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Cloning and Characterization of *pcd* Encoding Δ′-Piperideine-6-Carboxylate Dehydrogenase from *Flavobacterium lutescens* IFO3084

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The *pcd* gene from *Flavobacterium lutescens* IFO3084 encoding Δ′-piperideine-6-carboxylate dehydrogenase (PCD) was cloned, sequenced, and expressed in *Escherichia coli*. The deduced amino acid sequence of PCD from *F. lutescens* IFO3084 showed strong similarity to that from *Streptomyces clavuligerus*. The molecular mass of the recombinant PCD was estimated to be approximately 58,000 Da by SDS-PAGE and native PAGE, which indicated that the enzyme molecule is a monomer. The *in vitro* analysis of L-α-aminoadipic acid (L-AAA) production showed that L-AAA is synthesized from L-lysine in two steps catalyzed by L-lysine 6-aminotransferase (LAT) and PCD from *F. lutescens* IFO3084.

Key words: L-α-aminoadipic acid (L-AAA), *Flavobacterium lutescens* IFO3084, *lat*, *pcd*, Δ′-piperideine-6-carboxylate (P6C).

L-α-Aminoadipic acid (L-AAA), which is a rare amino acid and not observed in proteins, is a precursor of β-lactam antibiotics and various chemicals. In β-lactam antibiotics producing actinomycetes, L-AAA is synthesized, and L-lysine 6-aminotransferase (LAT) activity is required for the first step of the L-AAA biosynthetic pathway (*1*). LAT converts L-lysine into α-aminoadipic semialdehyde, which is spontaneously cyclized to form Δ′-piperideine-6-carboxylate (P6C) (*1*). The gene encoding LAT (*lat*) is located in the β-lactam antibiotics gene cluster in both *Nocardia lactamdurans* (*2*) and *Streptomyces clavuligerus* (*3*), whereas it is absent from the genome of most other actinomycetes, confirming that LAT is specific for secondary metabolism.

In the case of the gram-negative bacterium *Flavobacterium lutescens* IFO3084, L-AAA is also synthesized and LAT is essential for the first step of the L-AAA biosynthetic pathway (*4*). Recently, we cloned and sequenced the gene encoding LAT (*lat*) from *F. lutescens* IFO3084 (*5*). The native PAGE analysis of purified LAT showed a single band corresponding to a molecular mass of about 110 kDa. *lat* encodes a protein of 493 amino acids with a deduced molecular weight of 53,200, which is very close to that of purified LAT determined by SDS-PAGE. The expression in *Escherichia coli* revealed that *lat* encodes a single subunit protein with LAT activity. Thus, LAT from *F. lutescens* IFO3084, like most other aminotransferases, is derived from a single ORF and is active as a homodimer.

Recently, the enzyme responsible for catalyzing the conversion of P6C into L-AAA, named Δ′-piperideine-6-carboxylate dehydrogenase (PCD), was first identified in *S. clavuligerus* (*6*). The purified PCD, a monomer of 56.2 kDa, utilizes both P6C and NAD^+ efficiently as substrates. DNA sequencing of the *pcd* gene from *S. clavuligerus* revealed that PCD shows a strong similarity to various aldehyde dehydrogenases, especially to an aldehyde dehydrogenase from *Cenorhabditis elegans* (*7*). PCD activity was also found in other β-lactam antibiotics producers, but not in the non-producing actinomycetes (*6*). Furthermore, the gene encoding PCD (*pcd*) is located in the β-lactam antibiotics gene cluster (*7*). Thus, PCD is a specific enzyme in the biosynthetic pathway of L-AAA, a precursor of β-lactam antibiotics.

In this study, we cloned and sequenced *pcd* from *F. lutescens* IFO3084, and showed that L-AAA is synthesized from L-lysine in two steps catalyzed by LAT and PCD from *F. lutescens* IFO3084.

MATERIALS AND METHODS

Bacterial Strains—*F. lutescens* IFO3084 was used as an L-AAA–producing microorganism. A mutant strain designated HGN1, which is incapable of producing L-AAA, was derived from strain *F. lutescens* IFO3084 by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis (see below).

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 electro-transformation. Transformants of *F. lutescens* IFO3084 and *E. coli* 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) and grown in L-broth containing 20 μg/ml kanamycin sulfate. Some of the mutant cells of *F. lutescens* IFO3084 were spread on MEM-agar plates (polypepton 0.5%, yeast extract 0.2%, L-lysine HCl 1.0%, methylen blue 0.006%, eosin Y 0.04%, Bacto agar 1.5%, pH 7.2) for the isolation of L-AAA non-producing mutants. *F. lutescens* were grown in SC medium (polypepton 1.0%, yeast extract 0.2%, L-lysine HCl 1.0%, pH 7.2) for TLC analysis.

Construction of Plasmids—A vector plasmid pCF704 was constructed and used for the cloning experiments. Briefly, a

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

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95 bp fragment containing a multi-cloning site of pUC19 was amplified by PCR, with primers designed based on the sequences of pUC19 containing an *Eco*RI site at one end (5′-ACGAATTCGAGCTCGGTA-3′; the underlined sequence indicates an *Eco*RI site) and a *Nco*I site at the other end (5′-TCCCATGGACGACGTTGTA-3′; the underlined sequence indicates a *Nco*I site), and then cloned into the *Eco*RI–*Nco*I site of pBBR122 (Mo Bi Tec). The chromosomal DNA from *F. lutescens* IFO3084 partially digested with *Sau*3AI and fractionated by agarose gel electrophoresis to give fragments in the size range of 4–6 kb was ligated to the unique *Bam*HI site of pCF704 for the gene library. pCF213 was selected from the library by the cloning procedure. pCF235 was constructed by subcloning the 2,501 bp *Not*I fragment from pCF213, which had been blunt-ended with T4 polymerase, into the *Hinc*II site of pCF704.

