fitted with a Beckman An-60Ti analytical rotor. The subunit organization of the enzyme was analysed according to the procedure of Van Holde & Baldwin (1958).

Enzyme assays. Enzyme assays were carried out according to the method described by Nakatani *et al.* (1970). The temperature for the assay was set to 45 °C to avoid non-enzymic oxidation of NADH. The enzyme reaction was initiated by adding 10 μ l of the enzyme solution (0.25 mg ml⁻¹) to 1 ml reaction buffer (100 mM potassium phosphate buffer, pH 7.5, 50 mM NH₄Cl, 10–120 μ M 2-OA, 2.0 mM L-glutamate, 0.15 mM NADH, 10 μ M pyridoxal 5'-phosphate, 11.9 U glutamate dehydrogenase ml⁻¹), which was preincubated at 45 °C for 5 min. For measuring the activity of 2-oxoisocaproate (2-OIC), 2-oxoisovalerate (2-OIV) and 2-oxo-3-methylvalerate (2-OMV), 10–200 μ M 2-OIC, 10–120 μ M 2-OIV or 0.1–5 mM 2-OMV was added to the reaction buffer instead of 2-OA. The reaction was monitored at 45 °C by measuring the decrease in absorption at 340 nm. Kinetic parameters were calculated by using an initial velocity program, HYPER (Cleland, 1979). Specific activity was determined by using 20 mM L-glutamate as the amino donor, and 1 mM 2-OA, 2-OIC, 2-OIV or phenylpyruvate, or 10 mM 2-OMV, pyruvate or oxaloacetate as the amino acceptor. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μ mol NADH min⁻¹ at 45 °C in the reaction.

RESULTS

Strategy for cloning the *T. thermophilus* gene encoding AAAAT

We have previously reported that *T. thermophilus* synthesizes lysine through AAA. To determine the whole lysine biosynthetic pathway, we attempted to clone the gene that encodes the enzyme AAAAT, which catalyses the transamination reaction of 2-OA to produce AAA in lysine biosynthesis of *T. thermophilus*. However, a corresponding gene has not been identified even in fungi or yeast, although the lower eukaryotes are known to synthesize lysine through the same pathway up to the step for AAA synthesis. Furthermore, the gene for the aminotransferase is not contained in the putative gene cluster involved in lysine biosynthesis of any of the micro-organisms that were recently suggested to synthesize lysine in a manner similar to that in *T. thermophilus* (Brinkman *et al.*, 2002; Nishida *et al.*, 1999).

By searching in the literature for the enzyme that catalyses the transamination reaction, we found that the aminotransferase Kat2 (EC 2.6.1.7), which irreversibly transaminates a tryptophan metabolite, kynurenine, to form

kynurenine acid, a putative endogenous modulator of the glutamatergic neurotransmitter in mammals, catalyses the transamination reaction between 2-OA and glutamate to produce AAA and 2-oxoglutarate (Rico *et al.*, 1995), although mammals cannot synthesize lysine. We then analysed the distribution of *Kat2* homologues in micro-organisms and found that all the micro-organisms that have been suggested to possess the lysine biosynthesis pathway through AAA carry a gene encoding a protein that shows considerable identity to *Kat2* in amino acid sequence: YGL202W (32%) from *S. cerevisiae*, DR1588 (31%) from *Deinococcus radiodurans*, PH0207 (31%) from *Pyrococcus horikoshii*, ST1411 (32%) from *Sulfolobus tokodaii* and APE0169 (30%) from *Aeropyrum pernix*. We therefore decided to clone a *Kat2* homologue from *T. thermophilus*. Determination of the nucleotide sequence of the cloned full-length *Kat2* homologue revealed that the gene encodes a protein composed of 397 amino acid residues with a predicted molecular mass of 43 845 Da. The *Kat2* homologue showed significant identity in amino acid sequence to DR1588 (44%), PH0207 (35%), ST1411 (37%) and APE0169 (30%).

