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A Gene Encoding Lysine 6-Aminotransferase, Which Forms the β-Lactam Precursor α-Aminoadipic Acid, Is Located in the Cluster of Cephamycin Biosynthetic Genes in *Nocardia lactamdurans*

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A gene (*lat*) encoding lysine 6-aminotransferase was found upstream of the *pcbAB* (encoding α-aminoadipyl-cysteinyl-valine synthetase) and *pcbC* (encoding isopenicillin N synthase) genes in the cluster of early cephamycin biosynthetic genes in *Nocardia lactamdurans*. The *lat* gene was separated by a small intergenic region of 64 bp from the 5′ end of the *pcbAB* gene. The *lat* gene contained an open reading frame of 1,353 nucleotides (71.4% G+C) encoding a protein of 450 amino acids with a deduced molecular mass of 48,811 Da. Expression of DNA fragments carrying the *lat* gene in *Streptomyces lividans* led to a high lysine 6-aminotransferase activity which was absent from untransformed *S. lividans*. The enzyme was partially purified from *S. lividans*(pULBS8) and showed a molecular mass of 52,800 Da as calculated by Sephadex gel filtration and polyacrylamide gel electrophoresis. DNA sequences which hybridized strongly with the *lat* gene of *N. lactamdurans* were found in four cephamycin-producing *Streptomyces* species but not in four other actinomycetes which are not known to produce β-lactams, suggesting that the gene is specific for β-lactam biosynthesis and is not involved in general lysine catabolism. The protein encoded by the *lat* gene showed similarity to ornithine-5-aminotransferases and *N*-acetylornithine-5-aminotransferases and contained a pyridoxal phosphate-binding consensus amino acid sequence around Lys-300 of the protein. The evolutionary implications of the *lat* gene as a true β-lactam biosynthetic gene are discussed.

Antibiotics and a variety of other microbial products having different pharmacological activities (7) are synthesized from intermediates or final products of primary metabolism (e.g., amino acids) (10) which are converted by (i) a series of modification reactions into specific precursors of antibiotics and (ii) condensation or polymerization reactions that lead to formation of the skeleton of the antibiotic molecules. This basic structure is usually modified by late modification reactions (30).

The genes encoding the condensation (or polymerization) reactions and those for the late modifications of the antibiotic molecule are usually clustered together in the genomes of antibiotic-producing actinomycetes (32) and filamentous fungi (9, 17, 26). However, little information is available on the location of genes involved in the conversion of intermediates of primary metabolism into specific precursors of antibiotics. Only a few of those enzymes, i.e., *p*-aminobenzoic acid synthase (16), valine dehydrogenase (34, 35), and threonine dehydratase (41), which are involved in the formation of precursors of macrolide antibiotics, have been characterized. They were believed to be associated with primary metabolism, and therefore, the location in the chromosome of the genes encoding these enzymes with respect to the clusters of genes involved in antibiotic biosynthesis was unclear. α-Aminoadipic acid is a specific precursor of β-lactam antibiotics (Fig. 1) and is formed in actinomycetes by deamination of lysine by the enzyme lysine 6-aminotransferase (LAT) (21, 28). α-Aminoadipic acid is condensed with L-valine and L-cysteine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the action of the enzyme ACV synthetase, a multidomain peptide synthetase (1, 42, 43). The tripeptide ACV is cyclized into isopenicillin N (IPN) by the action of IPN synthase. IPN is later converted into cephalosporin or cephamycins (see the review by Martín and Liras [31]).

We previously cloned a fragment of DNA from *Nocardia lactamdurans* that carries the cluster of early cephamycin biosynthetic genes including *pcbAB* (encoding ACV synthetase) and *pcbC* (IPN synthase) (6). Other genes of the cephamycin pathway also appear to be located in the same cluster both in *N. lactamdurans* (14) and in *Streptomyces clavuligerus* (24, 39).

It was therefore of great interest to determine whether the gene encoding LAT (one of the two steps required to convert lysine into α-aminoadipic acid) was located in the same cluster of early cephamycin genes. We report here that the gene (*lat*) encoding this enzyme is, indeed, closely linked to the *pcbAB* and *pcbC* genes and should be considered an authentic cephamycin biosynthetic gene. The *lat* gene encodes a lysine:2-ketoglutarate 6-aminotransferase that accepts lysine as a substrate and is efficiently expressed in *Streptomyces lividans*.

