Characterization of L-Lysine 6-Aminotransferase and Its Structural Gene from *Flavobacterium lutescens* IFO3084

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L-Lysine 6-aminotransferase (LAT) is an enzyme involved in L-lysine catabolism in a wide range of living organisms. LAT from *Flavobacterium lutescens* IFO3084 was purified, and its structural gene (*lat*) was cloned, sequenced and expressed in *Escherichia coli*. Native PAGE analysis of purified LAT gave a single band corresponding to a molecular weight of about 110,000. *lat* encoded a protein of 493 amino acids with a deduced molecular weight of 53,200, which is very close to that of purified LAT determined on SDS-PAGE. Expression of *lat* in *E. coli* revealed that *lat* encodes a single subunit protein leading to LAT activity. These data suggested that LAT from *F. lutescens* IFO3084, like most other aminotransferases, is derived from a single ORF and is active as a homodimer.

Key words: *Flavobacterium lutescens* IFO3084, homodimer, LAT, *lat*.

L-Lysine 6-aminotransferase (LAT) is an enzyme involved in L-lysine catabolism, which is found in a wide range of living organisms, such as *Candida guilliermondii* (*1*), *Rodotorula gutinis* (*2*), *Flavobacterium lutescens* IFO3084 (*3*), *Flavobacterium* sp. SC 12,154 (*4*), *Pseudomonas aeruginosa* (*5*), and β-lactam–producing actinomycetes (*6*). LAT converts L-lysine into the α-aminoadipic semialdehyde, which is subsequently cyclized to form piperideine-6-carboxylate (P6C). In actinomycetes, LAT activity is specific to β-lactam antibiotic producers and is considered to be the first step in the β-lactam antibiotic biosynthetic pathway (*7*). The gene encoding LAT (*lat*) was shown to be located in the β-lactam antibiotic gene cluster in both *Streptomyces clavuligerus* (*8*) and *Nocardia lactamdurans* (*9*), whereas *lat* is absent from the genome of most other actinomycetes, confirming that this enzyme is specific for secondary metabolism. *lat* from *S. clavuligerus* was sequenced and revealed to contain a single open reading frame (ORF) encoding a protein with a molecular weight of 49,000 (*8*). Expression of this coding sequence in *Escherichia coli* led to the production of LAT activity, indicating that LAT from *S. clavuligerus*, like most aminotransferases, is active as either a monomer or a homopolymer (*10*).

In the case of Gram-negative bacteria, however, little information is available on LAT. LAT from *F. lutescens* IFO3084 has only been purified and characterized biochemically (*11*). Reportedly, the LAT from *F. lutescens* IFO3084 has a molecular weight of about 116,000 and is composed of four non-identical subunits, A, B1, B2, and C, with molecular weights of 24,000, 28,000, 28,000, and 45,000, respectively (*12*). This is in contrast with the fact that most aminotransferases are active as either a monomer or a homopolymer (*10*). Therefore, it is of great interest to characterize LAT from *F. lutescens* IFO3084 at the molecular level. In this study, we cloned and sequenced a structural gene (*lat*) of LAT from *F. lutescens* IFO3084, and demonstrated that LAT from *F. lutescens* IFO3084 is active not as a heterotetramer but as a homodimer.

MATERIALS AND METHODS

Bacterial Strains—*F. lutescens* IFO3084 was used for the isolation of LAT and genomic DNA. *E. coli* TOP10 (Invitrogen) was used as the LAT-expressing strain.

Media—*F. lutescens* IFO3084 and *E. coli* strains were cultivated at 32°C in L-broth (polypepton 1.0%, yeast extract 0.5%, NaCl 0.5%, glucose 0.1%, pH 7.2) for the isolation of chromosomal DNA and seed culture. Transformants of *E. coli* TOP10 were spread on L-agar plates (polypepton 1.0%, yeast extract 0.5%, NaCl 0.5%, glucose 0.1%, Bacto agar 1.5%, pH 7.2) containing 50 μg/ml ampicillin sodium and then grown in L-broth containing 50 μg/ml ampicillin sodium. *F. lutescens* IFO3084 was cultivated in FM9-medium (Na_2HPO_4 0.6%, KH_2PO_4 0.3%, NH_4Cl 0.1%, NaCl 0.2%, polypepton 1.0%, yeast extract 0.5%, L-lysine HCl 0.5%, Silicone KM75 0.005%, $CaCl_2$ 0.0015%, pH 7.2) for the preparation of cell-free extracts.

LAT Assay—LAT activity was determined by a method similar to that described by Soda (*3*). Briefly, 100 μl of the enzyme solution was added to 1.0 ml of 0.2 M phosphate buffer (pH 7.2) containing L-lysine HCl (40 μmol), 2-ketoglutarate (40 μmol), and pyridoxal phosphate (0.15 μmol). The mixture was incubated at 32°C for 60 min. The reaction was stopped by adding 550 μl of 5% trichloroacetic acid in ethanol. The precipitated proteins were removed by centrifugation at 14,000 ×*g* for 5 min. The reaction product (P6C) was quantified by adding 1.5 ml of 4 mM ortho-aminobenzaldehyde in 0.2 M phosphate buffer (pH 7.2) to 1 ml of the deproteinized reaction mixture, followed by incubation for 1h at 37°C for color development. The absorbance at 465 nm was converted to micromoles of P6C formed

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Abbreviations: LAT, L-lysine 6-aminotransferase; P6C, piperideine-6-carboxylate; AAA, L-α-aminoadipic acid.

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using ε_{466} = 2,800 (liter/mol/cm). LAT-specific activity (U) was defined based on P6C formed (μmol) per milligram of protein in 1 h.