Disruption of *Kat2* homologue in *T. thermophilus*

We next investigated the role of the *Kat2* homologue in *T. thermophilus*. For this purpose, we constructed a mutant of *T. thermophilus* with disruption in the chromosomal *Kat2* homologue as described in Methods. The *Kat2*-homologue disruptant, TM1001, required longer lag time for growth in the shift-down experiments from rich medium to minimal MP medium: the lag time was about 20 h for the mutant and 10 h for the wild-type (Fig. 1). The extended lag phase was significantly reversed by addition of 0.1 mM lysine or AAA, but not affected by addition of 0.1 mM 2-OA. Furthermore, the growth rate of the mutant (doubling time, 190 min) was found to be lower than that of the wild-type strain (84 min) in minimal medium. The doubling

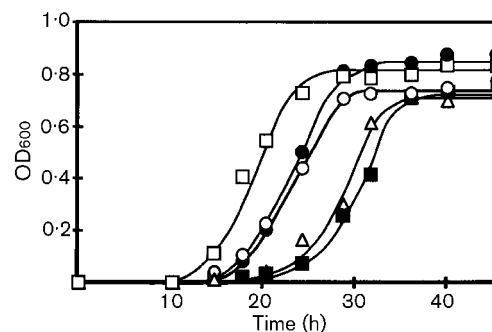


Fig. 1. Growth curves of the wild-type *T. thermophilus* HB27 (□) and the *lysN*-deficient mutant (Δ) in a minimal (MP) medium, and of the *lysN*-deficient mutant in minimal MP medium supplemented with 0.1 mM 2-OA (■), AAA (●) or lysine (○).

time of the mutant was not shortened by addition of 2-OA (192 min), but it was significantly improved by addition of AAA (121 min) and lysine (151 min). These results suggested the involvement of the *Kat2* homologue in lysine biosynthesis of *T. thermophilus*. Hereafter, we refer to this gene as *lysN*.

Expression and purification of LysN

To characterize the *lysN* gene product, we expressed the gene in *E. coli* and obtained a purified preparation of LysN by using a T7 promoter expression system. LysN was accumulated as insoluble inclusion bodies when the *E. coli* cells carrying pETLysN7 were cultured in the presence of 1 mM IPTG at 37 °C (data not shown). By lowering the temperature and the IPTG concentration to 25 °C and 0.1 mM, respectively, a portion of LysN proteins was produced in the soluble fraction. Through four steps of purification procedure, 2 mg LysN was prepared from 800 ml culture with more than 95 % purity on SDS-PAGE (data not shown). The apparent molecular mass of 44 kDa on SDS-PAGE coincided well with the molecular mass of 43 845 Da calculated from the amino acid sequence. To estimate the quaternary structure of LysN, we performed sedimentation equilibrium analysis. By using a partial specific volume of 0.7463 that was calculated from the amino acid composition of LysN, we found that the enzyme was in equilibrium as monomer–dimer with an equilibrium constant of 2.4×10^{-5} M. In this equilibrium, LysN was calculated to be present as a mixture of homodimer (75 %) and monomer (25 %) when the enzyme concentration was about 1 mg ml⁻¹. Therefore, LysN was considered to be a homodimer when characterizing its catalytic properties.

Catalytic properties of LysN

For an initial evaluation of the substrate specificity of LysN, we determined specific activities for several compounds in the crude extract prepared by heat treatment of the *E. coli* cell lysate and successive centrifugation steps. As expected, *T. thermophilus* LysN showed transamination of 2-OA to produce AAA with a specific activity of 6788 U (mg protein)⁻¹ (Table 2). When similar analysis was done in the reverse reaction at the same pH with AAA and 2-oxoglutarate as the amino donor and the amino acceptor, respectively, only negligible activity was detected. From this analysis, we concluded that LysN was a biosynthetic

Table 2. Specific activities of LysN for several 2-oxoacids

Data are shown as means ± SE.

