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The *pcd* Gene Encoding Piperideine-6-Carboxylate Dehydrogenase Involved in Biosynthesis of α-Aminoadipic Acid Is Located in the Cephamycin Cluster of *Streptomyces clavuligerus*

FRANCISCO J. PÉREZ-LLARENA,[1] ANTONIO RODRÍGUEZ-GARCÍA,[1] FRANCISCO J. ENGUITA,[1] JUAN F. MARTÍN,[1,2] AND PALOMA LIRAS[1,2]*

Area of Microbiology, Faculty of Biology, University of León, 24071 León,[1] and Institute of Biotechnology INBIOTEC, Scientific Park of León, 24006 León,[2] Spain

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Three open reading frames (ORFs) have been located downstream of *cefE* in the cephamycin C gene cluster of *Streptomyces clavuligerus*. ORF13 (*pcd*) encodes a 496-amino-acid protein (molecular weight [MW], 52,488) with an N-terminal amino acid sequence identical to that of pure piperideine-6-carboxylate dehydrogenase. ORF14 (*cmcT*) encodes a 523-amino-acid protein (MW, 54,232) analogous to *Streptomyces* proteins for efflux and resistance to antibiotics. ORF15 (*pbp74*) encodes a high molecular weight penicillin-binding protein (MW, 74,094).

Cephamycins are derived from the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine. In bacteria, L-α-aminoadipic acid is formed by deamination of lysine by the lysine-6-aminotransferase that converts lysine into piperideine-6-carboxylic acid (P6C). The activity of a second enzyme, P6C dehydrogenase, has recently been shown to be required for conversion of P6C into L-α-aminoadipic acid (6). The genes for biosynthesis of antibiotics are frequently clustered with antibiotic resistance genes and sometimes with genes involved in precursor biosynthesis (12). The *lat* gene encoding lysine-6-aminotransferase is located in the cephamycin cluster of *Streptomyces clavuligerus* and *Nocardia lactamdurans* (2, 11). The purpose of this study was to investigate whether the *pcd* gene required for the second step in the formation of α-aminoadipic acid was clustered together with *lat* and other genes involved in cephamycin formation.

Cloning of the region downstream from *cefE* in the *S. clavuligerus* cephamycin cluster. A genomic library of *S. clavuligerus* DNA in phage λGEM12 (15) was hybridized with plasmid pULF33 (inserting a 3.3-kb *Bgl*II-*Bam*HI DNA fragment containing *cefE* and part of the *cefD* gene of *S. clavuligerus*). Nine phages gave positive hybridization, and one of them, λGEM-C9, contained the unknown region downstream of *cefE* (Fig. 1). Three *Bam*HI DNA fragments from phage λGEM-C9 of 4.0, 3.2, and 1.7 kb were subcloned in pBSSK(+) to give plasmids pULF17, pULFP32, and pULF40. A total of 7.1 kb downstream from *cefE* was sequenced with the AutoRead Sequencing System (Pharmacia). Analysis of the nucleotide sequence of this 7.1-kb fragment (EMBL/GenBank/DDBJ accession no. AJ001743) with the Geneplot program (DNAstar) identified four open reading frames (ORFs). Three of them are in the same orientation as *cefE* (ORF13, ORF14, and ORF15), and ORF16, in the opposite orientation, corresponds to the *bla* gene (15).

ORF13 encodes the P6C-DH of *S. clavuligerus*. The predicted start codon of 1,488-bp ORF13 is only 15 bp downstream from the stop codon of *cefE*, and ORF13 encodes a protein of 496 amino acids with a deduced M_r of 52,488. A search of the Swissprot data base showed a high similarity between the protein encoded by ORF13 and the aldehyde dehydrogenases (Fig. 2). The identity among amino acids was maximal in the region between amino acids 33 and 425. The highest similarity (48% identity in 389 amino acids) is found with an aldehyde dehydrogenase from *Cenorhabditis elegans* and with the betaine dehydrogenase responsible for turgor in *Pisum sativum* (45% identity in 392 amino acids). Because of its similarity with other dehydrogenases, the sequence TGSTRMGR (amino acids 239 to 246 in ORF13) corresponds to the NADH binding motif as calculated with the Prosite program. The protein has lower homology with 1-pyrroline-5-carboxylate dehydrogenase from *Bacillus subtilis* (29.3% identity) (8), an enzyme of proline catabolism that converts 1-pyrroline-5-carboxylic acid into glutamic acid.

