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L-*allo*-Threonine Aldolase from *Aeromonas jandaei* DK-39: Gene Cloning, Nucleotide Sequencing, and Identification of the Pyridoxal 5′-Phosphate-Binding Lysine Residue by Site-Directed Mutagenesis

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We have isolated the gene encoding L-*allo*-threonine aldolase (L-*allo*-TA) from *Aeromonas jandaei* DK-39, a pyridoxal 5′-phosphate (PLP)-dependent enzyme that stereospecifically catalyzes the interconversion of L-*allo*-threonine and glycine. The gene contains an open reading frame consisting of 1,014 nucleotides corresponding to 338 amino acid residues. The protein molecular weight was estimated to be 36,294, which is in good agreement with the subunit molecular weight of the enzyme determined by polyacrylamide gel electrophoresis. The enzyme was overexpressed in recombinant *Escherichia coli* cells and purified to homogeneity by one hydrophobic column chromatography step. The predicted amino acid sequence showed no significant similarity to those of the currently known PLP-dependent enzymes but displayed 40 and 41% identity with those of the hypothetical GLY1 protein of *Saccharomyces cerevisiae* and the GLY1-like protein of *Caenorhabditis elegans*, respectively. Accordingly, L-*allo*-TA might represent a new type of PLP-dependent enzyme. To determine the PLP-binding site of the enzyme, all of the three conserved lysine residues of L-*allo*-TA were replaced by alanine by site-directed mutagenesis. The purified mutant enzymes, K51A and K224A, showed properties similar to those of the wild type, while the mutant enzyme K199A was catalytically inactive, with corresponding disappearance of the absorption maximum at 420 nm. Thus, Lys199 of L-*allo*-TA probably functions as an essential catalytic residue forming an internal Schiff base with PLP of the enzyme to catalyze the reversible aldol reaction.

The bioorganic synthesis of β-hydroxy-α-amino acids is attracting a great deal of attention because of their potential application as chiral building blocks for the synthesis of biologically active molecules (6, 7, 24, 32, 33). A variety of β-hydroxy-α-amino acids are present in complex natural compounds with interesting biological properties. 4-Hydroxy-L-threonine, for instance, is a precursor of rizobitoxine, a potent inhibitor of pyridoxal 5′-phosphate (PLP)-dependent enzymes (32). 3,4,5-Trihydroxy-L-aminopentanoic acid is a key component of polyoxins (32). β-Hydroxy-α-amino acids are also constituents of a range of antibiotics, for example, cyclosporin A, lysobactin, and vancomycin (30) and bouvardin and deoxybouvardin (9). Threonine aldolase (EC 4.1.2.5), which catalyzes the retro-aldol cleavage of certain β-hydroxy-α-amino acids, has also been shown to catalyze synthesis of the substituted amino acids from aldehyde and glycine (24, 32, 33).

Thus far, two types of threonine aldolases have been purified and characterized. Low-specific-activity L-threonine aldolase (L-TA), previously named L-threonine aldolase, catalyzes the cleavage of both L-threonine and L-*allo*-threonine to glycine and acetaldehyde, as well as the reverse aldol condensation. The enzymes have been purified from *Candida humicola* (13, 35) and *Pseudomonas* sp. strain NCIMB 11097 (5). L-TA activity has also been shown to exist in mammals (10, 20, 23) and a variety of other microbial species (4, 6, 16, 36). L-*allo*-Threonine aldolase (L-*allo*-TA), isolated and purified from *Aeromonas jandaei* DK-39 (11), stereospecifically catalyzes the reversible interconversion of L-*allo*-threonine and glycine. The enzyme is notable for the synthesis of certain homo-chiral β-hydroxy-α-amino acids (11). L-TA and L-*allo*-TA are commonly yellow, exhibiting an absorption maximum at 420 nm due to the PLP bound as an internal Schiff base. They also have comparable molecular masses, pH and temperature optima, and thermostabilities. However, the two types of aldolases catalyze the interconversion of a broad range of β-hydroxy-α-amino acids and glycine with distinct stereospecificities. We are interested in determining the structural and functional relationships of L-*allo*-TA and L-TA, so as to be able to engineer an artificial aldolase with varied stereospecificity.

In this paper, we describe the cloning, sequencing, and overexpression in *Escherichia coli* cells of the L-*allo*-TA gene (*ltaA*) of *A. jandaei* DK-39 and identification of the active-site lysine residue of the novel enzyme by site-directed mutagenesis. We also report the identification of two hypothetical proteins, GLY1 protein of *Saccharomyces cerevisiae* and GLY1-like protein of *Caenorhabditis elegans*, that show significant similarity to L-*allo*-TA in amino acid sequence. The functions and physiological roles of these proteins are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *A. jandaei* DK-39 was used as the source of chromosomal DNA (11). *E. coli* XL1-Blue MRF′ {*recA1 thi endA1 supE44 gyrA46 relA1 hsdR17 lac*/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15o::Tn*10* (Tet^r)]} (Toyobo, Osaka, Japan) was used as a host strain for gene cloning and in most DNA manipulations. *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1*/pCJ105 (F′

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Cam^r)] was used for the generation of a uracil-containing single-stranded DNA for site-directed mutagenesis. *E. coli* BMH71-18*mutS* [Δ(*lac-proAB*) *supE thi mutS215*::Tn*10*(Tet^r)/F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] (Takara Shuzo, Kyoto, Japan) was used as a host strain for site-directed mutagenesis. Plasmid pUC118 was used as a cloning vector. *A. jandaei* DK-39 was grown under conditions described previously (11). Recombinant *E. coli* cells were cultivated at 37°C in Luria-Bertani (LB) medium (1% polypeptone, 0.5% yeast extract, 1% NaCl [pH 7.2]) containing 100 μg of ampicillin per ml. For induction of the gene under the control of the *lac* promoter, 0.2 mM isopropyl-1-thio-β-D-galactoside (IPTG) was added to the LB medium. To select recombinant cells harboring pUC118 with inserted DNA, 0.01% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added to the LB agar (1.5%, wt/vol) medium.

General recombinant DNA technique. Plasmid DNA was isolated by the alkaline sodium dodecyl sulfate (SDS) method. Plasmid DNA from large-scale preparations was purified with a plasmid purification kit from Qiagen Inc. (Chatsworth, Calif.). Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo and Toyobo and used according to the manufacturers' protocols. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions, using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research Inc., San Diego, Calif.). Other general procedures were performed as described by Sambrook et al. (22).