Purification of LAT—*F. lutescens* IFO3084 from a frozen glycerol stock was grown for 17 h at 32°C in L-broth (polypepton 1.0%, yeast extract 0.5%, NaCl 0.5%, glucose 0.1%, pH 7.2), and then 50 ml of the culture was added to 10 liters of FM9-medium (Na_2HPO_4 0.6%, KH_2PO_4 0.3%, NH_4Cl 0.1%, NaCl 0.2%, polypepton 1.0%, yeast extract 0.5%, L-lysine HCl 0.5%, Silicone KM75 0.005%, $CaCl_2$ 0.0015%, pH 7.2). Cells grown at 32°C for 17 h were collected, washed with sterile 0.85% NaCl and then suspended in 0.01 M phosphate buffer (pH 7.2) containing 0.5 mM pyridoxal phosphate. A cell-free extract was obtained by sonication (5-s pulses at 1-min intervals) in a Branson sonifier and the supernatant was recovered by centrifugation at 20,000 ×*g* for 90 min. The supernatant was brought to 30% saturation with ammonium sulfate and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant solution to 70% saturation, and the resulting precipitate was collected by centrifugation and dissolved in 20 ml of 0.1 M phosphate buffer (pH 7.2), and then dialyzed against the same buffer. This enzyme solution was applied to PD10 columns (Amersham Pharmacia), and then the enzyme was eluted with 0.1 M Tris-HCl buffer (pH 7.4). This desalted enzyme solution was applied to a QAE-TOYOPEAL 550C column (6.0 cm × 5.5 cm) (TOSOH) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.5 mM pyridoxal phosphate. The column was washed with the Tris-HCl buffer, and then a linear 0–1 M NaCl gradient in 0.1 M Tris-HCl buffer (total volume, 2 liters) was applied. The LAT active fractions were pooled and ammonium sulfate was added to 1 M concentration. The enzyme solution was applied to a Phenyl-TOYOPERL 650S column (5.5 cm × 3.0 cm) (TOSOH) previously equilibrated with 0.01 M phosphate buffer (pH 7.2) containing 1 M ammonium sulfate. The column was washed with the phosphate buffer, and then a reverse linear 0.8-0 M ammonium sulfate gradient (total volume, 1.2 liters) was applied. The LAT active fractions were ultrafiltered with UP-20 (ADVANTEC). The enzyme solution was applied to PD10 columns, and then the enzyme was eluted with 0.1 M Tris-HCl buffer (pH 7.4). This desalted enzyme solution was applied to a MonoQ HR5/5 column (Amersham Pharmacia) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.4). The column was washed with the Tris-HCl buffer and then a linear 0–0.4 M NaCl gradient in 0.1 M Tris-HCl buffer (total volume, 20 ml) was applied. The resulting active fractions were subjected to protein analysis and amino acid sequencing.

Cloning of a DNA Fragment Encoding the N-Terminal End of LAT—The N-terminal 20 amino acid sequence of the purified LAT was analyzed as SLLAPLAPLRAHAGTRLTQG. To clone the DNA fragment containing this part of the N-terminal region of LAT and its 5′-flanking region, amplification by PCR was performed. As a template, chromosomal DNA from *F. lutescens* IFO3084 was completely digested with *Pst*I and then ligated with *Pst*I-Cassette (TaKaRa Biomedicals). Amplification by PCR was performed, *i.e.* 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 2 min), and extension (72°C, 1 min) with cassette primer C1 (5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3′) and degenerate primer N1 (5′-CCYTGIGTIARICKIGTICCIGCRTGIGCICG-3′ for RAHAGTRLTQG) designed on the basis of the N-terminal amino acid sequence of LAT. Using this PCR product as a template, additional amplification by PCR was performed, *i.e.* 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 2 min), and extension (72°C, 1 min) with cassette primer C2 (5′-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3′) and degenerate primer N2 (5′-CCIGCRTGIGCICGIARIGGIGCIARIGGIGC-3′ for APLAPLRAHAG) designed on the basis of the N-terminal amino acid sequence of LAT. This PCR product was ligated into the pT7 Blue T-vector (Novagen) and sequenced.

Inverse PCR for DNA Sequences that Flank a Region Encoding the N-Terminal End of LAT—To clone the 5′ and 3′-flanking regions of the PCR product, amplification by inverse PCR (*13*) was performed. Primer Inv1 (5′-TTGATTTGAGCAGATTCGCACTGCCATTT-3′) and primer Inv2 (5′-AAGGTTTTCGACAAAGTGACCATTTCCCA-3′) were designed on the basis of the nucleotide sequence encoding the N-terminal end of LAT. As a template, chromosomal DNA from *F. lutescens* IFO3084 was completely digested with *Pst*I or *Sal*I and then circularized with T4 DNA ligase. Amplification by inverse PCR was performed, *i.e.* 25 cycles of denaturation (98°C, 20 s), and annealing and extension (68°C, 6 min) with primers Inv1 and Inv2. The inverse PCR products were ligated into the pT7 Blue T-vector and sequenced.

Construction of an Expression Plasmid of pTrcLAT—Two primers, primers Ex1 and Ex2, were prepared for PCR to obtain open reading frame (ORF) 3. Primer Ex1 was designed on the basis of the N-terminal sequence of this ORF containing a *Bam*HI site at one end (5′-GCGGATCCCTTCTTGCCCCGCTCGCCC-3′; the underlined sequence indicates the *Bam*HI site). Primer Ex2 was designed on the basis of the downstream sequence of this ORF containing a *Pst*I site at the other end (5′-CTGCTGCAGCTGGTGCCGGGCAGCAAAGAG-3′; the underlined sequence indicates the *Pst*I site). Using *F. lutescens* IFO3084 chromosomal DNA as a template, amplification by PCR was performed, *i.e.* 25 cycles of denaturation the (98°C, 20 s), annealing (60°C, 30 s), and extension (68°C, 2 min) with primers Ex1 and Ex2. The PCR product was digested with *Bam*HI and *Pst*I, and then ligated into pTrcHisA (Invitrogen) digested with *Bam*HI and *Pst*I. The resulting plasmid, designated as pTrcLAT, was prepared from the transformed *E. coli* TOP10. pTrcLAT provided a six-His-tagged LAT, which contained an additional enterokinase-recognition sequence (DDDDK) between LAT and the six-His tag.