The piperideine-6-carboxylate dehydrogenase (P6C-DH), which converts 1-piperideine-6-carboxylic acid into α-aminoadipic acid, a precursor of cephamycin C, has recently been purified (6). This enzyme has an M_r of 56,200 as determined by gel filtration, which is similar to the deduced molecular weight (MW) of the protein encoded by ORF13. In order to test whether the protein encoded by ORF13 was the P6C-DH, we sequenced the N-terminal end of the pure protein with an Applied Biosystems protein sequencer (model 476A). The sequence found was VTAAISGTDEI(K/L)RARA, which matches perfectly the sequence of the protein encoded by ORF13 from amino acids 2 to 16, indicating that ORF13 encodes the P6C-DH in which the starting methionine residue is removed in vivo. Therefore, the gene was named *pcd* (for piperideine carboxylate dehydrogenase).

Hybridization of DNA from different *Streptomyces* species with a 1.3-kb *Bam*HI-*Kpn*I DNA fragment internal to *pcd* showed a positive hybridization signal with the cephamycin C producers *Streptomyces jumonjinensis* NRRL 5741, *Streptomyces lipmanii* NRRL 3584, and *Streptomyces cattleya* NRRL 8057 and with the *Streptomyces griseus* NRRL 3851 producer of cephamycins A and B, whereas no hybridizing band was found with the DNA of the nonproducer strain *Streptomyces lividans* JI1326, *Streptomyces cacaoi* subsp. *asoensis* ATCC 19094, or *Streptomyces niveus* NRRL 2466.

S. lividans lacks P6C-DH activity (6), and therefore, to confirm *pcd* function, the gene was expressed in *S. lividans* by fusing it to the *saf* promoter. The *pcd* gene was subcloned from plasmid pULF22, a pBSKS(+) derivative with a 2.2-kb insert

* Corresponding author. Mailing address: Area of Microbiology, Faculty of Biology, University of León, 24071 León, Spain. Phone: 34-987-291504. Fax: 34-987-291506. E-mail: degplp@unileon.es.

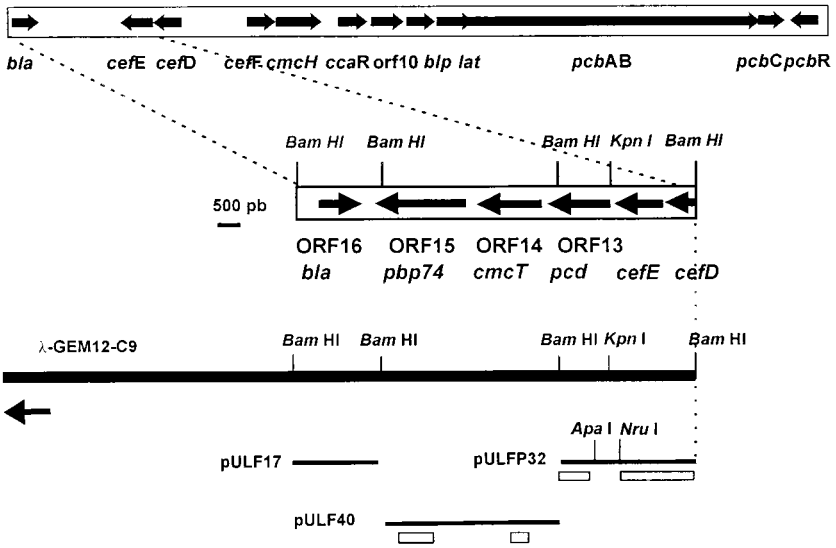

FIG. 1. Physical map of the DNA of *S. clavuligerus* encoding the cephamycin C gene cluster. A fragment of the phage λGEM-C9 DNA studied is indicated by a solid bar. The *S. clavuligerus* insert in the phage DNA follows in the direction of the arrows. DNA fragments in different plasmids used are indicated by thin lines, and the probes used for hybridization are shown as stippled bars.