Cloning of the *ltaA* gene. Two oligonucleotide primers were purchased from Hokkaido Biosystem Inc. (Sapporo, Japan), each with additional restriction sites (underlined in the following sequences) added to the 5′ end to facilitate cloning of the amplified product: primer I, 5′-CCGAAGCTTATGCGNTAYATHGAYYT-3′; and primer II, 5′-GCCGAATTCACRTCRTCNCCNACYTC-3′. Degenerate positions are indicated by Y for C or T, R for A or G, H for A, C, or T, and N for all bases. Primers I and II were based upon the NH_2-terminal amino acid sequence of the wild-type L-*allo*-TA from *A. jandaei* DK-39. PCR amplification was performed in 50 μl of 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.1 mM each deoxynucleotide–100 pmol of each primer–1 μg of *A. jandaei* chromosomal DNA–0.5 U of *Taq* DNA polymerase at 94°C for 1 min, 53°C for 1.5 min, and 72°C for 1.5 min in a total of 35 cycles. The amplified product was digested with *Eco*RI and *Hin*dIII, separated by agarose gel electrophoresis, and then purified with a Geneclean kit (Bio101 Inc., Vista, Calif.). The amplified DNA of 100 bp was then cloned into pUC118.

Chromosomal DNA isolated from *A. jandaei* DK-39 cells was partially digested with *Sau*3AI (21). The fragments in the molecular size range of 1 to 9 kb were separated by agarose gel electrophoresis and then purified with a Geneclean kit. The fragments were ligated into *Bam*HI-restricted pUC118, and the plasmids were introduced into *E. coli* XL1-Blue MRF′ cells to construct a genomic library of *A. jandaei* DK-39. The genomic library was screened by colony hybridization with the [α-^{32}P]dCTP-labeled 100-bp DNA fragment as a probe. The clone, pAJ3A1, carrying the 5.1-kb DNA fragment was selected for further analysis.

Sequence analysis. pAJ3A1 was used as a sequencing template. The nucleotide sequence was determined by the dideoxy-chain termination method with Cy5 AutoRead and Cy5 AutoCycle sequencing kits and a Pharmacia LKB ALFred DNA sequencer. A homology search was performed by means of the sequence similarity search programs Fasta (2) and Blast (18). The Clustal W method was used to align the sequences (29).

Overexpression of the *ltaA* gene in *E. coli*. To obtain the entire gene without excessive flanking parts, PCR amplification was carried out in 50 μl of 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.1 mM each deoxynucleotide–100 pmol of each primer–1 μg of *A. jandaei* chromosomal DNA–0.5 U of *Ex Taq* DNA polymerase (Takara Shuzo) at 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min in a total of 30 cycles. The 5′ primer (5′-GCCGAATTCACCaggagGGATGTC**ATG**C-3′) containing a Shine-Dalgarno sequence (lowercase) and an ATG initiation codon (boldface) and the 3′ primer (5′-CCGAAGCTTCATTCCATGAGATTG**TCA**CG-3′) with the complement of the TGA termination codon (boldface) were designed on the basis of the nucleotide sequence of the *ltaA* gene. To facilitate cloning, an additional restriction site (underlined) was incorporated into each primer. The amplified PCR product was digested with *Eco*RI and *Hin*dIII, separated by agarose gel electrophoresis, and then purified with a Geneclean kit. The amplified DNA of approximately 1.1 kb, which contained the complete coding sequences of the *ltaA* gene, was inserted downstream of the *lac* promoter of pUC118 and then transformed *E. coli* XL1-Blue MRF′ cells. The constructed plasmid was designated pAJPCR.

Feasible purification of L-*allo*-TA. (i) Preparation of cell extract. Cells of the *E. coli* transformant harboring overexpression plasmid pAJPCR were grown aerobically at 37°C for 14 h in 500 ml of LB medium containing 0.1 mg of ampicillin per ml and 0.2 mM IPTG. The cells were harvested and then rinsed with 100 mM potassium phosphate buffer (pH 7.5). After being suspended in 20 ml of the same buffer, the cells were disrupted by ultrasonic oscillation at 4°C for 20 min with a model 201M ultrasonic oscillator (Kubota, Tokyo, Japan). The cell debris was removed by centrifugation.

(ii) Hydrophobic column chromatography. The supernatant solution, brought to 30% saturation with ammonium sulfate, was applied to a butyl-Toyopearl 650M column (2.5 by 20 cm). Elution was carried out with a 600-ml linear gradient of 30 to 0% saturated ammonium sulfate in 50 mM potassium phosphate buffer (pH 7.5) at a flow rate of 5 ml/min. The active fractions eluted at an ammonium sulfate concentration of about 12 to 9% were concentrated by the addition of ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation and resuspended in a small volume of 100 mM potassium phosphate buffer (pH 7.5) containing 0.01 mM PLP. After dialysis against the same buffer, the purified enzyme was stored in 50 mM potassium phosphate buffer (pH 7.5) containing 20% (wt/vol) glycerol at −30°C.

Amino acid sequencing. The NH_2-terminal amino acid sequence was determined by the Edman degradation procedure with a 6625 ProSequencer (Milli-Gen, Milford, Mass.). To determine the internal amino acid sequence, the enzyme was digested with lysylendopeptidase (Wako Pure Chemical Industries, Osaka, Japan). The resultant peptide fragments were applied to a C_{18} column (4.6 by 150 mm; Tosoh Corp., Tokyo, Japan) and eluted with 0.05% trifluoroacetic acid for 10 min, followed by a linear gradient of 0 to 80% acetonitrile in 0.05% trifluoroacetic acid over 60 min at a flow rate of 1.0 ml/min. Elution was monitored at 215 nm with a UV detector, and fractions were collected. Four of the fractions were subjected to sequence analysis.

Site-directed mutagenesis. Mutants of L-*allo*-TA from *A. jandaei* DK-39 were prepared by the method of Kunkel et al. (14). The mutant enzymes and synthetic mutagenic primers were as follows (underlining indicates the mutagenized nucleotides): K51A, 5′-CAGCCTCTGCTCCCAGC-3′; K199A, 5′-CCAGCCCCGCGGAGAGGC-3′; K199C, 5′-CGCCCAGCCCGCAGGAGAGGCAGATGG-3′; and K224A, 5′-CCACCATCGCGCGCAGCC-3′. The substitutions were confirmed by DNA sequencing with AutoCycle sequencing kits and a Pharmacia LKB ALFred DNA sequencer. All of the mutant enzymes were produced by *E. coli* XL1-Blue MRF′ cells. The mutant enzymes were purified by the procedure used for the wild type except that the purification was monitored by SDS-polyacrylamide gel electrophoresis (PAGE) on a slab gel in the case of the nonactive mutant enzymes.