MATERIALS AND METHODS

Microorganisms and vectors used. *N. lactamdurans* LC411, an improved cephamycin-producing strain, was used as the source of DNA. *S. lividans* JI1326, a strain which does not produce β-lactam antibiotics (15), was used as the receptor for transformation experiments with the *N. lactamdurans* DNA. *Escherichia coli* DH5α was utilized for high-frequency plasmid transformation; *E. coli* WK6 and the helper bacteriophage M13K07 were used to obtain single-stranded DNA for sequencing.

Construction of a gene library of *N. lactamdurans* DNA in

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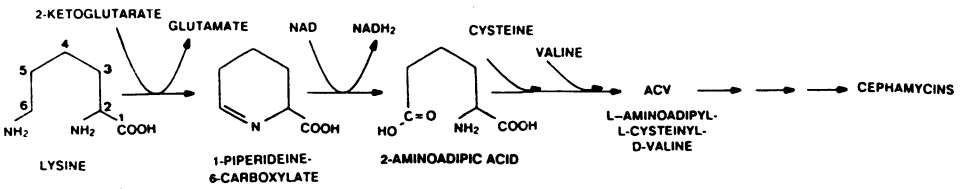

FIG. 1. Conversion of lysine into 2-aminoadipic acid and utilization of this amino acid for cephamycin biosynthesis. The first reaction is performed by LAT encoded by the *lat* gene.

vector λEMBL3 (13) was described previously (6). pBluescript KS(+), pUC118, and pUC119 were utilized to subclone DNA fragments for sequencing.

Plasmid pIJ702 (20) was used as a vector for studies of expression of the *lat* gene in *S. lividans*. In some constructions, inserts were first subcloned in pIJ2921 and rescued with *Bgl*II cohesive ends for insertion in pIJ702.

DNA isolation procedures. A 1.5-kb *Eco*RI fragment carrying the entire *lat* gene was subcloned in pBluescript KS(+) in both orientations from phage EMBL3-C8 (6).

For sequencing, ordered sets of nested DNA fragments were generated by sequential deletions using the Erase-a-base system (Promega, Madison, Wis.) by digestion with exonuclease III from appropriate ends and then S1 exonuclease removal of single-stranded DNA and treatment with Klenow DNA polymerase to fill the gaps introduced by the former enzymes (19). The DNA fragments were sequenced in both orientations by the dideoxynucleotide method (38), using *Taq* DNA polymerase (Promega) and 7-deaza-dGTP to avoid compressions.

Cell extracts and LAT assay. LAT was assayed in cell extracts obtained from clones of *S. lividans* transformed with the *lat* gene. Untransformed *S. lividans* and *N. lactamdurans* were used as controls. Cells were grown in minimal medium with glucose and lysine (28). Mycelia were collected at different times, washed with sterile 0.85% NaCl, and suspended in phosphate buffer (0.2 M, pH 7.3) containing pyridoxal phosphate (0.05 mM). Cell extracts were obtained by sonication (5-s pulses with 1-min intervals) in a Branson sonifier. LAT was recovered in the supernatant (S_{20}) after centrifugation at 20,000 × *g* for 90 min. The LAT assay was done in a reaction mixture containing L-lysine (40 μmol), 2-ketoglutarate (40 μmol), and pyridoxal phosphate (0.15 μmol) in a final volume of 1.1 ml. The mixture was incubated at 30°C for 60 min. The reaction was stopped by adding 0.5 volume of 5% trichloroacetic acid in ethanol. The precipitated proteins were removed by centrifugation at 14,000 × *g* for 5 min, and the reaction product, Δ^1-piperideine-6-carboxylic acid, was quantified by adding 1.5 ml of 4 mM *ortho*-aminobenzaldehyde in 0.20 M phosphate buffer (pH 7.3) to 1 ml of deproteinized reaction mixture and incubating for 1 h at 37°C to develop the color. The A_{465} was converted to nanomoles of product formed by using a molar extinction coeficient of 2,800 (12). Protein was quantified in all samples by the Bradford method (2a).

Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cell extracts of cultures of *S. lividans*(pULBS8) obtained as indicated above were fractionated by precipitation with ammonium sulfate. The LAT activity was recovered in the 30 to 60% ammonium sulfate saturation fraction. A 3-ml sample of this fraction was applied to a column of Sephadex G-75 (620 by 25 mm) equilibrated with phosphate buffer (0.2 M, pH 7.3) containing pyridoxal phosphate (0.05 mM). LAT was eluted with the same buffer at a flow rate of 9 ml/h. The column was calibrated with several proteins of known molecular mass (bovine serum albumin, 67,000 Da; ovalbumin, 63,000 Da; chymotrypsinogen A, 25,000 Da; and RNase A, 13,700 Da).

RESULTS

Characterization of ORF3 upstream of gene *pcbAB*. We have previously cloned a region of the *N. lactamdurans* DNA that carries the *pcbAB* and *pcbC* genes encoding, respectively, ACV synthetase and IPN synthase, the two first enzymes of the cephamycin biosynthetic pathway (6). To determine whether a gene(s) which synthesizes the α-aminoadipic acid precursor was located in the cluster of early cephamycin biosynthetic genes, we subcloned and sequenced the region upstream of the *pcbAB* gene. We found an open reading frame (ORF) of 1,353 bp (ORF3) located within an *Eco*RI fragment of 1,523 bp. This ORF is closely linked to the *pcbAB* gene in its 5′ region (Fig. 2).

Expression of DNA fragments carrying ORF3 in *S. lividans* leads to production of LAT. *S. lividans* lacks LAT, an enzyme found in *S. clavuligerus* that appears to be specific for secondary metabolism (28). Therefore, *S. lividans* was transformed with constructions carrying two different DNA fragments, both of which contained ORF3. The gene was subcloned in a 2.5-kb *Bam*HI-*Sac*I fragment that carried 1 kb of the sequences upstream of ORF3 and also in a 5.8-kb *Bam*HI-*Kpn*I fragment that also contained 3.2 kb of the 5′ region of the *pcbAB* gene (an unusually long gene extending over 10.95 kb) (6) (Fig. 2). Both fragments were subcloned in vector pIJ2921 and were later rescued with *Bgl*II cohesive ends to insert them in the *Streptomyces* vector pIJ702.

The recombinants were selected among thiostrepton-resistant transformants of *S. lividans* as white colonies resulting from insertional inactivation of the *mel* gene (for melanine biosynthesis). The presence of the inserts in the recombinant plasmids was confirmed by endonuclease restriction mapping.

Transformant clones carrying the expected constructions (plasmids pULBS8 and pULBK1) were grown in YEME medium (19a) supplemented with 34% sucrose and thiostrepton (final concentration, 5 μg/ml) for 48 h. The cells were collected and transferred to lysine-minimal medium as indicated in Materials and Methods. After cell disruption, definite LAT activity was observed in *S. lividans*(pULBS8) and *S. lividans*(pULBK1) that was not found in *S. lividans* (pIJ702) control cultures (Table 1). The enzymatic activity was very high at 48 h of incubation in lysine-minimal medium and decreased at 60 h. The LAT activity found in *S. lividans*(pULBK1) and *S. lividans*(pULBS8) cultures was 10-fold higher than that in cultures of *N. lactamdurans* LC411 (from which the gene was cloned) used as a control. These results indicated that ORF3 encodes a gene (named *lat*) for LAT.

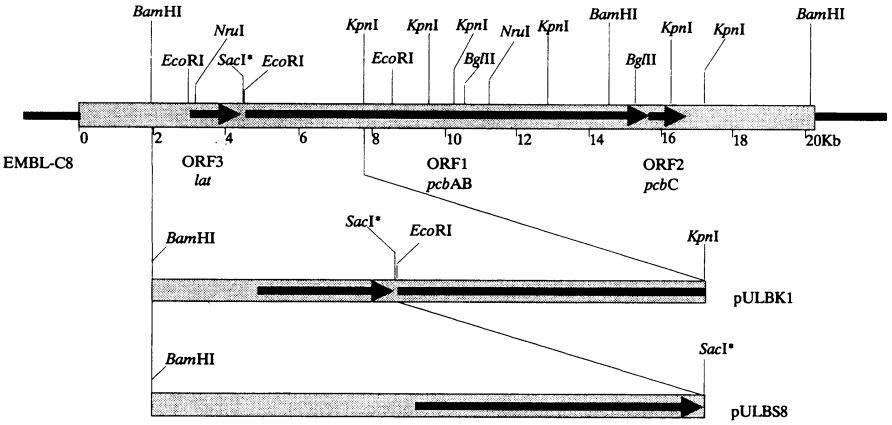

FIG. 2. Restriction endonuclease map of the cluster of early cephamycin biosynthetic genes cloned in phage lambda EMBL-C8. The three ORFs corresponding to the *lat*, *pcbAB*, and *pcbC* genes are indicated by solid arrows. The fragments subcloned in pULBK1 and pULBS8, used to express the *lat* gene in *S. lividans*, are shown in the lower part of the figure.