containing *pcd*, as an *Ecl*36II-*Hin*dIII fragment extending from 266 bp upstream of the ORF to 490 bp downstream of the TGA termination codon. Plasmid pULS699, which contains a 300-bp insert with the *saf* promoter (4) in pBSKS(+), was digested with *Sma*I and *Spe*I, the cohesive ends were filled in, and both constructions were ligated to give pULSpcd, in which the *pcd* gene is expressed from the *saf* promoter. Cultures of *S. lividans*(pULSpcd) and control *S. lividans*(pULS699) (lacking the *pcd* insert) were grown in YEME medium (0.3% yeast extract, 0.3% malt extract), and cells were harvested at 36 h. *S. clavuligerus* NRRL 3585 was grown under the same conditions. P6C-DH activity was measured in the cell extracts by the coupled radioactive method (6). The assay mixture was then analyzed by thin-layer chromatography, and the labelled compounds formed were detected by autoradiography.

Reaction samples with *S. clavuligerus* NRRL 3585 extracts from 36-h-old cells showed the formation of a compound (Fig. 3, lane 5) which was not found in the absence of either lysine dehydrogenase from *Agrobacterium tumefaciens* (forming P6C) (lane 6) or cell extracts of *S. clavuligerus* (lane 7). This compound with an R_f of 0.44 is derived from lysine and corresponds to α-aminoadipic acid. When control *S. lividans*(pULS699) extracts were used, no α-aminoadipic acid was formed (lanes 3

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PLTGADLFGLRAHTPEDVDRAVEAAHTAFLTWRTTPAPVRGALVKRFGELLTEHKQDLAD   98
PANNSPIANVQNGNVQDYEIAISEAKKAYNDWCEVPAPRRGEIVRQIGDKLRTQLQNLGK  103
PSTNQVIASVTEATLDDYEEGLRASSEAAKTWRTVPAPKRGEIVRQIGDALRAKLDPLGR  100
EIIG---YA-STADQELAEKAMQAALQAFDSWKKQRPEHRANILFKAAAILRRRKHEFSS  117

LVTIEAGKIRSEALGEVQEMIDICDFAVGLSRQLYGRTMPSERPGHRLMETWHPLGVVGV  158
LVSLEMGKISAEGVGEVQEYVDICDYATGLSRSLEGKIFPSERPGHALLEQWNPLGVVGV  163
LVALEMGKILAEGIGEVQEIIDMCDYSVGLSRQLNGSIIPSERPEHMMFEVWNPLGIVGV  160
YLVKEAGKPWKEADADTAEAIDFLEFYARQMLKLKEGAPVKSRAGEVNQYHYEALGVGIV  177

ISAFNFPVAVWAWNAAVALVCGDTVVWKPSELTPLNRAACAALLDLAIADAGAPKG-LNQ  217
ISAFNFPCAVYGWNNALALVTGNSVVWKPAPSTPLTAIAVTKLVEEVLVANNVNPA-LCS  222
ITAFNFPCAVLGWNACIALVGGNTVVWKGAPTTPLITVAVTKLIAEVFERNNLPGA-IFT  219
ISPFNFPLAIMAGTAVAAIVTGNTILLKPADAAPV---VAAKFVEV-MEEAGLPNGVLNY  233

VVVGAADVGERLVDSPRVPLVSATGSTRMG-----RAVGPRVAAR-FGRTILELGGNNAA  271
LVCGEGDVGQALVKDKRVNLVSFTGSSEIG-----KIVGQQVQAR-FGKLLLELGGNNAI  276
ALCGGADIGHAIAKDTRIPLVSFTGSSKVG-----ALVQQTVSQR-FGKTLLELSGNNAI  262
IPGDGAEIGDFLVEHPKTRFVSFTGSRAVGCRIYERAAKVQPGQKWLKRVIAEMGGKDTV  293