Enzyme and protein assays. L-*allo*-TA activity was assayed with L-*allo*-threonine as a substrate. The reaction mixture comprised 2.5 μmol of L-*allo*-threonine, 0.01 μmol of pyridoxal phosphate, 20 μmol of Tris-HCl buffer (pH 8.0), and the enzyme, in a total volume of 200 μl. The reaction was carried out at 30°C for 15 min and was terminated by the addition of 50 μl of 30% trichloroacetic acid. The acetaldehyde released was measured spectrophotometrically by the method of Paz et al. (17). One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of acetaldehyde per min under the assay conditions described. To determine the kinetic constants of the enzyme, threonine aldolase activity was measured spectrophotometrically at 340 nm by coupling the reduction of acetaldehyde (oxidation of NADH) with yeast alcohol dehydrogenase (Wako Pure Chemical Industries). The assay mixture comprised 100 μmol of Tris-HCl buffer (pH 8.0), 0.2 μmol of NADH, 30 U of yeast alcohol dehydrogenase, and appropriate amounts of the enzyme and substrate, in a final volume of 1 ml. One unit of aldolase activity is the amount of enzyme that catalyzes the formation of 1 μmol of acetaldehyde (1 μmol of NADH oxidized) per min at 30°C. The protein assay was performed with a Bio-Rad protein assay kit.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D87890.

RESULTS

Cloning of the *ltaA* gene. The primers used for cloning of the *ltaA* gene by PCR were based on the NH_2-terminal amino acid sequence of the purified enzyme from *A. jandaei* DK-39 as described in Materials and Methods. PCR with the primers and *A. jandaei* DK-39 chromosomal DNA as the template yielded an amplified band of 100 bp. Only this band was constantly amplified under different PCR conditions. The amplified DNA was then cloned into pUC118 in *E. coli*. Nucleotide sequencing of the 100-bp fragment showed the presence of an open reading frame (ORF) continuing over the entire sequence. The deduced amino acid sequence of the PCR fragment was in perfect agreement with the NH_2-terminal amino acid sequence determined from the purified L-*allo*-TA of *A. jandaei* DK-39 (11). We then directly performed colony hybridization with the 100-bp fragment as a probe against the established *A. jandaei* DK-39 genomic library; seven positive recombinant *E. coli* clones were obtained from about 10,000 transformants. The clone showing the highest L-*allo*-TA activity (0.3 U/mg) was chosen for further characterization.

The plasmid, pAJ3A1, in this clone contained a 5.1-kb insert. Southern hybridization with the 100-bp PCR fragment as the probe indicated that the *ltaA* gene is located between the *Sal*I and *Sac*I sites (Fig. 1).

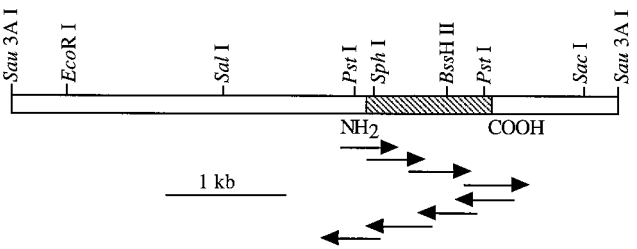

FIG. 1. Partial restriction map of and sequencing strategy for pAJ3A1. The coding region is shown by the hatched box, and the positions corresponding to the NH_2 and C termini are denoted by NH_2 and COOH, respectively. Arrows indicate the directions and extents of the sequenced fragments.

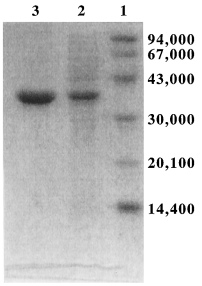

FIG. 3. Purification of the recombinant L-*allo*-TA from *A. jandaei* DK-39. Samples from each of the purification steps were loaded on an SDS–10% polyacrylamide gel and stained with Coomassie blue after electrophoresis. Lane 1, molecular weight standards; lane 2, cell extract (15 μg); lane 3, butyl-Toyopearl pool (15 μg). Numbers to the right are molecular weights of the standards.

Sequence analysis of the *ltaA* gene. Nucleotide sequence analysis revealed that the ORF consists of 1,014 bp starting with an initiation codon, ATG, and ending with a termination codon, TGA, at position 1249 (Fig. 2). A probable ribosome-binding sequence, AGGAG, is present seven bases upstream of the putative translational start codon (25). However, sequences similar to the *E. coli* −35 sequence (TTGACA) and −10 sequence (TATAAT) were not found. The ORF encodes a protein of 338 amino acid residues. The predicted molecular weight is 36,294, which is in good agreement with the value determined for the purified enzyme from *A. jandaei* DK-39 (11). The NH_2-terminal and internal amino acid sequences coincided with those of the purified enzyme determined by Edman degradation (Fig. 2).

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CCA GCC CTT TCT CCA TGC GCT GGC CCC CGC TAG TCT GCA GGA TCC TGC ATT GAT CGC CCC    60
CCG TTT ACT GGC TCT GAT CGC CGG TTT ATC GTT GGC GCA ATC CGG CGC CTT TGT GGA TCT   120
GCA GGG CAG CCG CTG CCC TGG TAA GCC GTC ACC GGG CTC ACA TTC CAG CGA CGC CCT GCT   180
TGC CAA GCC CGC CCG GGC TGG CTA ACG TCG TCC TCC TGC ACC AGG AGG GAT GTC ATG CGC   240
                                                                  M   R           2

TAT ATC GAT TTA CGA AGT GAT ACG GTC ACC CAG CCC ACG GAT GCC ATG CGT CAG TGC ATG   300
 Y   I   D   L   R   S   D   T   V   T   Q   P   T   D   A   M   R   Q   C   M    22

CTC CAT GCC GAG GTC GGG GAT GAT GTT TAT GGG GAG GAT CCG GGC GTC AAC GCG TTG GAA   360
 L   H   A   E   V   G   D   D   V   Y   G   E   D   P   G   V   N   A   L   E    42