Purification of LAT Expressed in E. coli TOP10—*E. coli* TOP10 harboring pTrcLAT from a frozen glycerol stock was grown for 17 h at 32°C in L-broth. One ml of the culture was added to 50 ml L-broth containing 50 μg/ml ampicillin sodium, followed by growth at 32°C. When the absorbance at 660 nm of the culture reached 1.0, the production of LAT was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM). Six hours later, cells were collected, washed with sterile 0.85% NaCl and then suspended in 2 ml of BugBuster (Novagen). The supernatant was recovered by centrifugation at 20,000 ×*g* for 20 min for the purification of LAT. Chromatography on a ProBond resin column (Invitrogen) was performed under native conditions by means of the protocol for the Xpress Protein Purification System (Invitrogen) except for the elution step. In the elution step, the column to which the sample had

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been applied was washed with Native Wash Buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0) containing 200 mM imidazole. The column was then eluted with Native Wash Buffer containing 650 mM imidazole. The enzyme was applied to PD10 columns and then eluted with 0.2 M phosphate buffer (pH 7.2). The resulting protein was used as the recombinant LAT.

Protein Analysis—SDS-PAGE of proteins was performed using a Multigel 10/20 (DAIICHI PURE CHEMICALS). Native PAGE of proteins was performed using a Multigel 4/20 (DAIICHI PURE CHEMICALS). Total protein was quantified with a Protein Assay Kit I (BIO-RAD).

Amino Acid Sequencing—The purified LAT were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). Then the N-terminal 20 amino acid sequences were determined with a HP G1005A Protein Sequencing System (HEWLETT PACKARD).

DNA Manipulation—Genomic DNA from *F. lutescens* IFO3084 was prepared using a Qiagen RNA/DNA Kit (Qiagen). Plasmids from *E. coli* strains were prepared using a Qiagen Plasmid Kit (Qiagen). All restriction enzymes and T4 ligase were obtained from TaKaRa Biomedicals. All amplifications by PCR and inverse PCR were performed in a reaction mixture (50 μl) containing template DNA (50 ng), both primers (25 pM each), $MgCl_2$ (2.5 mM), dNTPs (0.25 mM each), and TaKaRa LA Taq polymerase (1 U) in the buffer for LA Taq polymerase (TaKaRa Biomedicals).

RESULTS

Purification of LAT—LAT from *F. lutescens* IFO3084 was purified to determine its N-terminal amino acid sequence. *F. lutescens* IFO3084 cell-free extracts exhibited LAT activity. The ammonium sulfate fractionation (30–70% saturation) step gave 1.8-fold purification. LAT was eluted from a QAE-TOYOPEAL 550C column with 0.42 M NaCl, with 16-fold purification. The active fractions were applied to a Phenyl-TOYOPERL 650S column and the enzyme was eluted with 0.08 M ammonium sulfate, with 50-fold purification. The ultrafiltrated and desalted enzyme solution was applied to a MonoQ HR5/5 column and the enzyme was eluted with 0.13 M NaCl, with 104-fold purification. The resulting protein was used as the purified LAT. The LAT-specific activity of the purified LAT was calculated to be 560 (U). The purified LAT gave a single band corresponding to a molecular weight of about 110,000 on native PAGE (Fig. 1A), and also gave a single band corresponding to a molecular weight of about 53,000 on SDS-PAGE (Fig. 1B), suggesting that LAT from *F. lutescens* IFO3084 is active as a homodimer.

Molecular Cloning of lat—The N-terminal 20 amino acid sequence of the purified LAT was determind to be SLLAPLAPLRAHAGTRLTQG. To clone the DNA fragment containing this part of the N-terminal region of LAT and its 5′-flanking region, amplification by PCR was performed, and the PCR product obtained was cloned and sequenced. Then, to clone the 5′ and 3′-flanking regions of the PCR product, amplification by inverse PCR (*13*) was performed. The inverse PCR products were ligated into the pT7 Blue T-vector and sequenced. These sequences were connected with the known nucleotide sequence of the N-terminal region of LAT and its 5′-flanking region. Finally, the nucleotide sequence of 2,663 bp was revealed, as shown in Fig. 2. We found a potential ORF that begins at the ATG initiation codon (nucleotide position 801) and ends at the TGA stop codon (position 2279). This ORF encodes a protein of 493 amino acids with a calculated molecular weight of 53,200, which is very close to that of the purified LAT. Twenty of the deduced N-terminal amino acids were the same as those of the purified LAT, the exception being the first Met, indicating that the N-terminal Met was processed after translation. This ORF was proceeded by a possible ribosome binding site (positions 789 to 791), and −35 and −10 promoter sequences (positions 753 to 758 and 777 to 782).