VVTPSADLDLTVNAAVFAAAGTAGQRCTTLRRLIVHEDIADTVVERLTAAFERLP--IGD  329
IVNEDADLNMVVPATVFAAVGTAGQRCTTTRRLIVHDKVYDQVLERLKKAYAQFESRIGC  336
IVMDDADITLAVRSIFFAAVGTAGQRCTTCRRLYLHESVYANVLEQLTALYKQVK--IGN  333
LVDKDADLDLAASSIVYSAFGYSGQKCSAGSRAVIHQDVYDEVVEKAVALTKTLT--VGN  351

PFQDTTLVGPLVNEAAFGRMREAVERATAEGGTLCAGGERQFPDAAPGAYYVRPA-LVR-  388
PLDSNTIIGPLHNQQAVGKYKASVAEAVASGGKIEYGG-KVL-E-RDG-NFVLPT-IVTG  391
PLEEGTLVGPLHTRSAVENFKNGISAIKSQGGKIVTGGS-VL-E-SEGNFVV-PT-IVE-  386
PEDPDTYMGPVIHEASYNKVMKYIEIGKSEGKLL-AGGE----GDDSKGYFIQPTIFADV  406

MPAQTAVVREETFAPILYVLTYRDLDEAIRLNNEVPQ 425  P6C-DH S. clavuligerus
LKHDSPVVLRETFAPILYVLKFSTLEEAIAINNEVDQ 428  ADH C. elegans     48.0%
ISADAAVVKEELFAPVLYVMKFKDLEEAIALNNSVPQ 423  ADH P. sativum     45.0%
DENARLMQEEIFGPVVAICKARDFDHMLEIANNTEYG 443  P5C-DH B. subtilis 29.3%
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FIG. 2. Deduced amino acids sequence of the piperideine-6-carboxylate dehydrogenase encoded by the *pcd* gene. Comparison with aldehyde dehydrogenases from *C. elegans* and *P. sativum* and with the pyrroline-5-carboxylate dehydrogenase from *B. subtilis* is shown. Amino acids conserved in at least three of the sequences are indicated by gray shading. A bar indicates the putative NADH binding motif.

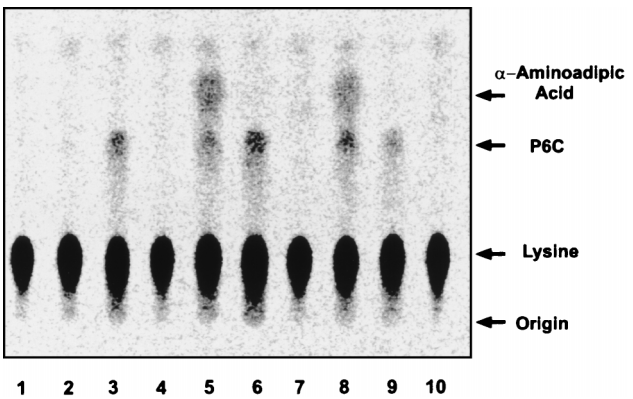

FIG. 3. Autoradiography of the products (separated by thin-layer chromatography) formed from ^{14}C-lysine by the coupled lysine-ε-dehydrogenase-piperideine-6-carboxylate dehydrogenase assay. Lanes: 1, ^{14}C-lysine (0.5 μCi); 2, ^{14}C-lysine (0.5 μCi) and assay cofactors lacking cell extracts; 3, complete reaction with *S. lividans*(pIJ699) extracts; 4, as in lane 3 but without lysine-ε-dehydrogenase; 5, complete reaction with *S. clavuligerus* NRRL 3585 cell extracts; 6, as in lane 5 but without cell extract; 7, as in lane 5 but without lysine-ε-dehydrogenase; 8, complete reaction with *S. lividans*(pULSpcd) cell extract; 9, as in lane 8 but without cell extract; 10, as in lane 8 but without lysine-ε-dehydrogenase.