Molecular weight of cloned LAT. To confirm the molecular weight of the enzyme deduced from the nucleotide sequence (see below), we partially purified LAT of *S. lividans* (pULBS8) by ammonium sulfate precipitation and gel filtration. Cell extracts were fractionated by ammonium sulfate precipitation. Most LAT activity was collected in the 30 to 60% ammonium sulfate fraction, and a small part precipitated in the 0 to 30% ammonium sulfate fraction. No activity was found in the 60 to 90% ammonium sulfate fraction.

The proteins in the 30 to 60% fraction were chromatographed through a Sephadex G-75 column previously equilibrated with 0.2 M phosphate buffer (pH 7.3) containing 0.05 mM pyridoxal phosphate. LAT eluted with the same buffer as a single peak (with a K_{av} value of 0.108, which corresponds to a logarithm of M_r of 4.7224) between bovine serum albumin (M_r 67,000) and ovalbumin (M_r 43,000) with a molecular mass of 52,800 ± 1,000 Da. A protein band of the expected size was enriched in the fractions with higher activity after gel filtration as observed in SDS-PAGE gels and is being further purified.

Nucleotide and deduced amino acid sequence. The nucleotide sequence of the *lat* gene is shown in Fig. 3. The gene showed a high G+C content (71.47%), which fits with the G+C content of species of the genus *Nocardia* (66.1 to 72.7%) (25), and encoded a protein of 450 amino acids with a deduced molecular mass of 48,811 Da, which is similar to the molecular size obtained by gel filtration.

The 3′ end of the ORF of the *lat* gene is separated by a small intergenic region of 64 bp from the 5′ end of the *pcbAB* gene. No inverted repeat sequences that may generate a transcription terminator were found by computer analysis of the intergenic region (see Discussion).

TABLE 1. LAT activity of *S. lividans* transformed with the *lat* gene of *N. lactamdurans*

Strain	Sp act at[a]:	
	48 h	60 h
N. lactamdurans LC411	0.24	ND[b]
S. lividans(pIJ702)	0	0
S. lividans(pULBS8)	2.43	1.86
S. lividans(pULBK1)	2.50	1.12

[a] Time of incubation of the culture in lysine-minimal medium as indicated in Materials and Methods. Enzyme specific activity is defined as nanomoles of piperidine 6-carboxylate formed per minute per milligram of protein.
[b] ND, not determined.

The *lat* gene occurs in β-lactam producers but not in other actinomycetes. To determine whether the *lat* gene was related to the biosynthesis of cephamycins or whether it was involved in catabolism of lysine, we hybridized *Sal*I (or *Bam*HI)-digested total DNA from eight different *Streptomyces* species with a 1.5-kb *Eco*RI fragment which contains the *lat* gene (Fig. 2). Clear bands of hybridization were seen in the DNA of *S. griseus* NRRL3851 (1.7 kb), *S. clavuligerus* NRRL3585 (1.1 kb) (Fig. 4), *S. lipmanii* NRRL3584 (a faint band of about 6.0 kb), and *S. cattleya* NRRL8037 (two bands of 0.7 and 2.7 kb); no hybridizing bands were observed in the DNA of *S. albus* G, *S. lividans* JI1326, *S. coelicolor* A3(2) JI2280, or *S. griseus* IMRU3570, none of which is known to produce β-lactam antibiotics (see Discussion).

LAT has a strong homology with ornithine 5-aminotransferases and acetylornithine 5-aminotransferases. In a computer search of the NBRF data base using the DNASTAR Program, we did not find the sequence of any LAT, but we did observe a strong homology in some regions of the protein with the ornithine aminotransferases of humans, rats, and yeasts (Fig. 5). The total homology at the amino acid level was 25.8% with the human, 26.7% with the rat, and 28.9% with the *Saccharomyces cerevisiae* ornithine aminotransferases, but the homology was very high around one of the regions known to be involved in the catalytic activity of ornithine aminotransferases.