Isolation of L-AAA Non-Producing Mutants—*F. lutescens* IFO3084, from a frozen glycerol stock, was grown overnight at 32°C in L-broth, and 100 μl of the broth was added to 50 ml L-broth. Cells grown at 32°C for 4.5 h were collected, washed, and suspended in 0.2 M phosphate buffer (pH 6.0). The cells were treated with NTG (final concentration 1.3 mg/ml) at 32°C for 20 min, added to 50 ml L-broth, and grown at 32°C for 17 h. Glycerol (final concentration 30%) was added and the culture was stored at –70°C as the mutant glycerol stock. Some of the mutant glycerol stock samples were diluted with 0.85% NaCl to 10,000 cells per ml and spread on MEM-agar plates. After incubation at 32°C for 3 days, white colonies were dispersed in 1 ml SC medium. After cultivation at 32°C for 2 days, 3 μl of each culture was transferred to a TLC plate (Merck Art. 13143) and the plates were developed with solvent (1-buthanol: acetic acid:H_2O = 3:1:1). The clones of interest, non-producing mutants, did not produce an L-AAA spot on the TLC plate, as detected by the ninhydrin reaction.

Cloning Procedure—Cells of the L-AAA non-producing mutant HGN1 harboring a recombinant plasmid were diluted with 0.85% NaCl to 10,000 cells per ml and spread on MEM-agar plates containing 20 μg/ml kanamycin sulfate. After incubation at 32°C for 3 days, red colonies, which were expected to result from the production of L-AAA, were dispersed in 1 ml SC medium containing 20 μg/ml kanamycin sulfate and subjected to TLC analysis. The clones of interest showed a spot of L-AAA on the TLC plate.

Electro-Transformation—*F. lutescens* IFO3084 from a frozen glycerol stock were grown overnight at 32°C in L-broth, and 100 μl of the broth was added to 50 ml L-broth. Cells grown at 32°C for 4.5 h were collected, washed, and suspended in 10% glycerol. These electroporation cells were thawed on ice and 1 μl of DNA solution in water (200 μg/ml) was added. The mixture was pulsed with a Gene Pulser II (Bio-Rad) at 2.4 kV with 25 μF and 200 Ω in chilled 0.2 cm electroporation cuvettes. Immediately after pulsing, the cells were transferred to Falcon 2059 tubes and mixed with 1 ml of L-broth. These cells were grown at 32°C for 2 h and spread on L-agar plates containing 20 μg/ml kanamycin sulfate. Colonies formed after incubation at 32°C for 2 days.

Construction of a PCD Expression Plasmid—Two primers, primer Ex1 and primer 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 ORF3, which contained a *Bam*HI site at one end (5′-GCGGATCCATGTCGTTTGAACTGCTCAAGG-3′; the underlined sequence indicates a *Bam*HI site). Primer Ex2 was designed on the basis of the downstream sequence of ORF3, which contained a *Pst*I site at the other end (5′-CTGCTGCAGAATTGCAGTCATGCAGTCACTC-3′; the underlined sequence indicates a *Pst*I site). Using *F. lutescens* IFO3084 chromosomal DNA as a template, a PCR amplification was performed, *i.e.*, 25 cycles of denaturation (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 pTrcPCD, was prepared from the transformed *E. coli* TOP10. pTrcPCD provided a six-His–tagged PCD, which contained an additional enterokinase-recognition sequence (DDDDK) between PCD and the six-His tag.

Purification of Recombinant LAT and Recombinant PCD Expressed in E. coli TOP10—Recombinant LAT and recombinant PCD were expressed in *E. coli* TOP10 and purified as described previously (*5*).

Analysis of In Vitro L-AAA Production—Fifty microliters of recombinant LAT solution (25.4 μg/ml) and 50 μl of recombinant PCD solution (6.8 μg/ml) were added to 1.0 ml of 0.2 M phosphate buffer (pH 7.2) containing L-lysine HCl (20 μmol), 2-ketoglutarate (20 μmol), pyridoxal phosphate (0.075 μmol), and β-NAD^+ (200 μmol). The mixtures were incubated at 32°C for 15 h. L-AAA was derivatized with phenylisothiocyanate and separated by reverse-phase high-performance liquid chromatography (*8*, *9*).

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

DNA Manipulation—Plasmids from *F. lutescens* and *E. coli* strains were prepared using a Qiagen Plasmid Kit (Qiagen). All restriction enzymes, T4 ligase, T4 DNA polymerase, and LA Taq polymerase were obtained from TaKaRa Biomedicals. DNA sequencing analysis was done using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (ABI).

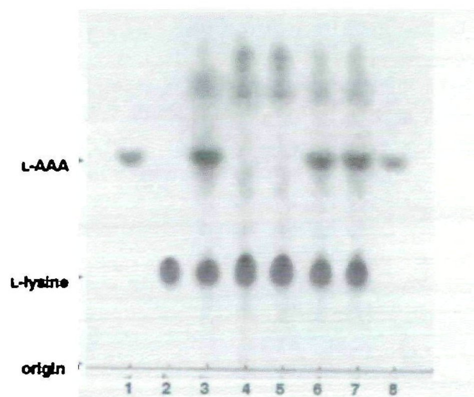

Fig. 1. **Complementation of the *pcd* mutant for L-AAA production.** TLC analysis of L-AAA production from L-lysine by *F. lutescens* IFO3084 and its mutant, HGN1, harboring various plasmids. Lanes: 1 and 8, L-AAA (500 μg/ml); 2, L-lysine (500 μg/ml); 3, *F. lutescens* IFO3084; 4, HGN1; 5, HGN1 (pCF704); 6, HGN1 (pCF213); 7, HGN1 (pCF235).