Substrate	Metabolism	Specific activity [U (mg protein) ⁻¹]*
2-OA (1 mM)	Lysine	6788 ± 120
2-OIC (1 mM)	Leucine	5079 ± 305
2-OIV (1 mM)	Valine	1102 ± 81
2-OMV (10 mM)	Isoleucine	561 ± 64
Pyruvate (10 mM)	Alanine	568 ± 32
Oxaloacetate (10 mM)	Aspartate	661 ± 50
Phenylpyruvate (1 mM)	Phenylalanine	513 ± 119

*Heat-treated lysate of *E. coli* without the recombinant plasmid had no activity.

enzyme for lysine biosynthesis. We next analysed the transamination reaction with several 2-oxoacids as the amino acceptor by using glutamate as an amino donor; we found that LysN recognized all these compounds as a substrate.

Kinetic parameters of LysN

Since, as shown above, LysN recognized branched 2-oxoacids as well as 2-OA as a substrate, we performed kinetic analysis of the purified enzyme using 2-OA, 2-OIC, 2-OIV or 2-OMV, which are intermediates of lysine, leucine, valine or isoleucine biosynthesis, respectively, as an amino acceptor, and glutamate as an amino donor. Double reciprocal plots indicated that the reaction catalysed by LysN proceeded through a ping-pong bi-bi mechanism (data not shown), similar to results obtained with other aminotransferases. Kinetic parameters indicate that although LysN recognizes 2-OA most efficiently, the enzyme can also utilize other compounds involved in biosynthesis of branched-chain amino acids with comparable efficiency (Table 3).

DISCUSSION

The gene encoding the fourth enzyme in the lysine biosynthesis pathway, which catalyses the conversion of 2-OA to AAA, has not previously been identified in any organism

Table 3. Kinetic parameters of LysN

Data are shown as means ± SE.

Substrate	Biosynthesis	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ M ⁻¹)
2-OA	Lysine	24.2 ± 1.3	22.3 ± 0.4	(9.2 ± 0.5) × 10 ⁵
2-OIC	Leucine	28.8 ± 3.4	17.8 ± 0.6	(6.2 ± 0.8) × 10 ⁵
2-OIV	Valine	13.3 ± 3.6	2.1 ± 0.1	(1.6 ± 0.4) × 10 ⁵
2-OMV	Isoleucine	148.3 ± 15.4	3.5 ± 0.1	(2.4 ± 0.3) × 10 ⁴

known to synthesize lysine through AAA. In this study, we cloned the *Kat2* homologue, *lysN*, and the gene product possessed AAAAT activity. Unexpectedly, the *lysN* mutant of *T. thermophilus*, TM1001, did not show a lysine-auxotrophic phenotype. However, the mutant required a longer lag time and showed slower growth in minimal MP medium, which was significantly reversed by exogenous addition of either 0.1 mM lysine or AAA. These results indicate that LysN plays a primary role in the conversion of 2-OA to AAA, which is the fourth reaction in lysine biosynthesis by *T. thermophilus*. When the evolutionary relationship of LysN to other aminotransferases was phylogenetically analysed, LysN was found to be outgrouped with aspartate aminotransferases but grouped with putative aminotransferases from organisms that have been suggested to synthesize lysine through the AAA pathway (Fig. 2). Therefore, this phylogenetic analysis suggests that all the putative aminotransferases related to LysN are involved in lysine biosynthesis in these micro-organisms.

Our previous studies suggested that *T. thermophilus* lysine biosynthesis has evolutionary relationships with leucine biosynthesis, arginine biosynthesis and the TCA cycle (Miyazaki *et al.*, 2003; Nishida *et al.*, 1999). The fourth reaction in the lysine biosynthesis pathway, the conversion of 2-OA to AAA, is thought to proceed in a way similar to that of the related reaction catalysed by branched-chain amino acid aminotransferase (BcAT) in leucine, valine and isoleucine biosyntheses, which are the conversion of 2-OIC to leucine, 2-OIV to valine and 2-OMV to isoleucine,

respectively (Fig. 3). The fourth reaction in the lysine biosynthesis pathway is also similar to this reaction, i.e. the conversion of 2-oxoglutarate to glutamate catalysed by aspartate aminotransferase, which connects the TCA cycle and arginine biosynthesis. As shown in Tables 2 and 3, LysN exhibits activity for branched-chain amino acids and aspartate. Therefore, these results support our previous assumption that lysine biosynthesis has functional and evolutionary relationships with branched-chain amino acid biosynthesis, arginine biosynthesis and the TCA cycle.