LAT also showed a significant homology with the acetylornithine aminotransferases of *E. coli* and *S. cerevisiae*, enzymes which carry the fourth step of the arginine biosynthetic pathway (18).

There are at least three regions of LAT (amino acids 230 to 245, 267 to 281, and 329 to 339) in which a stretch of at least 9 to 10 consecutive amino acids is conserved. Another conserved region in the LATs and the ornithine and acetylornithine aminotransferases is the active center for binding of pyridoxal phosphate, a well-known effector of a variety of aminotransferases. This cofactor interacts with an amino group of a lysine residue of the enzymes to form a Schiff base. Although it is not possible to assign a putative pyridoxal phosphate-binding site from an alignment of the sequences, comparison of the different aminotransferases with

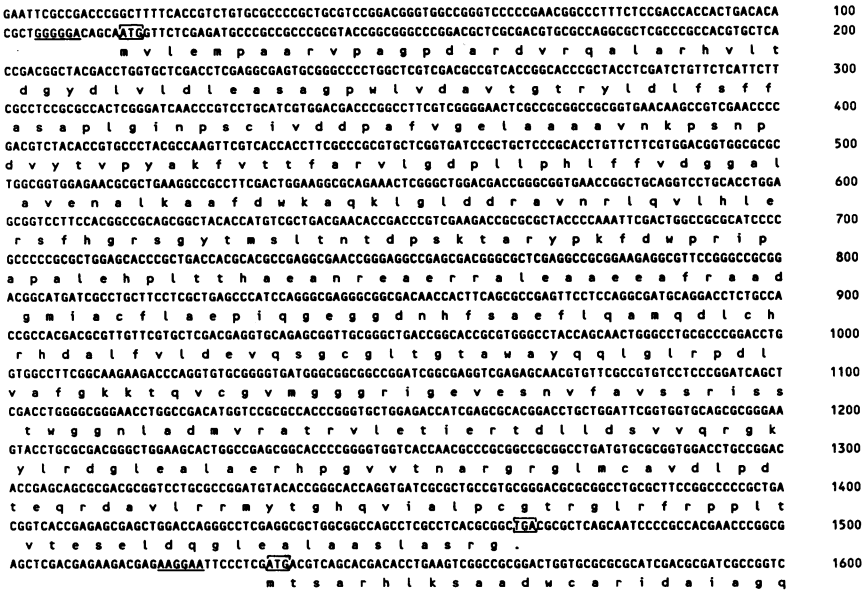

FIG. 3. Nucleotide and deduced amino acid sequence of a 1,600-bp DNA fragment containing the *lat* gene and the upstream and downstream regions. The ATG initiation triplets of the *lat* and *pcbAB* genes and the TGA termination codon of the *lat* gene are boxed. Putative ribosome-binding sites preceding the ATG initiation triplets of both genes are underlined. Note the small intergenic region between the *lat* and *pcbAB* genes.

the aspartyl aminotransferase from chicken heart, whose three-dimensional structure and catalytic site are known (23), led to the proposal that Lys-292 of the rat ornithine aminotransferase (33), Lys-271 of the yeast ornithine aminotransferase, Lys-255 of the *E. coli* acetylornithine aminotransferase, and Lys-276 of the yeast acetylornithine aminotransferase (18) are the respective pyridoxal phosphate-binding residues in these enzymes. Since this domain is highly conserved, Lys-300 of the LAT of *N. lactamdurans* is probably the pyridoxal phosphate-binding residue (Fig. 6).

DISCUSSION

Lysine catabolism proceeds in different organisms by an array of diverse mechanisms that involve decarboxylation,

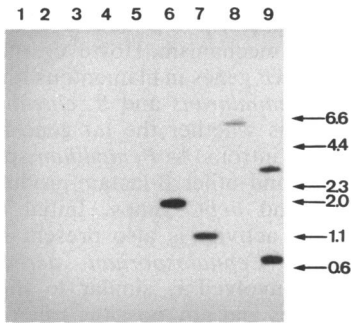

FIG. 4. Hybridization of total DNA (*Sal*I digested) of several *Streptomyces* species with a 1.5-kb *Eco*RI probe carrying the *lat* gene of *N. lactamdurans*. Lanes: 1, *S. albus* G (*Bam*HI digested); 2, *S. lividans* JI1326; 3, *S. coelicolor* A3(2); 4, *S. griseus* IMRU3570; 5, DNA size markers (λ *Hin*dIII plus φX174 *Hae*III); 6, *S. griseus* NRRL3851; 7, *S. clavuligerus* NRRL3585; 8, *S. lipmanii* NRRL 3584; 9, *S. cattleya* NRRL8057. Numbers on right show size in kilobases.