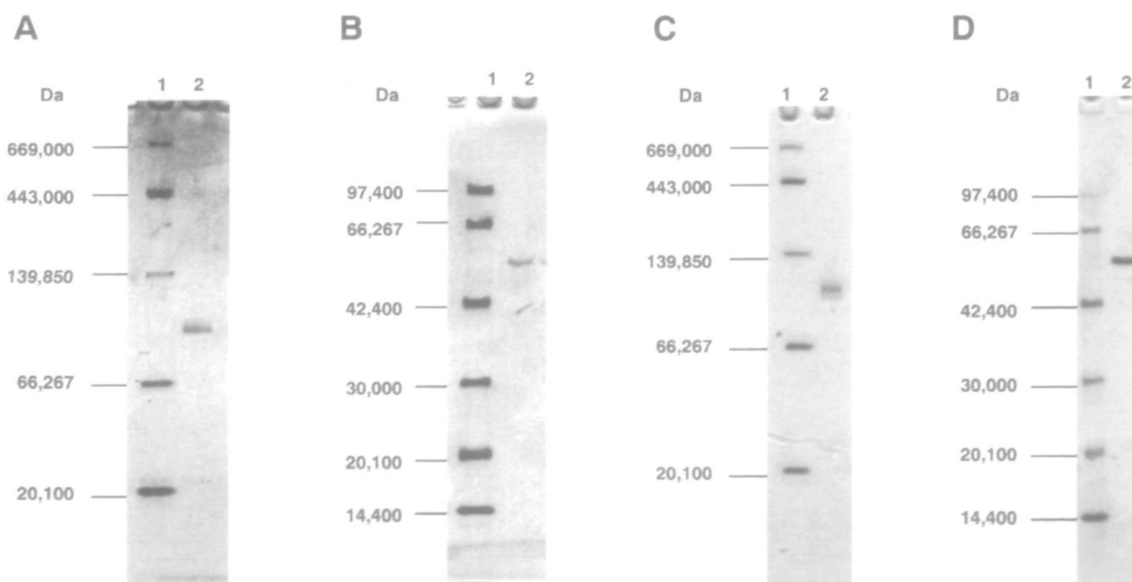

Fig. 1. **Purification of LAT from *F. lutescens* IFO3084.** (A) Native PAGE of purified LAT. (B) SDS-PAGE of purified LAT. (C) Native PAGE of recombinant LAT. (D) SDS-PAGE of recombinant LAT. Lane 1, molecular mass standards; lane 2, purified LAT.

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CCCGGGTGTC ATTGAATACC AGCAGGTCGC CAGGTTGCAG CAGCTGGTCC AGATCGCGCA     60
CCTGGCGATC CTCCAGCGCA GCCGGTGCCG GCGGCACCAG CAGCAGGCGG CTGGCCGAAC    120
GCTCCGGCAG CGGCGCCTGG GCAATCAGTT CGGGAGGCAG GTGGTAGGCA AAATCGGACT    180
TCTTCAACGC CGGCAGCTCG ATACAACGGG GGCGTCAGTT TACGCCCCTG TACCGCCTGT    240
GCCCTCACCG CTCGAACTTG GTGCCCAGGA TCACCGCCGT GGTGGTGCGC TCGACCCCAT    300
CAGTGGCGCC GATGGCATCG GTCAGCTCGT CCATCGCCGC CACGCCATCG ACGGCGGCCA    360
TCGCCACCAG GTCATGCGCG CCACTGACCG AATGCAGGCT GCGCACCGCA GCAATGGCCT    420
GCAGCGCCCG CACGACCGCC GGCATTTTCT TCGGCATCAC GGTGATGGAG ATATGCGCGC    480
GGACCTGCTG GCGCTCCATC GCCTGGCCAA GGCGCACGGT GTAGCCGGCG ATTATTCCGC    540
TGTGCTGCAG CCGCTCGATC CGGCTCTGCA CCGTGGTCCG CGACACCCCG AGCCGGCGCG    600
CCAGCGCCGC GGTCGAGGCG CGCGCATCCT CACGCAACAG GTCAAGCAAC TGTGCATCCG    660
CCTGGGAAAT GGTCACTTTG TCGAAAACCT TTCGTCAATC CGCCGAAACC GGCCATTGAT    720
TTGAGCAGAT TCGCACTGCC ATTTGTAGTG CGTTAACGGT TACAACTAAC ACTAGACACA    780
                                    -35                       -10
ATCAGCACGG ATTCAGC ATG TCC CTT CTT GCC CCG CTC GCC CCG CTC CGC       830
        RBS
                   Met Ser Leu Leu Ala Pro Leu Ala Pro Leu Arg
                    1               5                   10

GCC CAT GCC GGC ACC CGC CTT ACC CAG GGC CTG TCT GAC CCG CAG GTC      878
Ala His Ala Gly Thr Arg Leu Thr Gln Gly Leu Ser Asp Pro Gln Val
            15                  20                  25

GAG CAG CTG GCC GCC AAC CAC CCT GAC CTG CGC GCC GCC ATC GAC GCC      926
Glu Gln Leu Ala Ala Asn His Pro Asp Leu Arg Ala Ala Ile Asp Ala
        30                  35                  40

GCT GCC GAC GAA TAC GCG CGC ATC AAA CCG CAG GCC GCG GCA TTG CTG      974
Ala Ala Asp Glu Tyr Ala Arg Ile Lys Pro Gln Ala Ala Ala Leu Leu
    45                  50                  55

GAC CTG GAT GAA AGC GCG CAG ATC GCC GCC GTG CAG GAT GGC TTC GTC     1022
Asp Leu Asp Glu Ser Ala Gln Ile Ala Ala Val Gln Asp Gly Phe Val
60                  65                  70                  75

AAC TTC TAT GCC GAT GAT GCG GTG GTG CCC TAT ATC GCC CTG GCC GCC     1070
Asn Phe Tyr Ala Asp Asp Ala Val Val Pro Tyr Ile Ala Leu Ala Ala
                80                  85                  90

CGC GGG CCG TGG GTG GTC AGC CTG AAG GGC GCG GTG CTG TAT GAC GCC     1118
Arg Gly Pro Trp Val Val Ser Leu Lys Gly Ala Val Leu Tyr Asp Ala
            95                  100                 105

GGC GGC TAC GGC ATG CTC GGC TTC GGC CAT ACC CCG GCC GAT ATC CTG     1166
Gly Gly Tyr Gly Met Leu Gly Phe Gly His Thr Pro Ala Asp Ile Leu
        110                 115                 120