GCC TAT GGC GCC GAC CTG CTG GGA AAA GAG GCT GCG CTG TTC GTG CCA TCC GGC ACC ATG   420
 A   Y   G   A   D   L   L   G   K   E   A   A   L   F   V   P   S   G   T   M    62

TCC AAC TTG CTG GCG GTG ATG AGC CAT TGC CAG CGT GGG GAA GGG GCT GTG CTG GGC TCG   480
 S   N   L   L   A   V   M   S   H   C   Q   R   G   E   G   A   V   L   G   S    82

GCA GCC CAC ATC TAT CGC TAT GAG GCG CAG GGT TCT GCT GTG CTG GGG TCT GTG GCC CTG   540
 A   A   H   I   Y   R   Y   E   A   Q   G   S   A   V   L   G   S   V   A   L   102

CAA CCT GTG CCC ATG CAG GCT GAT GGC TCG CTG GCG CTG GCG GAT GTG CGG GCG GCC ATC   600
 Q   P   V   P   M   Q   A   D   G   S   L   A   L   A   D   V   R   A   A   I   122

GCC CCT GAC GAT GTC CAT TTT ACC CCG ACT CGC CTC GTC TGC CTC GAA AAC ACC CAT AAC   660
 A   P   D   D   V   H   F   T   P   T   R   L   V   C   L   E   N   T   H   N   142

GGC AAG GTG CTG CCC TTG CCT TAT CTG CGC GAG ATG CGC GAA CTG GTC GAT GAG CAT GGT   720
 G   K   V   L   P   L   P   Y   L   R   E   M   R   E   L   V   D   E   H   G   162

TTG CAG TTG CAT CTG GAC GGT GCT CGT CTG TTC AAT GCC GTG GTG GCG AGT GGC CAT ACG   780
 L   Q   L   H   L   D   G   A   R   L   F   N   A   V   V   A   S   G   H   T   182

GTG CGC GAA CTG GTC GCG CCG TTT GAC AGT GTC TCC ATC TGC CTC TCC AAG GGG CTG GGC   840
 V   R   E   L   V   A   P   F   D   S   V   S   I   C   L   S   K   G   L   G   202

GCG CCG GTG GGA TCG CTG CTG GTG GGT TCT CAT GCC TTT ATT GCC CGT GCC AGA AGG CTG   900
 A   P   V   G   S   L   L   V   G   S   H   A   F   I   A   R   A   R   R   L   222

CGC AAG ATG GTG GGG GGC GGT ATG CGT CAG GCG GGG ATC CTG GCT CAG GCT GGC CTG TTT   960
 R   K   M   V   G   G   G   M   R   Q   A   G   I   L   A   Q   A   G   L   F   242

GCG TTG CAG CAG CAT GTG GTA CGC CTC GCG GAC GAT CAT CGC CGT GCC AGA CAG TTG GCC  1020
 A   L   Q   Q   H   V   V   R   L   A   D   D   H   R   R   A   R   Q   L   A   262

GAG GGG CTG GCT GCG CTG CCT GGC ATC AGG CTG GAT CTG GCG CAG GTT CAG ACC AAC ATG  1080
 E   G   L   A   A   L   P   G   I   R   L   D   L   A   Q   V   Q   T   N   M   282

GTG TTC CTG CAA CTG ACG AGT GGC GAG AGT GCA CCC TTG CTG GCC TTC ATG AAG GCG CGG  1140
 V   F   L   Q   L   T   S   G   E   S   A   P   L   L   A   F   M   K   A   R   302

GGG ATC CTC TTC TCC GGT TAT GGT GAG CTG CGG TTG GTG ACC CAT CTG CAG ATC CAC GAT  1200
 G   I   L   F   S   G   Y   G   E   L   R   L   V   T   H   L   Q   I   H   D   322

GAT GAC ATT GAA GAG GTG ATT GAC GCC TTT ACC GAG TAT CTC GGG GCG TGA CAA TCT CAT  1260
 D   D   I   E   E   V   I   D   A   F   T   E   Y   L   G   A   •               388

GGA ATG AAC CAG GGC GCC GCG GCC CCC TGT TGG TTG                                  1296
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FIG. 2. Nucleotide and deduced amino acid sequences of the *ltaA* gene and its flanking regions. The asterisk marks a translational stop codon. A putative Shine-Dalgarno sequence is boxed. The four underlined amino acid sequences are identical to those determined for the purified enzyme by Edman degradation.

Overexpression of the *ltaA* gene in *E. coli*. The whole *ltaA* gene amplified by PCR directly from *A. jandaei* chromosomal DNA, with a putative Shine-Dalgarno sequence (AGGAG), an initiation codon (ATG), and a termination codon (TGA), was inserted into the *Eco*RI and *Hin*dIII sites of pUC118. The plasmid constructed, named pAJPCR, was introduced into *E. coli* XL1-Blue MRF′ cells. The nucleotide sequence of the whole amplified gene was further confirmed to have undergone no error matching during PCR by sequencing of the double strands. As shown in Fig. 3 and Table 1, the recombinant cells produced a large amount of L-*allo*-TA. Judging from the specific activity of the crude extract, L-*allo*-TA comprises about 40% of the total soluble protein. The protein was produced only in the presence of IPTG (data not shown), indicating that the *lac* promoter is essential for overexpression. L-*allo*-TA was rapidly purified by only one butyl-Toyopearl column chromatography step, with a yield of 62% (Table 1). The properties of the recombinant enzyme, such as molecular mass, thermostability, optimum pH, optimum temperature, and substrate specificity, were practically identical to those of the enzyme purified from *A. jandaei* DK-39.