The fact that *lysN*-knockout did not confer the complete lysine (AAA)-auxotrophic phenotype suggests the presence of another enzyme(s) capable of compensating for *lysN* deficiency of the mutant. BcAT may be a possible candidate to compensate the loss of LysN because BcAT is known to have broad substrate specificity (Hall *et al.*, 1993; Yvon *et al.*, 2000). Alternatively, an aromatic aminotransferase may be involved in the process. *YGL202W*, a *Kat2* homologue in *S. cerevisiae*, has another name, *ARO8*. *ARO8* encodes aromatic aminotransferase I involved in the last step of the reaction in phenylalanine and tyrosine biosyntheses, which transaminates amino groups of glutamate to phenylpyruvate and *p*-hydroxyphenylpyruvate. Interestingly, *Aro8p* was found to have the ability to convert 2-OA to AAA (Urrestarazu *et al.*, 1998). In that study, the authors reported that the action of the aromatic aminotransferase I accounted for half of the glutamate:2-OA aminotransferase activity in a cell-free extract of *S. cerevisiae* when the aminotransferase activity in the wild-type and an

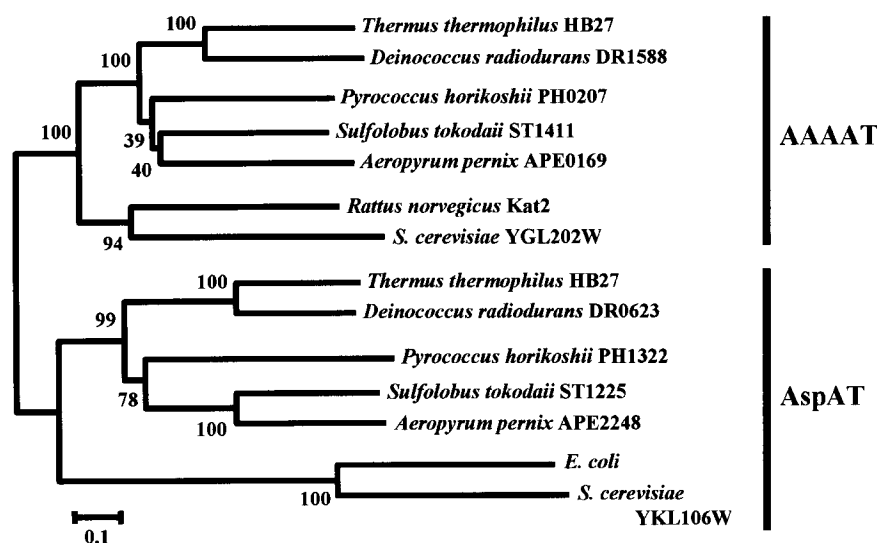


Fig. 2. Phylogenetic tree of LysN and related aminotransferases, constructed by using neighbour-joining methods. The amino acid sequences were aligned by using CLUSTAL W (Thompson *et al.*, 1994). Using the aligned data, a phylogenetic tree was constructed by the computer program MEGA (Kumer *et al.*, 2001). Numbers on selected nodes indicate bootstrap values. The figure includes AAAAT and aspartate aminotransferases (AspAT) from *T. thermophilus* HB27, *D. radiodurans* (NP_295311, NP_294346), *P. horikoshii* (NP_142204, NP_143210), *S. tokodaii* (NP_377369, NP_377158), *A. pernix* (NP_147018, NP_148487), *Rattus norvegicus* (NP_058889), *S. cerevisiae* (NP_011313, NP_012816) and *E. coli* (NP_415448). AspAT, aspartate amino transferase.