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Fig. 2. (continued)

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        10        20        30        40        50        60        70        80        90
GGATCGGGCCACTGGGCTCACTGCTGGACGCAATCCGAGTGCCGGGATGGCTCGGGTTGAAGGTGTTGCGGATCACGATCGGCATCTGCC
   I  P  G  S  P  E  S  S  S  A  I  R  T  G  P  H  S  P  N  F  T  N  R  I  V  I  P  M  Q  R

       100       110       120       130       140       150       160       170       180
GGGCGATGGCCGGGCTCATCGTCTGCGGGTGCACCACCTTGGCGCCGAAATAGGCCAGTTCGCAGGCCTCGTCATAGCTGAGCGTGGCCA
   A  I  A  P  S  M  T  Q  P  H  V  V  K  A  G  F  Y  A  L  E  C  A  E  D  Y  S  L  T  A  L

       190       200       210       220       230       240       250       260       270
GGGTCACCGCCTCGGGCACCACCCGCGGGTCGGCCGACAGCACACCGTCGACATCGGTCCAGATGTGCAGCTCGGCCGCCTCGAACAGCG
   T  V  A  E  P  V  V  R  P  D  A  S  L  V  G  D  V  D  T  W  I  H  L  E  A  A  E  F  L  A

       280       290       300       310       320       330       340       350       360
CGGCAAAGATCGCCCCGGAATAATCGCTGCCGTTGCGGCCCAGGGTGGTGATCCTGCCCTGGCCATCACGGGCGACAAAACCGGTGACCA
   A  F  I  A  G  S  Y  D  S  G  N  R  G  L  T  T  I  R  G  Q  G  D  R  A  V  F  G  T  V  V

       370       380       390       400       410       420       430       440       450
CCACCCGCGACTGCGGGTTGTCCACACGCCAGGCGGCCAGGTTGGCCGCACTGCGTTCCCAGTCGACGCTGACCCCCAGCTCGCCGTGTG
   V  R  S  Q  P  N  D  V  R  W  A  A  L  N  A  A  S  R  E  W  D  V  S  V  G  L  E  G  H  A

       460       470       480       490       500       510       520       530       540
CGACCACCAGCACATCGCGGGCATCGAGCACCGCGCAGGGGTGGCCGAGCCGGTTGAAATAGCGGCCCAGCAGCTGGGCCGAGAACACCT
   V  V  L  V  D  R  A  D  L  V  A  C  P  H  G  L  R  N  F  Y  R  G  L  L  Q  A  S  F  V  E

       550       560       570       580       590       600       610       620       630
CGCCCAGCCCCTGCACCCTTTCAAGCACCTCCTCGGGCAGGCCGCCGATCACCGCCAGCGCTTCCAGCAACCCGGCCAGCTTGTCAAAGC
   G  L  G  Q  V  R  E  L  V  E  E  P  L  G  G  I  V  A  L  A  E  L  L  G  A  L  K  D  F  R

       640       650       660       670       680       690       700       710       720
GTCCATCCAGCCACTGCAGCAGGTCGGCAGAATCCTCGCCCAGCAGTTCGGTGGCCGCTTCATGGTGGCGCTGGCGCAGGGCCTGCCAGG
   G  D  L  W  Q  L  L  D  A  S  D  E  G  L  L  E  T  A  A  E  H  H  R  Q  R  L  A  Q  W  A

       730       740       750       760       770       780       790       800       810
CATCACGCCAGCGCGGCTGACCGTGGGCGGCCAGGGTAGCCAGCTCGATCAAGGCATCGGTGACACCCTTCATCGCCGAGACCACCACCA
   D  R  W  R  P  Q  G  H  A  A  L  T  A  L  E  I  L  A  D  T  V  G  K  M  A  S  V  V  V  V

       820       830       840       850       860       870       880       890       900
CCTGGGTGGGTTCCGGGCGCTGCAGCAGCAACTCGGCGACATGGCGGTAGCGCTGCGCCGAGGCCACCGAGGTGCCGCCGAACTTGTGGG
   Q  T  P  E  P  R  Q  L  L  L  E  A  V  H  R  Y  R  Q  A  S  A  V  S  T  G  G  F  K  H  A

       910       920       930       940       950       960       970       980       990
CGATGACCTGGGCATCGGGCGCGGGAGCGGGAGCGGGTGCAGCGGCAGGCGATGACATCACAACAGACCTCTGGGGTTGAGGCCCGGCAC
   I  V  Q  A  D  P  A  P  A  P  A  P  A  A  A  P  S  S  M ORF1

      1000      1010      1020      1030      1040      1050      1060      1070      1080
CGCAGGTTGCGAAGTCCCGCAACCTGGTCGGTGCGGGGCCGTTGTTTTCGGGGGTTAGACGAATACGACGGGCCGCACCAGCCAAGTGGT

      1090      1100      1110      1120      1130      1140      1150      1160      1170
GGTGGTAATGATGGTCATGCCGGTGACGCCAGCAGGCGCCAGCAGGGCGGCAGTGGAATCAACGGTGGCGCGGCAGATCGACATGCAGCG

      1180      1190      1200      1210      1220      1230      1240      1250      1260
AGCAGACCGCACAGCGCCTGCTGCTGTCAACTGTTGCATTGCAAAATAATTTTCCGCGCATCATCGGCGAACATGCACCGATTTGGTTG

      1270      1280      1290      1300      1310      1320      1330      1340      1350
AAATGTGATCGTCAGCGATCTTCTGTCAAAACCCGCGGATCAAGCGGCCACAGCCGCTGCGGCAGCCGCGGACCACCGCGCGCCGATGCC

      1360      1370      1380      1390      1400      1410      1420      1430      1440
AGCGCCGGGCGGCAGAGCAAGCCGCCAGCGCAACCGGCCATTACCGCGGCCAGGCGCCGGGCCTGCGCGGCTCAACCGTGGATTTTTCC
                                                                               *  G  H  I  K  E

      1450      1460      1470      1480      1490      1500      1510      1520      1530
CAGCGGGCGTGGGCCTGCGCGGCCAGCACCACCCCGCCGACCAACAGCGCAATGGCCAGCAGCTCCAGCAGGGTCGGGCCACGCTGCTGC
 W  R  A  H  A  Q  A  A  L  V  V  G  G  V  L  L  A  I  A  L  L  E  L  L  T  P  G  R  Q  Q

      1540      1550      1560      1570      1580      1590      1600      1610      1620
CAGATGAAGCCATAAAGCAACGCGAACAGGGTTTCAAACACGATCAGCTGCCCGCCCAGGCTCAGCGGCAGGCTGCGCGTGGCCCGGTTC
 W  I  F  G  Y  L  L  A  F  L  T  E  F  V  I  L  Q  G  G  L  S  L  P  L  S  R  T  A  R  N