Sequence homology with other proteins. The predicted amino acid sequence showed no significant similarity to those of the currently known PLP-dependent enzymes. However, two hypothetical proteins, the GLY1 protein from *S. cerevisiae* (15) and the GLY1-like protein from *C. elegans* (34), were found to be significantly similar to L-*allo*-TA in primary structure upon a search of protein amino acid sequence databases (GenBank, EMBL, PIR, and SWISS-PROT) by means of the sequence similarity search programs Blast (2) and Fasta (18). The GLY1 protein was previously shown by gene disruption studies to possibly be a key enzyme, constituting an additional pathway for glycine synthesis in yeast, besides the well-known pathway for serine hydroxymethyltransferase synthesis, although its enzymatic activity was not elucidated (see Discussion). The GLY1-like protein of *C. elegans*, as its name indi-

TABLE 1. Purification of the recombinant L-*allo*-TA from *A. jandaei* DK-39

Step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Cell extract	620	182	3.4	1	100
Butyl-Toyopearl	385	46	8.4	2.5	62

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  1 ---------------------------------MRYIDLRSDTVTQPTDAMRQCMLHAEV L-allo-TA
  1 ----------------------MTEFELPPKYITAANDLRSDTFTTPTAEMMEAALEASI GLY1
  1 MTKVNGNSNGNGISASTDLLDTPSAYTQKSNKTHTSIDLRSDTVTVPSVEMRRAMAEAIV GLY1-like
                                      ****** * *.  *   .   * .
 28 GDDVYGEDPGVNALEAYGADLLGKEAALFVPSGTMSNLLAVMSHCQRGEGAVLGSA-AHI L-allo-TA
 39 GDAVYGEDVDTVRLEQTVARMAGKEAGLFCVSGTLSNQIAIRTHLMQPPYSILCDYRAHV GLY1
 61 GDDVYGEDTTTNRLEQRCAELFGKEAGLFVTSGTMGNLLAIMAHCQRGEEIIVGRY-NHI GLY1-like
    ** *****     **   * . **** **  ***. * .*. .*  .    ..     *.
 87 YRYEAQGSAVLGSVALQPVPMQADGSLALADVRAAIAPD-------------------D L-allo-TA
 99 YTHEAAGLAILSQAMVVPVVPSNGDYLTLEDIKSHYVPDDG-----------------D GLY1
120 HRWEQGNYAQFAGISATTLEVKPDGTMDLNDIEQAIRVKGGYVQIKDESCDDLEVTSIPD GLY1-like
     *  . *         .       . * *.                               *
127 VHFTPTRLVCLENTHN---GKVLPLPYLREMRELVDEHGLQLHLDGARLFNAVVASGHTV L-allo-TA
141 IHGAPTRLISLENTLH---GIVYPLEELVRIKAWCMENGLKLHCDGARIWNAAAQSGVPL GLY1
180 CHMPASKLICIENTHNYTGGKALPIEWMRSVKQLAERRDLKVHMDGARIYNAAVASNCSV GLY1-like
     *   ..*...*** .   *   *.  .  ..       *.** ****. **   *.  .
184 RELVAPFDSVSICLSKGLGAPVGSLLVGSHAFIARARRLRRMVGGGMRQAGILAQAGLFA L-allo-TA
198 KQYGEIFDSISICLSKSMGAPIGSVLVGNLKFVKKATHFRKQQGGGIRQSGMMARIALVN GLY1
240 SKIASFADTVQMCFSKGLGAPVGSIVVGPKDFIDRARHSRKALGGGWRQSGILAAAAHIA GLY1-like
         *.. .* ** .***.**..**   *. .* . **  *** **.*..*
244 LQ-QHVVRLADDHRRARQLAEGLAALP-----GIRLDLAQVQTNMVFLQLTSG-ESAPLL L-allo-TA
258 INNDWKSQLLYSHSLAHELAEYCEAKG-------IPLESPADTNFVFINLKAARMDPDVL GLY1
300 LD-HADATIRADHERAKTLARMINDATPEEFRTKVFAAEKDITNMVLVHCQNG-VTVQQL GLY1-like
 .     .   *  *. **                ** * .           *
297 AFMKARGILFSGYGELR---LVTHLQIHDDDIEEVIDAFTEYLGA--------------- L-allo-TA
311 VKKGLKYNVKLMGGRVS----F-HYQVTRDTLEKVKLAISEAFDYAK----EHPFDCNGP GLY1
358 TDFFQKHDILAMTFDARRIRMVLNWNVSDENLETIVEVYKKFLKQFDDKKRESTYVLPES GLY1-like
      .  .              . ..  . .* .
    ---------------------------------------------------------- L-allo-TA
362 TQIYRSESTEVDVDGNAIREIKTKYKY-------------------------------- GLY1
418 NNFFNPSTYRLYIHVLLTMRILLILFITVAYVTAFPGFPKTKWDPFVFDITVYCNDKTRD GLY1-like

    ---------------------------------------------------------- L-allo-TA
    ---------------------------------------------------------- GLY1
478 TYNLLIEWWEDDTMPGMEQITRPQFYHPKPGNFSFRMEGAMDGDESFSDGHKPVAYITHD GLY1-like

    ---------------------------------------------------------- L-allo-TA
    ---------------------------------------------------------- GLY1
538 CHKKAQQVELVLTVNTLCKTENSCHYRIIQDISGAQGEKNIQADALVVGGNFTSFPDFSL GLY1-like