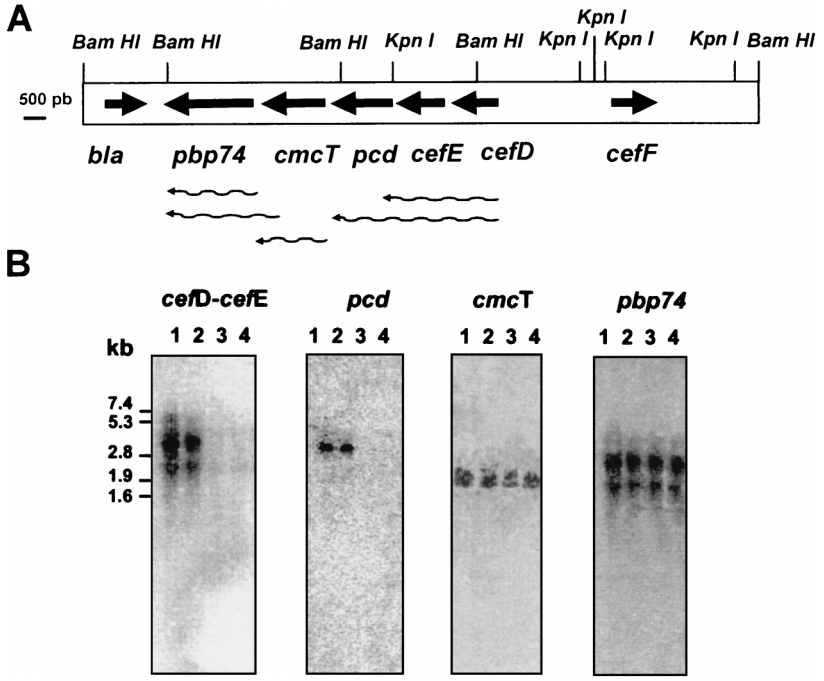

FIG. 4. (A) Physical map of a 15.1-kb *Bam*HI DNA fragment of *S. clavuligerus*. The putative transcription of the genes is indicated by wavy arrows. The DNA fragments used as probes are indicated by stippled bars in Fig. 1. (B) Hybridization of total RNA of *S. clavuligerus* NRRL 3585 grown in Trypticase soy broth medium for 24, 48, 72, and 96 h as shown in lanes 1, 2, 3, and 4, respectively. The markers correspond to the RNA type II of Boehringer-Mannheim.

and 4). Cell extracts from *S. lividans*(pULSpcd), in which the *pcd* gene is expressed, formed a labelled compound with an R_f of 0.44 that gave a positive reaction with ninhydrin. This compound cochromatographed with unlabelled α-aminoadipic acid when it was added to the assay after the reaction was stopped.

ORF14 encodes a protein with 14 transmembrane domains. ORF14 is 1,569 bp long and is predicted to start with a GTG codon 69 bp downstream from the TGA codon of *pcd*. The protein encoded by ORF14 has 523 amino acids (M_r, 54,232). Hydrophobicity analysis of the protein encoded by ORF14 with the SOAP program (PCGene) indicated that this protein contains 14 transmembrane segments and shows a high similarity with membrane proteins involved in proton-dependent drug efflux (14). Some of these proteins, as well as transmembrane proteins of the ABC transporter superfamily, confer resistance to antibiotics (5) and are encoded by genes present in antibiotic biosynthesis clusters. The highest identity was found with the CmcT protein of *N. lactamdurans* (3) (60% identity in 486 amino acids) followed by proteins belonging to cluster e of the family of 14 transmembrane segments, such as PurT, for puromycin exclusion in *Streptomyces alboniger* (27% identity in 503 amino acids) and proteins involved in the exportation of tetracenomycin, lincomycin, or methylenomycin.

ORF15 encodes a high-MW PBP with a proline-rich amino-terminal region. ORF15 contains 2,088 bp and is predicted to start with an ATG codon. Downstream from ORF15 there is an inverted sequence (nucleotides 5,856 to 5,870 and 5,884 to 5,899; ΔG_{25} of −38 kcal/mol) that may act as a transcriptional terminator.