     1630      1640      1650      1660      1670      1680      1690      1700      1710
CAGCAGGCATTGCCCAGCACCGAGGCACCGACGGCCAGCAGCGCACAGATGCCGGCAAAGTGCAGCCACTGGCCCTGGCTCTGCCCGAGC
 W  C  A  N  G  L  V  S  A  G  V  A  L  L  A  C  I  G  A  F  H  L  W  Q  G  Q  S  Q  G  L
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Fig. 2. (continued)

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       1720      1730      1740      1750      1760      1770      1780      1790      1800
GGCCCCAGCCACAGCGCCAGCGGCAGCAACAGCACGGCGATGGCCCCTGTGGCCACCCCGGTCAACAACGACCAGGCATGCCCGGACAGG
 P  G  L  W  L  A  L  P  L  L  L  V  A  I  A  G  T  A  V  G  T  L  L  S  W  A  H  G  S  L

       1810      1820      1830      1840      1850      1860      1870      1880      1890
TGCGGATAGCGCCGCATCCACACCACATTGGCGATCGAGTAGCCACTCCAGGCGGCCAGCGCGGCCAGCGCGCAGAGCAGGCCCAGCACC
 H  P  Y  R  R  M  W  V  V  N  A  I  S  Y  G  S  W  A  A  L  A  A  L  A  C  L  L  G  L  V

       1900      1910      1920      1930      1940      1950      1960      1970      1980
CGCTGACCGATGTCCTTGCCAGCATCGCCCGCCGCGCCCGCGCCGTGGCCGAGTGCAGCCCAGGCCACCAGCAGCGAGCCCAGCACACAC
 R  Q  G  I  D  K  G  A  D  G  A  A  G  A  G  H  G  L  A  A  W  A  V  L  L  S  G  L  V  C

       1990      2000      2010      2020      2030      2040      2050      2060      2070
AGGCACAGCGCCGGTGCCAGCTGACGCAACGGCAGGGCCGTTGGCCGCCGCGCATCCACCGCCGCCACCACCACCGGCACCATGCCCACG
 L  C  L  A  P  A  L  Q  R  L  P  L  A  T  P  R  R  A  D  V  A  A  V  V  V  P  V  M  G  V

       2080      2090      2100      2110      2120      2130      2140      2150      2160
ATCAGCGCGGCCGCCGCACCGCCAGCCCAGTGCACGGCCATCGCCAGAAACACGAAATAGACCAGGTTGCCGAGCAGGCTCAGCCCGGCC
 I  L  A  A  A  A  G  G  A  W  H  V  A  M  A  L  F  V  F  Y  V  L  N  G  L  L  S  L  G  A

       2170      2180      2190      2200      2210      2220      2230      2240      2250
AGGGCCAGCCAGGCGCGGCGATCGACCTGCGCACGCAGCGCCGGCCACAACGGCAGCAGCAACGCACAGGCCACCGCACCGTACAGCAGG
 L  A  L  W  A  R  R  D  V  Q  A  R  L  A  P  W  L  P  L  L  L  A  C  A  V  A  G  Y  L  L

       2260      2270      2280      2290      2300      2310      2320      2330      2340
TAGCGGCCCACGGCCAGCTGCAGCGCAGAAAATGCGGTGGTCAAGGCCGGCGCCAGGAACACCATGCCCCACAGGGCACCGGCGAGCACG
 Y  R  G  V  A  L  Q  L  A  S  F  A  T  T  L  A  P  A  L  F  V  M ORF2

       2350      2360      2370      2380      2390      2400      2410      2420      2430
CCGTTGAACAGTCCCCACGCGGTCTGGTTGTTGCGCTGGATCACGCTGCAAGGCCCTGCAATGAACAACAGGCCGGGGCGGCGCAGCGCA

       2440      2450      2460      2470      2480      2490      2500      2510      2520
TGGGCGCTGGCAGCTCTCCGACCTGTGCAAAGGTGGTGGCCCCGACACGATTCGAACGTGCGACCTGTCCCTTAGGAGGGGACCGCTCTA

       2530      2540      2550      2560      2570      2580      2590      2600      2610
TCCAGCTGAGCTACGGAGCCATGAGGCCGGCGATTCTAGCATCCGCTCTCCGTTCACGGCCATCGCCCGCAGCCGCAGTTCACAGTGCAG

       2620      2630      2640      2650      2660      2670      2680      2690      2700
GGCAACCGCAGCAAGCCCCCGCCCCGCTGCAACCCTCGCGCCCGCGCGCAACGTGACCGGCGCCGCGGCAGGCCCGGCCCCCACGGCCAC

       2710      2720      2730      2740      2750      2760      2770      2780      2790
TGGCGCCGGTTCCGCACCACGCCACCGGCAACACGCCCCCAGCCCTGCCCAACGTGGTGTTTCAGCGCTCTGTTAAGATGGCATGCCCAC

       2800      2810      2820      2830      2840      2850      2860      2870      2880
ATGCCACCTCCCCCCGGACGCGCCGCGGGTGCGTGACCTTTTCGTAACGTAATCTGGAGTTTCCATGTCGTTTGAACTGCTCAAGGCCTT
                -35                     -10                 SD ORF3 M  S  F  E  L  L  K  A  L

       2890      2900      2910      2920      2930      2940      2950      2960      2970
AGGGCTGGACGCCACCAATTCCGGCACCTACCTGGGTGATGGAGAATGGTCCAGCGCTACCGGTGCCGGGACCATCAGCCCGCGCAACCC
 G  L  D  A  T  N  S  G  T  Y  L  G  D  G  E  W  S  S  A  T  G  A  G  T  I  S  P  R  N  P

       2980      2990      3000      3010      3020      3030      3040      3050      3060
GACCACCGGCGAGGTCATTGCCCAGGTCCAGGCCACCACCGAGGCGGACTACGAAACCATCCTGGCCCGCGCCCAGCAGGCCTTCAAGGT
 T  T  G  E  V  I  A  Q  V  Q  A  T  T  E  A  D  Y  E  T  I  L  A  R  A  Q  Q  A  F  K  V