GAG GCG GTC GGC AAG CCG CAG GTG ATG GCC AAC ATC ATG ACT CCC TCG     1214
Glu Ala Val Gly Lys Pro Gln Val Met Ala Asn Ile Met Thr Pro Ser
    125                 130                 135

CTG GCC CAG GGC CGC TTC ATT GCC GCA ATG CGC CGC GAA ATC GGC CAT     1262
Leu Ala Gln Gly Arg Phe Ile Ala Ala Met Arg Arg Glu Ile Gly His
140                 145                 150                 155

ACC CGC GGC GGC TGC CCG TTC TCG CAC TTC ATG TGC CTG AAC TCC GGC     1310
Thr Arg Gly Gly Cys Pro Phe Ser His Phe Met Cys Leu Asn Ser Gly
                160                 165                 170

TCC GAA GCG GTC GGG CTG GCC GCG CGC ATC GCC GAC ATC AAC GCC AAG     1358
Ser Glu Ala Val Gly Leu Ala Ala Arg Ile Ala Asp Ile Asn Ala Lys
            175                 180                 185

CTG ATG ACC GAC CCG GGC GCC CGG CAT GCC GGC GCC ACG ATC AAG CGC     1406
Leu Met Thr Asp Pro Gly Ala Arg His Ala Gly Ala Thr Ile Lys Arg
        190                 195                 200

GTG GTG ATC AAG GGC AGT TTC CAC GGC CGT ACC GAC CGT CCG GCG CTG     1454
Val Val Ile Lys Gly Ser Phe His Gly Arg Thr Asp Arg Pro Ala Leu
    205                 210                 215

TAT TCC GAT TCC ACC CGC AAG GCC TAC GAT GCG CAT CTG GCC AGC TAC     1502
Tyr Ser Asp Ser Thr Arg Lys Ala Tyr Asp Ala His Leu Ala Ser Tyr
220                 225                 230                 235
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Fig. 2 (continued on next page)

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CGC GAC GAG CAC AGC GTC ATT GCC ATC GCC CCG TAT GAC CAG CAG GCC        1550
Arg Asp Glu His Ser Val Ile Ala Ile Ala Pro Tyr Asp Gln Gln Ala
                240                 245                 250

CTG CGC CAG GTG TTT GCC GAT GCC CAG GCC AAC CAC TGG TTC ATC GAG        1598
Leu Arg Gln Val Phe Ala Asp Ala Gln Ala Asn His Trp Phe Ile Glu
            255                 260                 265

GCG GTG TTC CTG GAG CCG GTG ATG GGC GAA GGC GAC CCG GGC CGT GCG        1646
Ala Val Phe Leu Glu Pro Val Met Gly Glu Gly Asp Pro Gly Arg Ala
        270                 275                 280

GTG CCG GTG GAC TTC TAC CGC CTG GCC CGT GAG CTG ACC CGC GAA CAC        1694
Val Pro Val Asp Phe Tyr Arg Leu Ala Arg Glu Leu Thr Arg Glu His
    285                 290                 295

GGC AGC CTG CTG CTG ATC GAT TCG ATC CAG GCC GCG CTG CGC GTG CAC        1742
Gly Ser Leu Leu Leu Ile Asp Ser Ile Gln Ala Ala Leu Arg Val His
300                 305                 310                 315

GGC ACC CTG TCC TTC GTC GAC TAC CCC GGC CAC CAG GAG CTG GAG GCA        1790
Gly Thr Leu Ser Phe Val Asp Tyr Pro Gly His Gln Glu Leu Glu Ala
                320                 325                 330

CCG GAC ATG GAG ACC TAC TCC AAG GCC CTG AAC GGC GCC CAG TTC CCG        1838
Pro Asp Met Glu Thr Tyr Ser Lys Ala Leu Asn Gly Ala Gln Phe Pro
            335                 340                 345

CTG TCG GTA GTG GCC GTG ACC GAG CAC GCC GCC GCG CTG TAC CGC AAG        1886
Leu Ser Val Val Ala Val Thr Glu His Ala Ala Ala Leu Tyr Arg Lys
        350                 355                 360

GGC GTG TAC GGC AAC ACC ATG ACC ACC AAC CCG CGG GCG CTG GAC GTG        1934
Gly Val Tyr Gly Asn Thr Met Thr Thr Asn Pro Arg Ala Leu Asp Val
    365                 370                 375

GCC TGC GCC ACC CTG GCA CGC CTG GAT GAG CCG GTC CGC AAC AAT ATC        1982
Ala Cys Ala Thr Leu Ala Arg Leu Asp Glu Pro Val Arg Asn Asn Ile
380                 385                 390                 395

CGC CTG CGT GGC CAG CAG GCG ATG CAG AAG CTG GAA GCA TTG AAG GAA        2030
Arg Leu Arg Gly Gln Gln Ala Met Gln Lys Leu Glu Ala Leu Lys Glu
                400                 405                 410

CGG CTG GGG GGC GCG ATC ACC AAG GTG CAG GGC ACC GGC CTG CTG TTC        2078
Arg Leu Gly Gly Ala Ile Thr Lys Val Gln Gly Thr Gly Leu Leu Phe
            415                 420                 425

TCC TGC GAG CTG GCC CCG CAG TAC AAG TGC TAC GGG GCC GGC TCC ACC        2126
Ser Cys Glu Leu Ala Pro Gln Tyr Lys Cys Tyr Gly Ala Gly Ser Thr
        430                 435                 440

GAG GAG TGG CTG CGC ATG CAC GGG GTC AAT GTG ATC CAC GGC GGC GAG        2174
Glu Glu Trp Leu Arg Met His Gly Val Asn Val Ile His Gly Gly Glu
    445                 450                 455