deamination, or transamination reactions (reviewed by Bhattacharjee [2]). LAT is an enzyme known to be involved in lysine catabolism in *Candida guilliermondii* (8), *Rhodotorula glutinis* (22), *Flavobacterium fuscum* (40), *Pseudomonas aeruginosa* (12), and *Achromobacter liquidum* (40).

Lysine catabolism in *S. lividans* and other actinomycetes proceeds by the action of L-lysine decarboxylase, which converts lysine to cadaverine (28). However, a few actinomycetes, including *N. lactamdurans* (21) and *S. clavuligerus* (28, 37), are also known to possess LAT, which might be a second system for catabolism of lysine or a biosynthetic enzyme involved in the formation of α-aminoadipic acid, a precursor of cephamycin biosynthesis. Our results (37) suggested that LAT was involved in cephamycin biosynthesis since mutants of *S. clavuligerus* having abnormally low levels of LAT were impaired in cephamycin biosynthesis. This view was supported by the finding of Vining and coworkers (28), who observed that *S. lividans* does not have LAT activity. We found (Fig. 4) that the cloned *lat* gene hybridizes strongly with the DNA of *S. griseus* NRRL3851, *S. clavuligerus* NRRL3585, and *S. cattleya* NRRL8057 and weakly with the DNA of *S. lipmanii* 3585, all of which are known to be producers of β-lactam antibiotics, but not with the DNA of *S. albus* G, *S. lividans* JI1326, *S. coelicolor* A3(2), and *S. griseus* IMRU3570, which are not producers of β-lactams. The *lat* gene seems, therefore, to encode an enzyme exclusively used for cephamycin biosynthesis since it occurs only in the genome of cephamycin-producing actinomycetes.

A similar LAT gene has been cloned and found to be associated with the cluster of cephamycin biosynthetic genes in *S. clavuligerus*, another cephamycin-producing actinomycete (5, 27), which indicates that this gene is conserved in cephamycin biosynthetic clusters.

These results suggest that the *lat* gene can be used as a specific probe for screening β-lactam-producing actinomycetes. Other genes involved in the biosynthesis of ceph-