       3070      3080      3090      3100      3110      3120      3130      3140      3150
CTGGCGCACCACCCCGGCACCGCGCCGCGGCGAGGCCATCCGCCTGTGTGGCGAGGCCCTGCGCCGCCACAAGGACGCGCTGGGTTCGCT
 W  R  T  T  P  A  P  R  R  G  E  A  I  R  L  C  G  E  A  L  R  R  H  K  D  A  L  G  S  L

       3160      3170      3180      3190      3200      3210      3220      3230      3240
GGTCGCGCTGGAAATGGGCAAGTCCAAGCCGGAAGGCGACGGCGAAGTCCAGGAAATGATCGACATCGCCGACTTTGCCGTCGGCCAGAG
 V  A  L  E  M  G  K  S  K  P  E  G  D  G  E  V  Q  E  M  I  D  I  A  D  F  A  V  G  Q  S

       3250      3260      3270      3280      3290      3300      3310      3320      3330
CCGCATGCTGTATGGCTACACCATGCACAGCGAGCGCCCCGGCCACCGCATGTACGAGCAGTACCAGCCGCTGGGCATCGTCGGCATCAT
 R  M  L  Y  G  Y  T  M  H  S  E  R  P  G  H  R  M  Y  E  Q  Y  Q  P  L  G  I  V  G  I  I

       3340      3350      3360      3370      3380      3390      3400      3410      3420
CTCGGCCTTCAACTTCCCGGTCGCGGTCTGGGCCTGGAACAGCTTCCTGGCCGCGATCTGTGGTGATGTCTGCATCTGGAAGCCGTCCAA
 S  A  F  N  F  P  V  A  V  W  A  W  N  S  F  L  A  A  I  C  G  D  V  C  I  W  K  P  S  N
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Fig. 2. (continued)

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       3430      3440      3450      3460      3470      3480      3490      3500      3510
CAAGACCCCGCTGACCGCGATCGCGTCCATGCGCATCTGCAACGAAGCACTGCGCGAAGGCGGCTTCCCGGACATCTTCTTCCTGATCAA
 K  T  P  L  T  A  I  A  S  M  R  I  C  N  E  A  L  R  E  G  G  F  P  D  I  F  F  L  I  N

       3520      3530      3540      3550      3560      3570      3580      3590      3600
CGACGCCGGCACCGCGTTGTCGGAGAAGCTGGTCGAGGACAAGCGCGTGCCGCTGATCTCCTTCACCGGCTCGACCCAGGTCGGCGCAT
 D  A  G  T  A  L  S  E  K  L  V  E  D  K  R  V  P  L  I  S  F  T  G  S  T  Q  V  G  R  I

       3610      3620      3630      3640      3650      3660      3670      3680      3690
CGTCAACCAGAAGGTCGCCGCCCGCCTGGGCCGCTGCCTGCTCGAGCTGGGCGGCAACAACGCGATCATCCTGGACGAAACCGCCGACCT
 V  N  Q  K  V  A  A  R  L  G  R  C  L  L  E  L  G  G  N  N  A  I  I  L  D  E  T  A  D  L

       3700      3710      3720      3730      3740      3750      3760      3770      3780
GAAGCTGGCCGTGCCGGGCATCGTCTTCGGCGCCGTCGGCACCGCCGGCCAGCGCTGCACCACCACCCGCCGCCTGATCGTGCACGAATC
 K  L  A  V  P  G  I  V  F  G  A  V  G  T  A  G  Q  R  C  T  T  T  R  R  L  I  V  H  E  S

       3790      3800      3810      3820      3830      3840      3850      3860      3870
GATCTACGACAACGTGCTGGCCACCTTGATCAAGGCCTACAAGCAGGTCGAAGGCAAGATCGGCGATCCGCTGGATGCCGCCAACCTGAT
 I  Y  D  N  V  L  A  T  L  I  K  A  Y  K  Q  V  E  G  K  I  G  D  P  L  D  A  A  N  L  M

       3880      3890      3900      3910      3920      3930      3940      3950      3960
GGGCCCGCTCAACAGCCCCGAAGCGGTGCAGCAGTTCCTGGCCTCGATCGAGAAAGCCAAGGCCGCTGGCGGCACCGTTCAAACCGGTGG
 G  P  L  N  S  P  E  A  V  Q  Q  F  L  A  S  I  E  K  A  K  A  A  G  G  T  V  Q  T  G  G

       3970      3980      3990      4000      4010      4020      4030      4040      4050
TACCGCGATCGACCGCCCGGGCAACTTCGTGCTGCCGGCCATCGTCACCGGCCTGAAGAACAGCGATGAGGTGGTCCAGCACGAGACCTT
 T  A  I  D  R  P  G  N  F  V  L  P  A  I  V  T  G  L  K  N  S  D  E  V  V  Q  H  E  T  F

       4060      4070      4080      4090      4100      4110      4120      4130      4140
CGCCCCGATCCTGTACGTAATGAAGTACTCCACCCTGGACGAAGCCATCGAGATGCAGAACGGCGTGCCGCAGGGCCTGTCCTCGTCGAT
 A  P  I  L  Y  V  M  K  Y  S  T  L  D  E  A  I  E  M  Q  N  G  V  P  Q  G  L  S  S  S  I

       4150      4160      4170      4180      4190      4200      4210      4220      4230
CTTCACCACGAACCTGAAGGCAGCCGAGAAGTTCCTGTCGGCGGCCGGCAGCGACTGCGGCATTGCCAACGTCAACATCGGCACTTCCGG
 F  T  T  N  L  K  A  A  E  K  F  L  S  A  A  G  S  D  C  G  I  A  N  V  N  I  G  T  S  G

       4240      4250      4260      4270      4280      4290      4300      4310      4320
TGCCGAGATCGGCGGCGCCTTCGGTGGCGAGAAGGAAACCGGCGGTGGCCGTGAGTCCGGCTCGGATGCGTGGAAGGTCTACATGCGCCG
 A  E  I  G  G  A  F  G  G  E  K  E  T  G  G  G  R  E  S  G  S  D  A  W  K  V  Y  M  R  R