AAT TCG CTG CGC TTC ACC CCG CAC TTC GGC ATG GAC GAG GCC GAA CTG        2222
Asn Ser Leu Arg Phe Thr Pro His Phe Gly Met Asp Glu Ala Glu Leu
460                 465                 470                 475

GAC CTG CTG GTG GAG ATG GTC GGG CGT GCG CTG GTC GAA GGC CCA CGC        2270
Asp Leu Leu Val Glu Met Val Gly Arg Ala Leu Val Glu Gly Pro Arg
                480                 485                 490

CGG GCC TGA TCCGCACCCG CAGGACGGAA GGCACGAGCC CACCGTGAGG CGGGCTCTT      2328
Arg Ala Stop


TGCTGCCCGG CACCAGCGGC AACAGGCCGC GCTGTCACCG GCCAGGCGGG GCGCCGGCAG      2388
TGGGTTTCAG CCGCAGGGGT CCGCCCTGCC AGCGCCTGCG GCGGGGCACA GGCTTGCGGG      2448
CATTGCGGCC TCTGCCACGG GCACGCAGCC GGAGATCAGG CTGACAAGGG GGCTGCCCCG      2508
GGTGGCAGTA CACGACCAGC CAGTTGACTG CCGGTATTTG CTTGATCAGC GCTGCATCCA      2568
GAACAGCACC ATCGGTTGCG TGACTGACGC GCCGCTGGCC GTTGCGGGAC AGCAGCCTTT      2628
GCGTCACACG TGGCCCGCAC CTGCCTGCAC TGCAG                                 2663
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Fig. 2. **Nucleotide sequence of *lat* and its deduced amino acid sequence.** A potential ribosome binding site (RBS), and –35 and –10 promoter sequences (–35 and –10) are double underlined and underlined, respectively. The N-terminal 20 amino acids determined are broken underlined. The DNA sequence of *lat* from *F. lutescens* IFO3084 has been deposited in GenBank (accession no. AB035478).

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Comparison of the deduced amino acid sequence of LAT from *F. lutescens* IFO3084 was performed using the BLAST computer algorithm (Fig. 3). LAT from *F. lutescens* IFO-3084 showed strong similarity to other aminotransferases, especially to acetylornithine aminotransferase from *E. coli*, succinylornithine aminotransferase from *E. coli* and ornithine aminotransferase from *Saccharomyces cerevisiae* (27, 27, and 25% identity, respectively). It also showed high similarity to other LATs, *i.e.* LAT from *N. lactamdurans*, hypothetical LAT from *Mycobacterium tuberculosis* and LAT from *S. clavuligerus* (23, 23, and 24% identity, respectively). The sequence alignment of these aminotransferases allowed assignment of the following conserved lysine residue: Lys-255 of acetylornithine aminotransferase from *E. coli*, Lys-253 of succinylornithine aminotransferase from *E. coli*, Lys-271 of ornithine aminotransferase from *S. cerevisiae*, Lys-300 of LAT from *N. lactamdurans*, Lys-300 of LAT from *M. tuberculosis* and Lys-304 of LAT from *S. clavuligerus*. Each of these lysine residues is considered to be the active center for binding of pyridoxal phosphate, a well-known effector of a variety of aminotransferases (*9*). Since this domain is highly conserved, Lys-339 of LAT from *F. lutescens* IFO3084 is probably the pyridoxal phosphate binding lysine residue. Thus, the ORF obtained here was structurally related to other aminotransferases and thought to be a structural gene (*lat*) for LAT activity.

Expression of lat in E. coli—Most aminotransferases are homopolymeric proteins composed of identical subunits with a molecular weight of about 50,000 (*10*). To determine whether or not *lat* from *F. lutescens* IFO3084 codes for a single subunit leading to LAT activity, *lat* was expressed in *E. coli* and then examined for LAT activity. The resulting

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E.coli-ACOAT            1 --------------------------------------------------------------------------------   1
E.coli-SUOAT            1 --------------------------------------------------------------------------------   1
S.cerevisiae-OAT        1 -------------------------------------------------------------MSEATLSSK-QTIEWENKY  18
F.lutescens-LAT         1 MSLLAPLAPLRAHAGTRLTQGLSDPQVEQLAANHP-DLRAAIDAAADEYARIKPQAAALLDLDESAQIAAVQD-GFVNFY  78
M.tuberculosis-LAT      1 --------------------------------------MAAVVKSVALAGRPTTP-DRVHEVLGRSMLVDGLDIVLDLTR  41
N.lactamdurans-LAT      1 -------------------------------------MVLEMPAARVPAGPDAR--D-VRQALARHVLTDGYDLVLDLEA  40
S.clavuligeruns-LAT     1 ----------------------------------MGEAARH-PDGDFSDVGNLHAQD-VHQALEQHMLVDGYDLVLDLDA  44

E.coli-ACOAT            1 ---------MAIEQTAITRATFDEVILPIYAPAEFIPVKGQGSRIWDQQGKEYVDFAGG-IAVTALGHCHPALVNALKTQ  70
E.coli-SUOAT            1 -----------MSQPI-TRENFDEWMIPVYAPAPFIPVRGEGSRLWDQQGKEYIDFAGG-IAVNALGHAHPELREALNEQ  67
S.cerevisiae-OAT       19 SAHNYHPLPVVFHKAKGAHVWDPEGKLYLDFLSA-YSAVNQGHCHPHIIKALTEQAQTLT---LSSRAFHNDVYAQFAKF  94
F.lutescens-LAT        79 ADDAVVPYIALAA--RGP--WVVSLKGAVLYDAGGYGMLGFGHTPADILEAVGKPQV-MAN-IMTP-SLAQGRFIAAMRR 151
M.tuberculosis-LAT     42 SGGSYLVDAITGRRYLD-MFTFVASSALGMNPPALVDDREFHAELMQAALNKPSNSDVYSVAMARFVETFARVLGDPALP 120
N.lactamdurans-LAT     41 SAGPWLVDAVTGTRYLD-LFSFFASAPLGINPSCIVDDPAFVGELAAAAVNKPSNPDVYTVPYAKFVTTFARVLGDPLLP 119
S.clavuligeruns-LAT    45 SSGVWLVDAVTQKRYLD-LFSFFASAPLGINPPSIVEDPAFMRELAVAAVNKPSNPDLYSVPYARFVKTFARVLGDPRLP 123