    ---------------------------------------------------------- L-allo-TA
    ---------------------------------------------------------- GLY1
598 M                                                            GLY1-like
```

FIG. 4. Sequence alignment of L-*allo*-TA from *A. jandaei* DK-39 with the GLY1 protein of *S. cerevisiae* and the GLY1-like protein of *C. elegans*. Identical residues are marked with asterisks; similarly functional amino acid residues are denoted by dots. The three conserved lysine residues are boxed. Numbers on the left are residue numbers.

cates, was previously shown to be similar to the GLY1 protein of *S. cerevisiae* in primary structure, but its function remained unknown (34). Figure 4 shows the alignment of these proteins with L-*allo*-TA, the identities of the proteins to L-*allo*-TA being as follows: 40% for GLY1 protein and 41% for GLY1-like protein. Interestingly, three of four lysine residues of the PLP-dependent L-*allo*-TA are conserved in the two hypothetical proteins and L-*allo*-TA, one of which likely functions as the PLP-binding site of the enzyme.

Identification of the active-site lysine residue. To identify the PLP-binding lysine residue of L-*allo*-TA, specific mutant enzymes were constructed by site-directed mutagenesis as described in Materials and Methods. The enzymatic activities and kinetic constants of the mutant L-*allo*-TA molecules are summarized in Table 2. Substitution at either Lys51 or Lys224 showed the invariant enzymatic activity of the wild-type enzyme. The similar kinetic constants of the mutants K51A and K224A and the wild-type enzyme further showed that Lys51 and Lys224 are not essential for the enzymatic reaction (Table 2). In contrast, substitution of Lys199 by Ala or Cys abolished all detectable enzymatic activity, and the mutant K199A enzyme showed the disappearance of both the absorption maximum at 420 nm (Fig. 5) and the positive circular dichroism band at 420 nm (Fig. 6) (the absorption and circular dichroism spectra of the mutant K199C protein were superimposed on those of K199A [data not shown]), indicating that the Schiff base linkage between the ε-amino group of the active-site lysine residue and the PLP cofactor aldehyde group of the wild type is not present in the K199A and K199C mutant enzymes. To determine whether the isolated inactive polypeptides are

TABLE 2. Relative activities and kinetic constants of the wild-type and mutant enzymes with respect to cleavage of L-*allo*-threonine[a]

Enzyme	Relative activity (%)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Wild type	100	3.0 ± 0.1	0.37 ± 0.03	8.1 ± 0.5
K51A	99	2.9 ± 0.2	0.36 ± 0.04	8.1 ± 1.3
K224A	100	3.0 ± 0.1	0.38 ± 0.06	7.9 ± 1.3
K199A	0			
K199C	0			

[a] Enzyme activity was determined spectrophotometrically at 340 nm by coupling the reduction of acetaldehyde released from L-*allo*-threonine with yeast alcohol dehydrogenase as described in Materials and Methods. The values of the kinetic constants are averages ± standard deviations of three different determinations.

the mutant threonine aldolases and whether the alternation might result from global variation in protein structure and not a specific effect of the side chains of the new amino acid, several different aspects were considered. First, the two mutant polypeptides, like the wild-type enzyme, were produced only in the presence of IPTG, and their expression levels were similar to that of the wild-type enzyme, as judged from SDS-PAGE of the cell extracts (data not shown). The modified enzymes migrated on SDS-PAGE and native PAGE like the wild-type enzyme (Fig. 7). Second, the superimposed circular dichroism spectra (200 to 300 nm) of the wild-type and K199A mutant enzymes suggested that no drastic change in the secondary structure of the mutant molecule had occurred (Fig. 6). Third, the native molecular weights of the wild-type and mutant K199A and K199C proteins were determined by a HiLoad Superdex 200 gel filtration column (Pharmacia Biotech, Uppsala, Sweden) to be 150,000, which makes extensive conformational changes unlikely. All of these data suggest that the proteins isolated from *E. coli* are the mutant threonine aldolases and that the tetrameric quaternary structure of the wild-type L-*allo*-TA is also characteristic of the mutant proteins.

DISCUSSION

A novel PLP-dependent enzyme, L-*allo*-TA, which shows stereospecific activity toward L-*allo*-threonine was previously purified and characterized (11). In this study, we cloned the *ltaA* gene from *A. jandaei* DK-39 by PCR, determined the primary structure of the encoded protein, and compared its structure with those of other proteins. To our knowledge, this is the first report of the primary structure of a threonine aldolase.

The gene has a putative Shine-Dalgarno sequence but not a

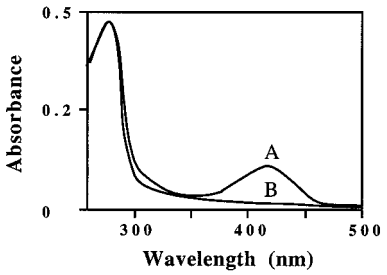

FIG. 5. Absorption spectra of the wild-type (A) and K199A mutant (B) enzymes. The absorption spectra were taken in 20 mM potassium phosphate buffer (pH 7.0) containing 0.01% 2-mercaptoethanol at a protein concentration of 1.5 mg/ml.

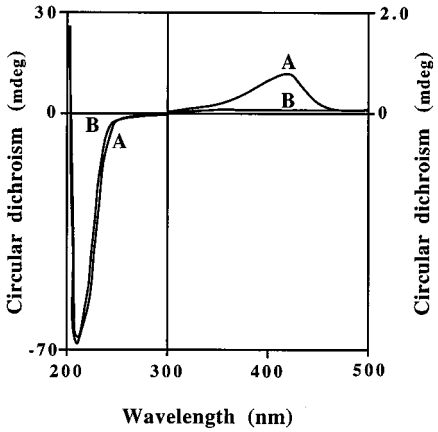

FIG. 6. Circular dichroism spectra of the wild-type (A) and K199A mutant (B) enzymes. The spectra were taken in 20 mM potassium phosphate buffer (pH 7.0) containing 0.01% 2-mercaptoethanol at a protein concentration of 1.0 mg/ml. mdeg, 10^{-3} degree.

σ^{70}-type promoter. However, L-*allo*-TA is produced efficiently by *E. coli* clone cells under regulation of the *lac* promoter in the presence of IPTG, leading to feasible purification of the enzyme by only one hydrophobic column chromatography. This overexpression-purification system provides us with sufficient L-*allo*-TA to study the structural and functional relationships of the enzyme and its application.