       4330      4340      4350      4360      4370      4380      4390      4400      4410
CCAGACCAACACCATCAACTACTCCGACTCGCTGCCGCTGGCCCAGGGCATCAAGTTCGACCTGTAAGCCGCTCGCCACGGCCCGCCTTC
 Q  T  N  T  I  N  Y  S  D  S  L  P  L  A  Q  G  I  K  F  D  L  *

       4420      4430      4440      4450      4460      4470      4480      4490      4500
CCCGGAAGCAGGCCGTGGCTGTTGCACCAGCCAGAGGAGTGACTGCATGACTGCAATTGAATCGACTGCCGCACGCACCACCAACACTTG

       4510      4520      4530      4540      4550      4560      4570      4580      4590
CGCCATCCTGTCGCTGGTACTGGCACTGCTGGGCTGGAATCTTTTGCCGGTGATTGGCTTTGTCGGCGCCATCATCTGCGGCCGCATCGC

       4600      4610      4620      4630      4640      4650      4660      4670      4680
CCAGCGCCAGCTCAAGCAGCCCGGCAATACCCAGGACGGTCACGGCCTGGCAAGGGCGGGCATCTGGATCAGTTGGATCAGCCTGATCCT

       4690      4700      4710      4720      4730      4740      4750      4760      4770
GGTTGCGCTGCTGATCGGCGTCGTGATCCCGTGGTTGACCGCCCCGATCACGATCAACCTGCCCGTTTCCACCTGACCCTCCTCCCTGCC

       4780      4790      4800      4810      4820      4830      4840      4850      4860
AGTCGCCCATGCGCTGACAGGCCAACCCGTTTCCTGCCTGGACCAGACCATGCTCCCGCCCGACCATCCGGCTCCACCATCGCCCATTGC

       4870      4880      4890      4900      4910      4920      4930      4940      4950
CGGCACCACAACCTCGACCAATGGCTATGCGGTGGCCTCGCTGGTGATGGGCATCCTTGGCTGGTCGATGATCCCGCTGTTGGGCAGCAT

       4960      4970      4980      4990      5000      5010      5020      5030      5040
CGGCGCCATCGTGTTCGGGCATCTGGCCCGGGCGCAGATCCGTCGCCAGCCCCAGCAGGGCGATGGCCTGGCACTGGCCGGGCTGATCCT

       5050      5060      5070      5080      5090      5100      5110      5120      5130
GGGCTGGATCTCGATTGCGCTGTGGATCCTCGGGATCCTGGCGTTTTTCCTCTTCTTTGGCGGGCTGGCCATGCTGCTCAGCCTGAACGC

      5140      5150      5160      5170      5180      5190      5200      5210      5220
CTGACCCGAGCCTTGCCGTATGTATTCCCTGCTCCGTCCCGCCCTGTTCTGCATGGATGCCGAGCGCGCCCATGGCGCCGGCCTGCGCGC
                  ORF4  M  Y  S  L  L  R  P  A  L  F  C  M  D  A  E  R  A  H  G  A  G  L  R  A
```


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RESULTS

Isolation of L-AAA Non-Producing Mutants—To search for *pcd* in the *F. lutescens* IFO3084 genome, mutant strains incapable of producing L-AAA were isolated by NTG mutagenesis. We hypothesized that *F. lutescens* IFO3084 with PCD activity would precipitate eosin Y when the pH of the media had fallen, resulting in red colony formation. Although most colonies of strains treated with NTG were red on the MEM-agar plates, some white colonies, thought to be L-AAA non-producing mutants, were obtained. As a result of TLC analysis of approximately 1,000 white colonies, six L-AAA non-producing mutants were obtained. Four of the six non-producing mutants showed little LAT activity, indicating a mutation in *lat*. The other two non-producing mutants had the same level of LAT activity as the parent strain, suggesting that they had mutations in *pcd*. One of the possible *pcd* mutants was named HGN1, and the mutation in this strain was named *pcd1* (Fig. 1).

Cloning of a DNA Fragment Containing the Gene Complements of the pcd1 Mutation—To clone the gene complements of *pcd1*, a gene library was constructed and introduced into a *pcd* mutant, HGN1. Although most of the transformant colonies on the MEM-agar plates were white, some red colonies, in which *pcd1* is considered to be complemented, were obtained. As a result of TLC analysis of approximately 500 red colonies, two L-AAA producing strains were obtained. Restriction analysis revealed that plasmids isolated from these two L-AAA producing strains

```
      5230      5240      5250      5260      5270      5280      5290      5300      5310
CCTGGATCTTGCCTACCGCAGCGGTACGCTGGGGCTGCTGGCCAGCCGGCCAGCACCGCTTCCAACCCGCGCTTTCGGCCTGGAATTCCC
 L  D  L  A  Y  R  S  G  T  L  G  L  L  A  S  R  P  A  P  L  P  T  R  A  F  G  L  E  F  P

      5320      5330      5340      5350      5360      5370      5380      5390      5400
CAACCCGGTGGGCCTGGCGGCCGGCCTGGACAAGAACGGCGAGCATATCGATGCACTGTTCGCGCTGGGCTTTGGCTATGTCGAAATCGG
 N  P  V  G  L  A  A  G  L  D  K  N  G  E  H  I  D  A  L  F  A  L  G  F  G  Y  V  E  I  G

      5410      5420      5430      5440      5450      5460      5470      5480      5490
CACGGTGACCCCGCGCCCGCAGGCCGGCAATCCGCAGCCACGGCTGTTCCGCGTGCCCGAGCACCTGGGCGTGATCAACCGCATGGGTTT
 T  V  T  P  R  P  Q  A  G  N  P  Q  P  R  L  F  R  V  P  E  H  L  G  V  I  N  R  M  G  F

      5500      5510      5520      5530      5540      5550      5560      5570      5580
CAACAATGCCGGCGTCGATGCGCTGGTGGCCAATGTGCGCGCGGCACGGCGTGACCGCGGCATCCTCGGCATCAACATCGGCAAGAACAA
 N  N  A  G  V  D  A  L  V  A  N  V  R  A  A  R  R  D  R  G  I  L  G  I  N  I  G  K  N  K