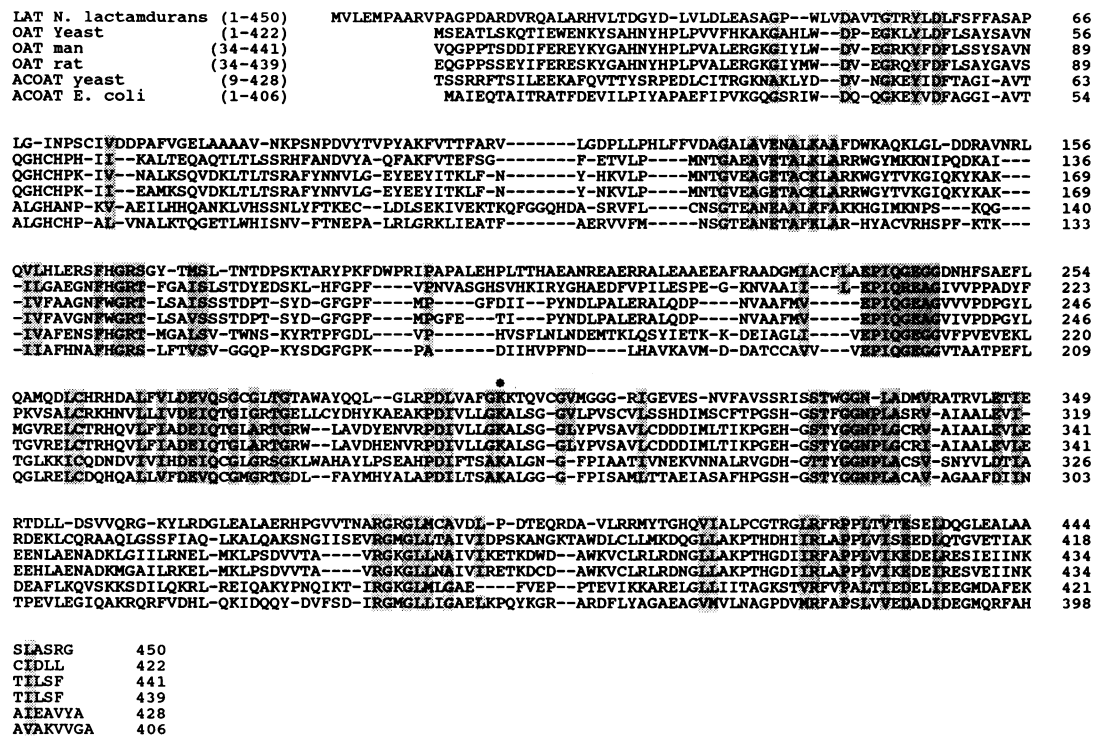

FIG. 5. Comparison of LAT with ornithine aminotransferases (OAT) and *N*-acetylornithine aminotransferase (ACOAT) (see text for details). Some gaps were introduced to obtain maximal alignment of the amino acids. Identities and conservative replacements have been shaded. Conservative replacements are R-K, D-E, S-T, G-A, F-Y, and I-L-V-M. Numbers at the end of the lines refer to the position in the original sequence. Lys-300 is indicated by an asterisk.

amycins (e.g., *pcbC*, encoding IPN synthase) may be even better probes for screening for the production of β-lactam antibiotics, since the cyclization reaction carried out by IPN synthases is very infrequent in nature (31).

The *lat* gene is closely linked to the *pcbAB* and *pcbC* genes (Fig. 2), which encode the first two steps of the cephamycin biosynthetic pathway (6). Expression of these three genes and other late genes of the pathway is probably coordinated (3). We are studying at present whether this coordinated regulation proceeds through formation of a single polycistronic mRNA. The lack of recognizable transcription termination signals between the *lat*, *pcbAB*, and *pcbC* genes supports this hypothesis.

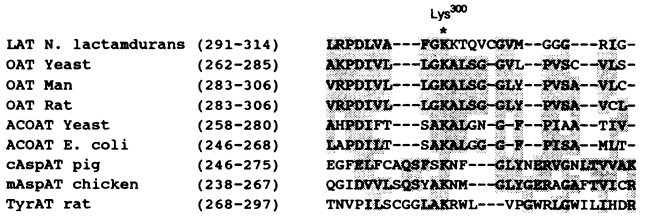

FIG. 6. Comparison of the amino acid sequences around the pyridoxal phosphate-binding sequence of several aminotransferases. Dashes indicate gaps made to optimize the alignment. The numbers in parentheses refer to the position in the original sequence. Conserved or conservatively replaced amino acids have been shaded. The asterisk indicates the site of the putative pyridoxal phosphate-binding Lys residue (see text). OAT, ornithine aminotransferase; ACOAT, *N*-acetylornithine aminotransferase; cAspAT, cytosolic aspartyl aminotransferase; mAspAT, mitochondrial aspartyl aminotransferase; TyrAT, tyrosine aminotransferase.

Synthesis of α-aminoadipic acid in a coordinated form with its conversion to ACV by ACV synthetase is clearly advantageous for the cephamycin-producing strains. The evolutionary mechanisms by which these genes have become linked is intriguing. Once they were linked together by DNA reorganization events, they probably remained clustered because of the ecological advantage that antibiotic production confers to the producing strain (32). It has been proposed (4, 36; see the review by Martín et al. [29]) that the genes of the cephamycin biosynthetic pathway moved from β-lactam-producing bacteria to β-lactam-producing fungi by a horizontal transfer mechanism. However, the organization of the *pcbC* and *pcbAB* genes in filamentous fungi is different from that in *N. lactamdurans* and *S. clavuligerus* (6). An intriguing question is whether the *lat* gene has been also transferred (without introns) to *Penicillium* species, *Cephalosporium* species, and other β-lactam-producing fungi, as have the *pcbAB* and *pcbC* genes. Initial evidence (11) indicates that LAT activity is also present in *Penicillium chrysogenum* and *Cephalosporium acremonium*, but whether the gene involved is similar to the *lat* gene of *Streptomyces* species and any possible role of this enzyme activity in biosynthesis of β-lactam antibiotics by fungi remain to be elucidated.

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