Like L-*allo*-TA, many enzymes catalyzing manifold reactions in the metabolism of amino acids require PLP as an essential cofactor. Upon recent computer analysis, these PLP-dependent enzymes were classified into the regiospecific α, β, and γ families (1). (i) The α enzymes, with a few exceptions, catalyze the transformation of amino acids in which the covalency changes are limited to the α carbon atom that carries the amino group forming the aldimine linkage to the coenzyme, such as serine hydroxymethyltransferase, 5-aminolevulinate synthase, and 8-amino-7-oxononanoate synthase. (ii) The β enzymes catalyze β-replacement or β-elimination reactions. (iii) The γ enzymes catalyze γ-replacement or γ-elimination reactions. The deduced amino acid sequence of L-*allo*-TA showed no significant homology to those of the currently known function proteins, including serine hydroxymethyltransferase, which also catalyzes the cleavage of L-*allo*-threonine to glycine, in addition to the conversion of serine to glycine. Accordingly, L-*allo*-TA may represent another B_6 enzyme family.

On the other hand, PLP-dependent enzymes were proposed to have a common PLP-binding motif. As judged from the primary structure around the PLP-binding Lys of a number of enzymes, the partial structure, -Ser-X-X-Lys (PLP)-, was suggested to be a structural feature common to pyridoxal enzymes as a whole (3, 8, 28, 31). It was also shown that many PLP enzymes have the sequence -His-Lys (PLP)- (12, 19, 27). However, L-*allo*-TA shows no such typical PLP-binding motifs. By means of site-directed mutagenesis, Lys199 of L-*allo*-TA was identified as the binding PLP of the enzyme, serving as the catalytic base in the reversible interconversion of threonine and glycine. On inspection of the sequence alignment of the L-*allo*-TA, GLY1, and GLY1-like proteins (Fig. 4), we found that the serine residue proximate to the active-site lysine is conserved. To understand whether the sequence -Ser-Lys (PLP)- determines a new type of PLP-dependent enzyme, a study on the role of Ser198 of L-*allo*-TA is in progress.

Glycine biosynthesis is an important metabolic pathway in both procaryotes and eucaryotes. The glycine synthesis pathway in *E. coli* has been comprehensively studied, and the hydroxymethyltransferase-catalyzed conversion of serine to glycine has been concluded to be the cell's only source of glycine (26). As for other microorganisms, the glycine-forming pathway has not been fully elucidated. The results of a recent study by McNeil et al. implied the existence of a pathway more important than the serine-glycine one for cellular glycine synthesis in yeast (15). In their study, the complementation of a glycine auxotrophic strain led to the isolation of two *SHM* genes, encoding two serine hydroxymethyltransferases, and an additional *GLY1* gene from *S. cerevisiae*, which is at a separate locus from the two *SHM* genes. Growth of the wild-type and mutant yeast strains with the two *SHM* and *GLY1* genes disrupted showed that the disruption of *GLY1* alone affects the growth rate, whereas disruption of both *SHM* genes does not. Therefore, they concluded that the GLY1 pathway, rather than the conventional serine-glycine one, might be the major source of glycine in yeast. However, the catalytic activity of the GLY1 protein was unidentified.

The significant similarity in primary structure between the GLY1 protein and L-*allo*-TA found in the present study suggests that the *GLY1* gene possibly encodes a threonine aldolase, catalyzing the conversion of threonine to glycine for cell growth. To verify this, we amplified the *GLY1* gene from the genomic DNA of *S. cerevisiae* S288C (ATCC 26108) by PCR and further cloned the gene into pUC118; the transformant *E. coli* cells displayed obvious threonine aldolase activity, while the host *E. coli* cells showed no enzymatic activity. The purified enzyme was found to be active toward not only L-*allo*-threonine but also L-threonine (14a). The stereospecificity toward threonine isomers of the GLY1 enzyme is similar to those of the threonine aldolases from *Candida humicola* and *Pseudomonas* sp. strain NCIMB 11097 (5, 13). Accordingly, the enzyme should be placed in the low-specific-activity L-TA aldolase family. The present results together with those of the previous study indicate that GLY1 threonine aldolase is responsible for the synthesis of cellular glycine from threonine in *S. cerevisiae*. Likewise, the GLY1-like protein of *C. elegans*, which is significantly similar to L-*allo*-TA and GLY1 in primary structure, might also be a threonine aldolase. We therefore speculate that the synthesis of glycine from threonine catalyzed

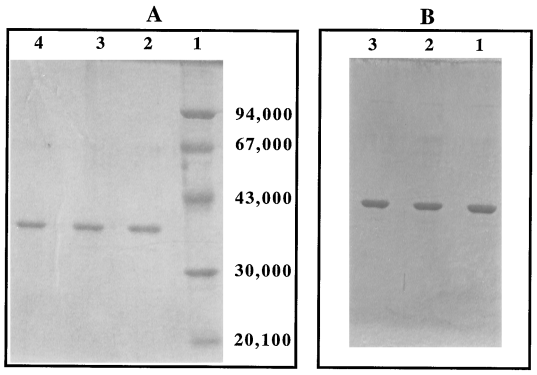

FIG. 7. Electrophoresis of the wild-type and mutant enzymes. (A) SDS-PAGE of purified enzymes. Lane 1, molecular weight standards; lane 2, wild-type enzyme (5 μg); lane 3, K199A mutant enzyme (5 μg); lane 4, K199C mutant enzyme (5 μg). Numbers to the right are molecular weights of the standards. (B) Native PAGE of wild-type and mutant enzymes. Lane 1, wild-type enzyme (15 μg); lane 2, K199A mutant enzyme (15 μg); lane 3, K199C mutant enzyme (15 μg).

by threonine aldolase might be a general pathway in both procaryotes and eucaryotes.

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REFERENCES

1. **Alexander, F. W., E. Sandmeier, P. K. Mehta, and P. Christen.** 1994. Evolutionary relationships among pyridoxal-5′-phosphate-dependent enzymes: regio-specific α, β and γ families. Eur. J. Biochem. **219:**953–960.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Angelaccio, S., S. Pascarella, E. Fattori, F. Bossa, W. Strong, and V. Schirch.** 1992. Serine hydroxymethyltransferase: origin of substrate specificity. Biochemistry **31:**155–162.
4. **Bell, S. C., and J. M. Turner.** 1977. Bacterial catabolism of threonine: threonine degradation initiated by L-threonine acetaldehyde-lyase (aldolase) in species of *Pseudomonas*. Biochem. J. **166:**209–216.
5. **Diaz-diaz, M., O. P. Ward, J. Honek, and G. Lajoie.** 1995. Enzymes from *Pseudomonas* sp. strain NCIB 11097 participating in biotransformation of acetaldehyde and glycine to threonine isomers. Can. J. Microbiol. **41:**438–443.
6. **Herbert, R. B., B. Wilkinson, G. J. Ellames, and E. K. Kunec.** 1993. Stereospecific lysis of a range of β-hydroxy-α-amino acids catalysed by a novel aldolase from *Streptomyces amakusaensis*. J. Chem. Soc. Chem. Commun. **1993:**205–206.