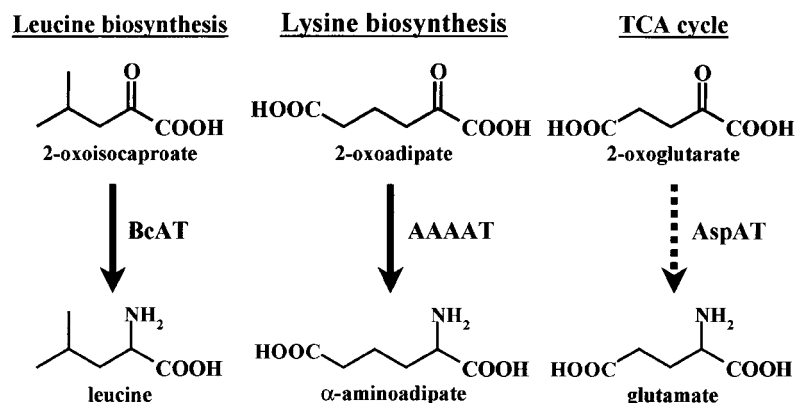


Fig. 3. The fourth reaction of the AAA lysine pathway and related reactions.

aro8 strain defective in *ARO8* was measured (Urrestarazu *et al.*, 1998). Therefore, glutamate:2-OA aminotransferase II, which was previously characterized by Matsuda & Ogur (1969), might be identical to the aromatic aminotransferase encoded by *ARO8*. In any case, it should be noted that the yeast *aro8* strain does not show the phenotype to require phenylalanine or tyrosine for growth without simultaneous knockout of *ARO9*, which encodes a catabolic enzyme mainly involved in tryptophan degradation (Iraqi *et al.*, 1998). Similarly, it is possible to postulate that *T. thermophilus* might have another enzyme that could compensate for LysN inactivation. However, another candidate enzyme involved in lysine metabolism has not yet been found in *T. thermophilus*. *T. thermophilus* genome projects that are currently in progress will provide a better understanding of the auxotrophy of the *lysN* mutant.

The addition of AAA or lysine to the minimal MP medium indeed shortened lag phase for growth of the *lysN*-knockout mutant and improved its growth rate, but it did not completely reverse the extended lag and the slow growth. This observation may suggest some other roles of LysN that are required for the normal growth of the cells as suggested in the case of Aro8p (Urrestarazu *et al.*, 1998; Zabriskie & Jackson, 2000). We would like to note that the expression of the *lysN* gene is not regulated by lysine in *T. thermophilus* cells (data not shown), which is in contrast to many other genes involved in amino acid biosynthesis. This might be evidence, though indirect, suggesting the possible involvement of LysN in several unrelated metabolic pathways. It is noteworthy that LysN recognizes 2-OIC more efficiently than oxaloacetate, as is the case for BcAT, which is involved in biosynthesis of branched-chain amino acids, although no significant identity in amino acid sequence has been found between LysN and BcAT (data not shown). Thus, since LysN has aminotransferase activity not only for AAA, but also for the branched-chain amino acids, there is the possibility that LysN might be involved at least in both lysine and branched-chain amino acid biosyntheses. There is a unique aminotransferase group, COG1167, in which a GntR-type DNA-binding HTH helix domain is fused at the N-terminus of type I aminotransferase. *T. thermophilus* AAAAT also belongs to the COG1167 group, but it lacks the

N-terminal GntR-type HTH domain as found in normal aminotransferases. Therefore, *T. thermophilus* LysN does not function as a transcription regulator. However, it should be noted that the corresponding protein of *Corynebacterium glutamicum*, PdxR (cg0897), is involved in valine biosynthesis as well as pyridoxine biosynthesis (McHardy *et al.*, 2003), which suggests a functional relationship between LysN and the MocR-type aminotransferase in that both the proteins play a putative role in branched-chain amino acid biosynthesis. In any case, such broad substrate specificity is a typical feature found in the enzymes involved in other reactions in lysine biosynthesis (Miyazaki *et al.*, 2001, 2002, 2003; Wulandari *et al.*, 2002). It should be noted that addition of branched-chain amino acids, isoleucine, leucine and valine, remarkably accelerated the growth of *T. thermophilus* cells in minimal medium and no difference was detected in growth between the wild-type and the *lysN* mutant (data not shown), suggesting that the amino group of the branched-chain amino acid might be, directly or indirectly, transferred to 2-OA to form AAA. Considering the similarity in amino acid sequence between LysN and YGL202W (*ARO8*), it might be possible to consider that LysN is involved in aromatic amino acid biosynthesis; however, this is unlikely because the activity of LysN for phenylpyruvate was quite low (Table 2) and addition of both phenylalanine and tyrosine had no effect on the growth of the *T. thermophilus* mutant lacking the *lysN* gene in the minimal medium (data not shown). To further elucidate the role of LysN in *Thermus* cells, detailed analysis of the nutrition required for the normal growth of the mutant will be necessary.