      5590      5600      5610      5620      5630      5640      5650      5660      5670
GGACACCCCCAACGAGCTGGCCCATACCGATTACCTGACCTGCCTGGAAAAGGTGTACGCGCTGGCCGACTACATCACCGTCAACATCTC
 D  T  P  N  E  L  A  H  T  D  Y  L  T  C  L  E  K  V  Y  A  L  A  D  Y  I  T  V  N  I  S

      5680      5690      5700      5710      5720      5730      5740      5750      5760
CTCGCCCAACACCGCCGGGCTGCGCGAGCTGCAGGAAGAACAGGCCCTGCGCGAGCTGGTCAGCCGCCTGCGCGAGGGCCAGGAAACCCT
 S  P  N  T  A  G  L  R  E  L  Q  E  E  Q  A  L  R  E  L  V  S  R  L  R  E  G  Q  E  T  L

      5770      5780      5790      5800      5810      5820      5830      5840      5850
GGCCGCACGCCATGGCAAGCGGGTGCCGATGCTGGTCAAGGTCGCGCCGGACCTGAGCGATGCCGATGTCGATGCCGCCGCCCGTGTGCT
 A  A  R  H  G  K  R  V  P  M  L  V  K  V  A  P  D  L  S  D  A  D  V  D  A  A  A  R  V  L

      5860      5870      5880      5890      5900      5910      5920      5930      5940
GGCAGAGCTGCAGGTGGACGGGGTGATCGCCACCAACACCACCATCGCGCGCGTGGGCATGGAAAACCACCCACTGGCCAGCGAGGCCGG
 A  E  L  Q  V  D  G  V  I  A  T  N  T  T  I  A  R  V  G  M  E  N  H  P  L  A  S  E  A  G

      5950      5960      5970      5980      5990      6000      6010      6020      6030
CGGCCTGTCCGGGGCACCGGTGATGGCGCGCTCCACCGCGGTGCTGCGCCGCCTGCGCACCCGGCTGCCGGAGTCGATCCCGCTGATCGG
 G  L  S  G  A  P  V  M  A  R  S  T  A  V  L  R  R  L  R  T  R  L  P  E  S  I  P  L  I  G

      6040      6050      6060      6070      6080      6090      6100      6110      6120
CGTCGGCGGCATCTGTTCCGGGGCTGATGCGGCGGCCAAGATGAGTGCCGGCGCGACCATGGTGCAGCTCTACAGCGGGCTGGTTTACCG
 V  G  G  I  C  S  G  A  D  A  A  A  K  M  S  A  G  A  T  M  V  Q  L  Y  S  G  L  V  Y  R

      6130      6140      6150      6160      6170      6180      6190      6200      6210
CGGCCCGGCACTGGTCGGCGAATGCGTCGAATCGATCCGCCGCCGGCGCGAAGCGCCCTCCAGCGGGGTAGCCCATCTGTGAGTACGCCG
 G  P  A  L  V  G  E  C  V  E  S  I  R  R  R  R  E  A  P  S  S  G  V  A  H  L  *

      6220      6230      6240      6250      6260      6270      6280      6290      6300
GGCTGGCAGCTGCACCACGATGTCGCACTGCAATCAATGAACACCTTCGGGGTAGCGGCCACCGCGCCGCGCCTGCTGCGCGTGCACGAC

      6310      6320      6330      6340      6350      6360
AGCCAGGCCCTGCCGGCGGCGCTGGCGCACCCGGAAGTAGCCGGACAGCCGTTGATC
```

Fig. 2. **Nucleotide sequence of *pcd* and its deduced amino acid sequence.** A potential ribosome binding site (RBS) and –35, –10 promoter sequences (–35 and –10) of *pcd* are double underlined and underlined, respectively. *Not*I sites are boxed. The DNA sequence in this region has been deposited in GenBank (accession no. AB042983).

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```
PCD F.lutescens       1  [illegible]  80
ADH C.elegans         1  [illegible]  85
PCD S.clavuligerus    1  [illegible]  80

PCD F.lutescens      81  [illegible]  170
ADH C.elegans        86  [illegible]  175
PCD S.clavuligerus   81  [illegible]  170

PCD F.lutescens     171  [illegible]  259
ADH C.elegans       176  [illegible]  264
PCD S.clavuligerus  171  [illegible]  259

PCD F.lutescens     260  [illegible]  349
ADH C.elegans       265  [illegible]  354
PCD S.clavuligerus  260  [illegible]  347

PCD F.lutescens     350  [illegible]  431
ADH C.elegans       355  [illegible]  436
PCD S.clavuligerus  348  [illegible]  436

PCD F.lutescens     432  [illegible]  501
ADH C.elegans       437  [illegible]  506
PCD S.clavuligerus  437  [illegible]  496

PCD F.lutescens     502  [illegible]DL  510
ADH C.elegans       507  [illegible]E-  514
PCD S.clavuligerus  496  ---------  496
```

Fig. 3. **Comparison of the deduced amino acid sequence of PCD from *F. lutescens* IFO3084 with other aldehyde dehydrogenases.** Alignment of the deduced amino acid sequence of PCD from *F. lutescens* IFO3084 (PCD F.lutescens) with a hypothetical aldehyde dehydrogenase from *C. elegans* (ADH C.elegans) and PCD from *S. clavuligerus* (PCD S.clavuligerus). The solid and broken bars indicate the probable NADH binding motif (TGSTQVGR) and the aldehyde dehydrogenases glutamic acid active motif (LELGGNNA), respectively.

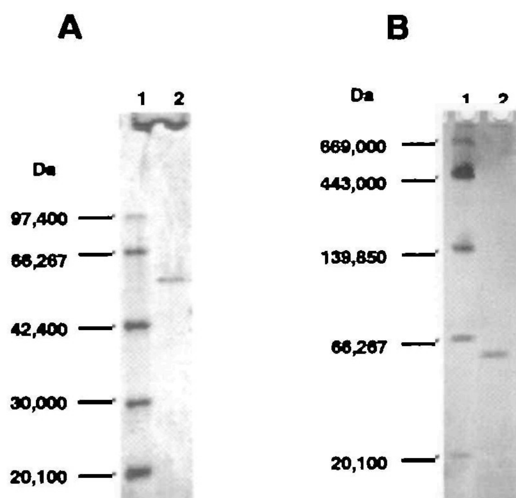

Fig. 4. **Estimation of molecular mass.** (A) Native PAGE of recombinant PCD. (B) SDS-PAGE of recombinant PCD. Lanes: 1, molecular mass standards; 2, recombinant PCD.

contained the same insert DNA. One of the plasmids was named pCF213 (Fig. 1).