7. **Herbert, R. B., and B. Wilkinson.** 1994. Preparation of (2R, 3S)-β-hydroxy-α-amino acids by use of a novel *Streptomyces* aldolase as a resolving agent for racemic material. Can. J. Chem. **72:**114–117.
8. **Ishikawa, K., E. Kaneko, and A. Ichiyama.** 1996. Pyridoxal 5′-phosphate binding of a recombinant rat serine: pyruvate/alanine: glyoxylate aminotransferase. J. Biochem. **119:**970–978.
9. **Jolad, S. D., J. J. Hoffmann, S. J. Torrance, R. M. Wiedhopf, J. R. Cole, S. K. Arora, R. B. Bates, R. L. Gargiulo, and G. R. Kriek.** 1977. Bouvardin and deoxybouvardin, antitumour cyclic hexapeptides from *Bouvardia ternifolia* (Rubiaceae). J. Am. Chem. Soc. **99:**8040–8044.
10. **Karasek, M. A., and D. M. Greenberg.** 1957. Studies on the properties of threonine aldolases. J. Biol. Chem. **227:**191–205.
11. **Kataoka, M., K. Nishi, S. Shimizu, H. Yamada, M. Ikemi, T. Morikawa, and T. Miyoshi.** 1993. Purification and properties of L-*allo*-threonine aldolase of *Aeromonas jandaei*. Nippon Nogeikagaku Kaishi **67:**549.
12. **Kobayashi, Y., D. L. Kaufman, and A. J. Tobin.** 1987. Glutamic acid decarboxylase cDNA: nucleotide sequence encoding an enzymatically active fusion protein. J. Neurosci. **7:**2768–2772.
13. **Kumagai, H., T. Nagatae, H. Yoshida, and H. Yamada.** 1972. Threonine aldolase from *Candida humicola*: purification, crystallization and properties. Biochim. Biophys. Acta **258:**779–790.
14. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.

14a. **Liu, J.-Q., S. Nagata, T. Dairi, H. Misono, S. Shimizu, and H. Yamada.** The *Gly1* gene of *Saccharomyces cerevisiae* encodes a low-specific L-threonine aldolase that catalyzes cleavage of L-*allo*-threonine and L-threonine to glycine: expression of the gene in *Escherichia coli* and purification and characterization of the enzyme. Eur. J. Biochem., in press.

15. **McNeil, J. B., E. V. Mcintosh, B. V. Taylor, F.-R. Zhang, S. Tang, and A. L. Bognar.** 1994. Cloning and molecular characterization of three genes, including two genes encoding serine hydroxyltransferases, whose inactivation is required to render yeast auxotrophic for glycine. J. Biol. Chem. **269:**9155–9165.
16. **Morris, J. G.** 1969. Utilization of L-threonine by a *Pseudomonad*: a catabolic role for L-threonine aldolase. Biochem. J. **115:**603–605.
17. **Paz, M. A., O. O. Blumenfeld, M. Rojkind, E. Henson, C. Furfine, and P. M. Gallop.** 1964. Determination of carbonyl compounds with N-methyl benzothiazolone hydrazone. Arch. Biochem. Biophys. **109:**548–559.
18. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
19. **Poulin, R., L. Lu, B. Ackermann, P. Bey, and A. E. Pegg.** 1992. Mechanism of the irreversible inactivation of mouse ornithine decarboxylase by alpha-difluoromethylornithine: characterization of sequences at the inhibitor and coenzyme binding sites. J. Biol. Chem. **267:**150–158.
20. **Roberto, P., L. Roberto, T. Lucia, C. John, P. Maria, and M. Enrico.** 1991. DL-Allothreonine aldolase in rat liver. Biochem. Soc. Trans. **19:**346–347.
21. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Schirch, L. V., and T. Gross.** 1968. Serine transhydroxymethylase: identification as the threonine and allothreonine aldolase. J. Biol. Chem. **243:**5651–5655.
24. **Shibata, K., K. Shingu, V. P. Vassilev, K. Nishide, T. Fujita, M. Node, T. Kajimoto, and C.-H. Wong.** 1996. Kinetic and thermodynamic control of L-threonine aldolase catalyzed reaction and its application to the synthesis of mycestericin D. Tetrahedron Lett. **37:**2791–2794.
25. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature **154:**34–38.
26. **Stauffer, G. V.** 1987. Biosynthesis of serine and glycine, p. 412–418. *In* F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology. American Society for Microbiology, Washington, D.C.
27. **Stover, P., M. Zamora, K. Shostak, M. Gautam-Basak, and V. Schirch.** 1992. *Escherichia coli* serine hydroxymethyltransferase: the role of histidine 228 in determining reaction specificity. J. Biol. Chem. **267:**17679–17687.
28. **Tanizawa, K., K. Masu, S. Asano, H. Tanaka, and K. Soda.** 1989. Thermostable D-amino acid aminotransferase from a thermophilic Bacillus species. Purification, characterization, and active site sequence determination. J. Biol. Chem. **264:**2445–2449.
29. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 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.
30. **Tymiak, A., T. J. McCormick, and S. E. Unger.** 1989. Structure determination of lysobactin, a macrocyclic peptide lactone antibiotic. J. Org. Chem. **54:**1149–1157.
31. **Vaaler, G. L., and E. E. Snell.** 1989. Pyridoxal 5′-phosphate dependent histidine decarboxylase: overproduction, purification, biosynthesis of soluble site-directed mutant proteins, and replacement of conserved residues. Biochemistry **28:**7306–7313.
32. **Vassilev, V. P., T. Uchiyama, T. Kajimoto, and C.-H. Wong.** 1995. L-Threonine aldolase in organic synthesis: preparation of novel β-hydroxy-α-amino acids. Tetrahedron Lett. **36:**4081–4084.
33. **Vassilev, V. P., T. Uchiyama, T. Kajimoto, and C.-H. Wong.** 1995. An efficient chemo-enzymatic synthesis of α-amino-β-hydroxy-γ-butyrolactone. Tetrahedron Lett. **36:**5063–5064.
34. **Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, A. Coulson, M. Craxton, S. Dear, Z. Du, R. Durbin, A. Favello, A. Fraser, L. Fulton, A. Gardner, P. Green, T. Hawkins, L. Hillier, M. Jier, L. Johnston, M. Jones, J. Kershaw, J. Kirsten, N. Laister, P. Latreille, J. Lightning, C. Lloyd, B. Mortimre, M. O'Callaghan, J. Parsons, C. Percy, L. Rifken, A. Roopra, D. Saunders, R. Shownkeen, M. Sims, N. Smaldon, A. Smith, M. Smith, E. Sonnharnmer, R. Staden, J. Sulston, J. Thierry-Mieg, K. Thomas, M. Vaudin, K. Vaughan, R. Waterston, A. Watson, L. Weinstock, J. Wilkinson-Sproat, and P. Wohldman.** 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature **368:**32–38.
35. **Yamada, H., H. Gumagai, T. Nagate, and H. Yoshida.** 1970. Crystalline threonine aldolase from *Candida humicola*. Biochem. Biophys. Res. Commun. **39:**53–58.
36. **Yamada, H., H. Gumagai, T. Nagate, and H. Yoshida.** 1971. Formation of threonine aldolase by bacteria and yeasts. Agric. Biol. Chem. **35:**1340–1345.