On the other hand, the *Pyrococcus furiosus* aminotransferase, PF0121, a possible orthologue of LysN and PH0207 of *P. horikoshii*, has been characterized biochemically and referred to as aromatic amino acid aminotransferase (Andreotti *et al.*, 1995; Schut *et al.*, 2003). Expression of PF0121 was upregulated in peptide-grown cells, suggesting its role in catabolism in the archaeon (Schut *et al.*, 2003). On the other hand, we observed that the catabolic activity of LysN to convert AAA to 2-OA was negligible and addition of lysine had no effect on the expression profile of *lysN* in *T. thermophilus* (data not shown). These results could

suggest that LysN plays a role that is different from that of PF0121 in *P. furiosus*. Nevertheless, it is of interest to analyse the substrate specificity of PF0121 for these amino acids.

In this study, we have shown that LysN catalyses the fourth reaction of lysine biosynthesis through the AAA pathway. Detailed analysis of the mechanism used by LysN to recognize several compounds as substrates may provide a clue to elucidate the evolution of aminotransferases. For this purpose, it is necessary to determine the three-dimensional structure of LysN and the complexes it forms with substrate analogues.

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REFERENCES

- Andreotti, G., Cubellis, M. V., Nitti, G., Sanna, G., Mai, X., Adams, M. W. W. & Marino, G. (1995). An extremely thermostable aromatic aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochim Biophys Acta* **1247**, 90–96.
- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brinkman, A. B., Bell, S. D., Lebbink, R. J., de Vos, W. M. & van der Oost, J. (2002). The *Sulfolobus solfataricus* Lrp-like protein LysM regulates lysine biosynthesis in response to lysine availability. *J Biol Chem* **277**, 29537–29549.
- Broquist, H. P. (1971). Lysine biosynthesis (yeast). *Methods Enzymol* **17**, 112–119.
- Cleland, W. W. (1979). Statistical analysis of enzyme kinetic data. *Methods Enzymol* **78**, 103–138.
- Cunin, R., Glandsorff, N., Oierard, A. & Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev* **50**, 314–352.
- Hall, T. R., Wallin, R., Reinhart, G. D. & Hutson, S. M. (1993). Branched chain aminotransferase isozymes. *J Biol Chem* **268**, 3092–3098.
- Hidaka, Y., Hasegawa, M., Nakahara, T. & Hoshino, T. (1994). The entire population of *Thermus thermophilus* cells is always competent at any growth phase. *Biosci Biotechnol Biochem* **58**, 1338–1339.
- Hoseki, J., Yano, T., Koyama, Y., Kuramitsu, S. & Kagamiyama, H. (1999). Directed evolution of the thermostable kanamycin-resistance gene: a convenient selection marker for *Thermus thermophilus*. *J Biochem* **126**, 951–956.
- Iraqui, I., Vissers, S., Cartiaux, M. & Urrestarazu, A. (1998). Characterization of *Saccharomyces cerevisiae* ARO8 and ARO9 genes encoding aromatic aminotransferase I and II reveals a new aminotransferase subfamily. *Mol Gen Genet* **257**, 238–248.
- Irvin, S. D. & Bhattacharjee, J. K. (1998). A unique fungal lysine biosynthesis enzyme shares a common ancestor with tricarboxylic acid cycle and leucine biosynthetic enzymes found in diverse organisms. *J Mol Evol* **46**, 401–408.
- Kobashi, N., Nishiyama, M. & Tanokura, M. (1999). Aspartate kinase-independent lysine synthesis in an extremely thermophilic bacterium, *Thermus thermophilus*: lysine is synthesized via α -amino adipic acid, not via diaminopimelic acid. *J Bacteriol* **181**, 1713–1718.