E.coli-ACOAT           71 GET-LWHISNVFTNEPALR--LGRKLIEATFAERVVF-MNSGTEANETAFKLARHYACVRHSPF--KTKIIA-FHNAFHG 143
E.coli-SUOAT           68 ASK-FWHTGNGYTNEPVLR--LAKKLIDATFADRVFF-CNSGAEANEAALKLARKFAHDRYGSH--KSGIVA-FKNAFHG 140
S.cerevisiae-OAT       95 -VTEFF---GFETVLPMNTGAEAVET--ALKLARRWGYMKKNIPQDK-AI--I---LGAEGNFHGRTFGAISLSTDYEDS 162
F.lutescens-LAT       152 EIGHTRGGCPFSHFMCLNSGSEAVGL--AARIADINAKLMTDPGA-RHAGATIK-RVVIKGSFHGRTDRP-ALYSDSTRK 226
M.tuberculosis-LAT    121 HLFFVEGG-ALAVENA-LKAAFDWKSRHNQAHGIDPA-LGTQVL--HL-RGAFHGRSGYTLSLTNTKPTITARFPK-FDW 193
N.lactamdurans-LAT    120 HLFFVDGG-ALAVENA-LKAAFDWKAQKLGLDDRAVNRL--QVL--HLER-SFHGRSGYTMSLTNTDPSKTARYPK-FDW 191
S.clavuligeruns-LAT   124 RLFFVDGG-ALAVENA-LKAALDWKAQKLGLAEPDTDRL--QVL--HLER-SFHGRSGYTMSLTNTEPSKTARFPK-FGW 195

E.coli-ACOAT          144 -RSLFTVSVGGQPKYSDGFGPKPADIIHVPFNDLHAVKAVMDDHTCAVVVEPIQGEGGVTAATP--EFLQGLRELCDQHQ 220
E.coli-SUOAT          141 -RTLFTVSAGGQPAYSQDFAPLPADIRHAAYNDINSASALIDDSTCAVIVEPIQGEGGVVPASN--AFLQGLRELCNRHN 217
S.cerevisiae-OAT      163 KLHFGPFVPNVASGHSVHKIR---YGHAEDFVPILESPEGKN--VAAIILEPIQGEA--GIVVPPADYFPKVSALCRKHN 235
F.lutescens-LAT       227 A--YDAHLASYRDEHSVIAIA--PYDQQA-LRQVFADAQANHWFIEAVFLEPVMGEGDPGRAVPV-DFYRLARELTREHG 300
M.tuberculosis-LAT    194 PR--IDAPYMRPGLDEPAMAALEAE--ALRQARAAFETRPHD-IACFVA-EPIQGEGGDRHFRP--EFFAAMRELCDEFD 265
N.lactamdurans-LAT    192 PR--IPAPALEHPLTTHAEANREAERRALEAAEEAFRAAD-GMIACFLA-EPIQGEGGDNHFSA--EFLQAMQDLCHRHD 265
S.clavuligeruns-LAT   196 PR--ISSPALQHPPAEHTGANQEAERRALEAAREAFAAAD-GMIACFIA-EPIQGEGGDNHLSA--EFLQAMQRLCHEND 269
                                                                    *
E.coli-ACOAT          221 ALLVFDEVQCGMGRTGDLFA----YMHYGVT--PDILTSAKA--LGGGFPISAMLTTAEIASAFHPGSHGSTYGGNPLAC 292
E.coli-SUOAT          218 ALLIFDEVQTGVGRTGELYA----YMHYGVT--PDLLTTAKA--LGGGFPVGALLATEECARVMTVGTHGTTYGGNPLAS 289
S.cerevisiae-OAT      236 VLLIVDEIQTGIGRTGEL-LCYDHYKAEAK---PDIVLLGKALSGGVLPVSCVLSSHDIM-SCFTPGSHGSTFGGNPLAS 310
F.lutescens-LAT       301 SLLLIDSIQAAL-RVHGTL-SFVDYPGHQELEAPDMETYSKALNGAQFPLSVVAVTEHAAAL-YRKGVYGNTMTTNPRAL 377
M.tuberculosis-LAT    266 ALLIFDEVQTGCGLTGTAWA----YQQLDV--APDIVAFGKKTQVCGVMAG-RRVDEVADNVFAVPSRLNSTWGGNLTDM 338
N.lactamdurans-LAT    266 ALFVLDEVQSGCGLTGTAWA----YQQLG-L-RPDLVAFGKKTQVCGVMGG-GRIGEVESNVFAVSSRISSTWGGNLADM 338
S.clavuligeruns-LAT   270 ALFVLDEVQSGCGITGTAWA----YQQLG-L-QPDLVAFGKKTQVCGVMGG-GRIDEVPENVFAVSSRISSTWGGNLADM 342