Nucleotide Sequencing—The nucleotide sequence of the insert DNA of pCF213 is shown in Fig. 2. We found four potential ORFs in the sequenced region (6,357 bp). Although ORF1 had a deletion in the C-terminal region, it started at ATG (nucleotide position 958) and may end in the upstream unknown region of the insert DNA. Using the BLAST computer algorithm, the deduced amino acid sequence of the protein encoded by ORF1 showed strong similarity to the aspartate kinase-homoserine dehydrogenase from *Arabidopsis thaliana* (39% identity in 296 amino acids). ORF2 started at ATG (position 2315) and ended at TGA (position 1422). The protein encoded by ORF2 showed strong similarity to a hypothetical 35.5 kDa protein from *E. coli* (32% identity in 301 amino acids). ORF3 started at ATG (position 2855), ended at TAA (position 4387), and was proceeded by a possible ribosome binding site (positions 2846 to 2849) and –35, –10 promoter sequence (positions 2805 to 2810 and 2829 to 2835). This ORF encoded a protein of 510 amino acids with a deduced molecular weight of 54,400 and showed strong similarity to a hypothetical aldehyde dehydrogenase from *C. elegans* (51% identity in 508 amino acids) and PCD from *S. clavuligerus* (48% identity in 485 amino acids) (Fig. 3). The Prosite program revealed that the sequence TGSTQVGR (amino acids 241 to 248) corresponds to the NADH binding motif and the sequence LELGGNNA (amino acids 263 to 270) corresponds to the aldehyde dehydrogenase glutamic acid active motif. Furthermore, pCF235, containing ORF3 as a unique complete ORF, complemented *pcd1*. Thus ORF3 was thought to be a structural gene (*pcd*) for PCD activity. ORF4 started at ATG (position 5150) and ended at TGA (position 6202). The protein encoded by ORF4 showed a strong similarity to dihydroorotate dehydrogenase from *E. coli* (51% identity in 335 amino acids).

TABLE I. **L-AAA production analysis *in vitro*.**

Enzyme added		Concentration (μg/ml)	
LAT	PCD	L-Lysine	L-AAA
+	+	1,829	57
+	−	2,943	N.D.
−	+	3,597	N.D.
−	−	4,183	N.D.

N.D., not detected.

Expression of pcd in E. coli and L-AAA Production Analysis In Vitro—To determine whether ORF3 codes for a single subunit with PCD activity, the gene was expressed in *E. coli* and tested for PCD activity. The entire ORF3 was amplified and ligated into pTrcHisA to construct a recombinant PCD expression vector of pTrcPCD. The expressed and purified recombinant PCD (six-His–tagged PCD) gave

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a single band on SDS-PAGE corresponding to a molecular mass of about 58,000 Da (Fig. 4A), which is in good agreement with that (58,500 Da) estimated from the deduced amino acid sequence. Using recombinant PCD and recombinant LAT (*5*), L-AAA production analysis *in vitro* was performed. L-AAA was detected only in the presence of both the recombinant LAT and the recombinant PCD (Table I). Since LAT catalyzes the conversion of L-lysine to P6C (*4, 5*), it is obvious that the recombinant PCD catalyzed the conversion of P6C to L-AAA. Taken together, it is concluded that ORF3 is a structural gene for PCD activity and ORF3 is identified as *pcd*.

Molecular Mass of PCD—The molecular mass of the recombinant PCD was estimated to be about 58,000 Da by native PAGE (Fig. 4B), which is in good agreement with the value obtained by SDS-PAGE. These results indicate that the enzyme is active as a monomer.

DISCUSSION

Here we cloned and sequenced the *pcd* gene from a gram-negative bacterium, *F. lutescens* IFO3084, encoding PCD, and demonstrated that L-AAA is synthesized from L-lysine in two steps catalyzed by LAT and PCD. The deduced amino acid sequence revealed that PCD from *F. lutescens* IFO3084, as well as LAT, shows strong similarity to that from *S. clavuligerus*. In addition we showed that PCD from *F. lutescens* IFO3084 is active as a monomer, as is the case for PCD from *S. clavuligerus* (*6*).

In actinomycetes, PCD activity was found only in β-lactam antibiotics producers, not in non-producers (*6*), suggesting that this enzyme is involved in secondary metabolism, *i.e.* β-lactam antibiotics biosynthesis. In fact, the *pcd* gene from *S. clavuligerus* was located in the β-lactam antibiotics gene cluster (*7*). In the present study, we cloned and sequenced four ORFs, including *pcd* from *F. lutescens* IFO3084. The protein encoded by ORF1 showed strong similarity to aspartate kinase–homoserine dehydrogenase, which is known to be a key enzyme in lysine biosynthesis (*10*). The protein encoded by ORF4 showed strong similarity to dihydroorotate dehydrogenase, which is known to be related to pyrimidine biosynthesis (*11*). This suggests that *pcd* in *F. lutescens* IFO3084, as ORF1 and ORF4, may be involved in primary metabolism.

L-AAA is a rare amino acid and has been widely used as a precursor for various beneficial chemicals including β-lactam antibiotics. We have been manufacturing L-AAA by the bioconversion technique using *F. lutescens* IFO3084. It is noteworthy that the bioconversion of L-lysine to L-AAA, unlike chemical synthesis, preserves chirality. Therefore, it is highly useful to establish genetically-engineered *F. lutescens* IFO3084 or recombinant *E. coli* using *lat* and *pcd*, which would dramatically improve the L-AAA manufacturing efficiency. On the basis of these ideas, we are currently constructing L-AAA producing microorganisms and will realize a high productivity of L-AAA in the near future.

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