ORF15 encodes a 696-amino-acid protein with a deduced MW of 74,094. This protein showed two different regions. Amino acids 1 to 300 showed homology to specific regions of proteins rich in proline such as glycoproteins for cell wall synthesis of *Clostridium thermocellum* (29.7% identical amino acids in 276 residues) (7) or with a protein secreted by *Xenopus laevis* skin (27.7% identity in 277 amino acids) (9). The C-terminal region (amino acids 301 to 696) showed a high similarity with penicillin-binding proteins (PBP) such as the PBP encoded by *dacF* of *B. subtilis* (27% identity) (16), *Escherichia coli* Pbp6 encoded by *dacC* (26% identity), and Pbp5 (25% identity) encoded by *dacA* (1). These proteins are D-alanyl-carboxypeptidases, produced during sporulation of *B. subtilis* or needed for cell wall synthesis in *E. coli*. The conserved motifs present in β-lactamases and the PBPs STAK, SGN, and KTG were found in the second half of the ORF15-encoded protein (amino acids 310 to 696). Hydropathy analysis of the protein with the RAOARGOS program (PCGene) detected the presence of an α-helix transmembrane motif (amino acids 286 to 309; LAMIAIPLAALLLVIAFVAVQLL). This region separates the proline-rich half of the protein and the PBP-like C-terminal half. This gene has been named *pbp74*.

A different gene (*pbpR*) encoding a PBP with a MW of 57,346, which confers partial resistance to benzylpenicillin, has been already located in the *S. clavuligerus* cephamycin C-clavulanic acid gene cluster (13). However, there is no substantial homology between both PBPs outside of the three well-conserved motifs found in all PBPs. The molecular weight of Pbp74 agrees quite well with the PBP with an MW of 79,000 detected by Horikawa et al. (10) in *S. clavuligerus* membranes.

Transcriptional analysis of the *pcd-cmc*T-*pbp74* cluster. RNA from 24-, 48-, 72-, and 96-h cultures of *S. clavuligerus* was isolated from cultures in Trypticase soy broth medium as reported previously (15), separated by electrophoresis, and hybridized with (i) an *Apa*I-*Bam*HI 0.56-kb DNA probe internal to *pcd*; (ii) a 0.4-kb *Sma*I-*Sac*I DNA fragment internal to *cmcT*; (iii) a *Sac*I-*Hin*dIII 1-kb DNA fragment internal to *pbp74*; and (iv) a 1.5-kb *Bam*HI-*Nru*I DNA fragment containing the 3′ end of *cefD* and the nearly complete *cefE* gene. As shown in Fig. 4A, when probes corresponding to the *cefE-cefD* region were used, transcripts of 2.6 and 4.1 kb were observed.

The 2.6-kb mRNA corresponds to the transcription of *cefE-cefD*. The 4.1-kb transcript also contains information from the *pcd* gene, since a probe internal to *pcd* also hybridized with the 4.1-kb transcript. This larger transcript provides a mechanism to coordinate the expression of enzymes for both the early and middle steps of the pathway.

cmcT was transcribed as a single mRNA of 1.9 kb, while the probe corresponding to *pbp74* revealed the presence of two abundant transcripts of 1.9 and 3.1 kb. This result suggests that *pbp74* is transcribed from two promoters, one of which is likely present inside the coding sequence of *cmcT* since the corresponding *pbp74* ORF is 2.0 kb long. The lack of a 3.1-kb hybridizing band when the mRNA was probed with a fragment internal to *cmcT* may be due to the fact that this 0.4-kb probe corresponds to the 5′ region of the gene. The presence of a strong transcription termination signal 37 bp downstream from *pbp74* and the presence of the *bla* gene in the opposite orientation make it less probable that the 3.1-kb transcript corresponds to regions downstream of *pbp74*.

The structural genes *cefE*, *cefD*, and *pcd* were transcribed early in the fermentation (at 24 and 48 h) (Fig. 4B), preceding the phase of intense cephamycin biosynthesis, which is maximal at 60 h of culture. A very small amount of transcript for these three genes (less than 20% as quantified by densitometry performed with an Instant Imager [Packard]) was observed thereafter. This rise and decline in the steady-state levels of transcripts for *cefE*, *cefD*, and *pcd* agrees well with the previously reported profile of cephamycin biosynthesis in *S. clavuligerus* and indicates that the decline in the rate of antibiotic synthesis is due to the decay of the mRNA for the biosynthetic genes. Interestingly, the *cmcT* and *pbp74* genes are transcribed throughout the fermentation (until at least 96 h).

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