E.coli-ACOAT          293 AVAGAAFDI-INTPEVLEGIQAKRQRFVDHLQK-IDQQYDVFSDI-RGMGLLIGAELKPQYKGRARDFLYAGAEA---GV 366
E.coli-SUOAT          290 AVAGKVLEL-INTPEMLNGVKQRHDWFVERLNT-INHRYGLFSEV-RGLGLLIGCVLNADYAGQAKQISQEAAKA---GV 363
S.cerevisiae-OAT      311 RVAIAALEVIRDEKLCQRAAQLGS-SFIAQLKALQAKSNGIISEVR-GMGLLTAIVIDPS-KANGKTAWDLCLLMKDHGL 387
F.lutescens-LAT       378 DVACATLARLDEP-VRNNIR-LRGQQAMQKLEALKERLGGAITKVQ-GTGLLFSCELAPQYKCYGAGSTEEWLRM--HGV 452
M.tuberculosis-LAT    339 -VRARRILEVIEAEGLF-ERAVQHGKYLRARLDELAADFPAVVLDPRGRGLMCAFSLPTTADRDELIRQLWQRAV---IV 413
N.lactamdurans-LAT    339 -VRATRVLETIERTDLL-DSVVQRGKYLRDGLEALAERHPGVVTNARGRGLMCAVDLPDTEQRDAVLRRMYTGHQ----V 412
S.clavuligeruns-LAT   343 -VRATRLLETIERTQVF-DTVVQRGKYFRDGLEDLAARHPSVVTNARGRGLMCAVDLPDTRTRNEVLRLMYTEHQ----V 416

E.coli-ACOAT          367 MVLNAGPDVMRFAPSLVVEDADIDEGM-------------QRFAHAVAKVV---GA--------                 406
E.coli-SUOAT          364 MVLIAGGNVVRFAPALNVSEEEVTTGL-------------DRFAAACEHFVSRGSS--------                 406
S.cerevisiae-OAT      388 LAKPTHDHIIRLAPPLVISEEDLQTGVETIAKC---------------------------IDLL                 424
F.lutescens-LAT       453 NVIHGGENSLRFTPHFGMDEAELDLLVEMVGRA---------------------LVEGPRRA--                 493
M.tuberculosis-LAT    414 --LPAGADTVRFRPPLTVSTAEIDAAIAAVRSALPVVT--------------------------                 449
N.lactamdurans-LAT    413 IALPCGTRGLRFRPPLTVTESELDQGLEALAASLA----SRG----------------------                 450
S.clavuligeruns-LAT   417 IALPCGGRSLRFRPALTIAEHEIDQALQALASSVTAVAESV-----------------------                 457
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Fig. 3. **Comparison of the deduced amino acid sequence of LAT from *Flavobacterium lutescens* IFO3084 with those of other aminotransferases.** The deduced amino acid sequence of LAT from *Flavobacterium lutescens* IFO3084 (F.lutescens-LAT) was aligned with those of acetylornithine aminotransferase from *Escherichia coli* (E. coli-ACOAT), succinylornithine aminotransferase from *Escherichia coli* (E. coli-SUOAT), ornithine aminotransferase from *Saccharomyces cerevisiae* (S.cerevisiae-OAT), LAT from *Nocardia lactamdurans* (N. lactamdurans-LAT), hypothetical LAT from *Mycobacterium tuberculosis*, and LAT from *Streptomyces clavuligerus* (S. clavuligerus-LAT) The probable pyridoxal phosphate binding lysine residue is indicated by an asterisk.

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protein, the recombinant LAT, gave a major band corresponding to a molecular weight of about 110,000 and a minor band under the major band on native PAGE (Fig. 1C). Since this minor band increased with time, the recombinant LAT may be partially denatured during purification and analysis. The recombinant LAT also gave a single band corresponding to a molecular weight of about 57,000 on SDS-PAGE (Fig. 1D), which is in good agreement with the value (57,100) estimated from the deduced amino acid sequence of the recombinant LAT. The molecular weight of the recombinant LAT on SDS-PAGE was greater than that of purified LAT because the recombinant LAT has an added His-tag and enterokinase-recognition sequence at its N-terminal end. The recombinant LAT also showed LAT specific activity, which was calculated to be 70 (U). The observed lower LAT-specific activity of the recombinant LAT may be explained by protein denaturation or a difference in protein folding between *F. lutescens* IFO3084 and *E. coli*. Thus, the recombinant LAT is also active as a homodimer.

DISCUSSION

In this study, LAT from *F. lutescens* IFO3084 was purified, and its structural gene (*lat*) was cloned, sequenced and expressed in *E. coli*. The purified LAT gave a single band corresponding to a molecular weight of about 110,000 on native PAGE (Fig. 1A), and also gave a single band corresponding to a molecular weight of about 53,000 on SDS-PAGE (Fig. 1B), suggesting that LAT from *F. lutescens* IFO3084 is active as a homodimer. The recombinant LAT also gave a single band on SDS-PAGE (Fig. 1D) and showed LAT-specific activity, supporting that the *lat* from *F. lutescens* IFO3084 codes for a protein composed of a single subunit with LAT activity.

Yagi *et al.* reported that LAT from *F. lutescens* IFO3084 was composed of four non-identical subunits (*12*), which is not consistent with our present results. The inconsistency is not explained by a difference in the enzymes purified between Yagi *et al.* and us, because we did not observe any other fractions with LAT activity throughout the purification in the present study. Although we have no direct evidence at present, it is possible that the LAT purified by Yagi *et al.* was partially cleaved during the process of purification and analysis. The amino acid sequence of LAT purified by Yagi *et al.* will give a good account of the inconsistency.

In *F. lutescens* IFO3084 and β-lactam antibiotic-producing species of actinomycetes, the transamination by LAT is essential for the first step of the L-α-aminoadipic acid (L-AAA) biosynthetic pathway (*3, 7*). The activity of the second enzyme, P6C dehydrogenase, is probably required for conversion of α-aminoadipic semialdehyde into AAA, as reported for *S. clavuligerus* (*14*). L-AAA is a rare amino acid, and an important precursor of β-lactam antibiotics and useful chemicals. Therefore, we have previously attempted the bioconversion of L-lysine into L-AAA through fermentation with *F. lutescens* IFO3084. To improve the L-AAA productivity of *F. lutescens* IFO3084, using the *lat* gene we cloned, genetically engineered strains are currently being established in our laboratory.

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