- Koyama, Y., Hoshino, T., Tomizuka, N. & Furukawa, K. (1986). Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. *J Bacteriol* **166**, 338–340.
- Kumer, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA: molecular evolutionary genetics analysis, version 2.1. University Park, PA: Pennsylvania State University.
- Matsuda, M. & Ogur, M. (1969). Separation and specificity of the yeast glutamate- α -keto adipate transaminase. *J Biol Chem* **244**, 3352–3358.
- McHardy, A. C., Tauch, A., Rückert, C., Pühler, A. & Kalinowski, J. (2003). Genome-based analysis of biosynthetic aminotransferase genes of *Corynebacterium glutamicum*. *J Biotechnol* **104**, 229–240.
- Miyazaki, J., Kobashi, N., Nishiyama, M. & Yamane, H. (2001). Functional and evolutionary relationship between arginine biosynthesis and prokaryotic lysine biosynthesis through α -amino adipate. *J Bacteriol* **183**, 5067–5073.
- Miyazaki, J., Kobashi, N., Fujii, T., Nishiyama, M. & Yamane, H. (2002). Characterization of a *lysK* gene as an *argE* homolog in *Thermus thermophilus* HB27. *FEBS Lett* **512**, 269–274.
- Miyazaki, J., Kobashi, N., Nishiyama, M. & Yamane, H. (2003). Characterization of homoisocitrate dehydrogenase involved in lysine biosynthesis of an extremely thermophilic bacterium, *Thermus thermophilus* HB27, and evolutionary implication of beta-decarboxylating dehydrogenase. *J Biol Chem* **278**, 1864–1871.
- Nakatani, Y., Fujioka, M. & Higashino, K. (1970). α -Amino adipate aminotransferase of rat liver mitochondria. *Biochim Biophys Acta* **198**, 219–228.
- Nishida, H., Nishiyama, M., Kobashi, N., Kosuge, T., Hoshino, T. & Yamane, H. (1999). A prokaryotic gene cluster involved in synthesis of lysine through the amino adipate pathway: a key to the evolution of amino acid biosynthesis. *Genome Res* **9**, 1175–1183.
- Rico, B., Alberati-Giani, D., Malherbe, P., Kohler, C., Broger, C. & Cesura, A. M. (1995). Cloning and functional expression of a soluble form of kynurenine/ α -amino adipate aminotransferase from rat kidney. *J Biol Chem* **270**, 29330–29335.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, E. F. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.
- Schut, G. J., Brehm, S. D., Datta, S. & Adams, M. W. W. (2003). Whole-genome DNA microarray analysis of a hyperthermophile and an archaeon: *Pyrococcus furiosus* grown on carbohydrates or peptides. *J Bacteriol* **185**, 3935–3947.
- Tanaka, T., Kawamoto, N. & Oshima, T. (1981). Cloning of 3-isopropylmalate dehydrogenase gene of an extreme thermophile and partial purification the gene products. *J Biochem* **89**, 677–682.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Urrestarazu, A., Vissers, S., Iraqui, I. & Grenson, M. (1998). Phenylalanine- and tyrosine-auxotrophic mutants of *Saccharomyces cerevisiae* impaired in transamination. *Mol Gen Genet* **257**, 230–237.
- Van Holde, K. E. & Baldwin, R. L. (1958). Rapid attainment of sedimentation equilibrium. *J Phys Chem* **62**, 734–743.

Vogel, H. J. (1964). Distribution of lysine pathway among fungi: evolutionary implications. *Am Nat* **98**, 446–455.

Wulandari, A. P., Miyazaki, J., Kobashi, N., Nishiyama, M., Hoshino, T. & Yamane, H. (2002). Characterization of bacterial homocitrate synthase involved in lysine biosynthesis. *FEBS Lett* **522**, 35–40.

Yvon, M., Chambellon, E., Bolotin, A. & Roudot-Algaron, F. (2000). Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. *Appl Environ Microbiol* **66**, 571–577.

Zabriskie, T. M. & Jackson, M. D. (2000). Lysine biosynthesis and metabolism in fungi. *Nat Prod Rep* **17**, 85–97.