Molecular Cloning and Expression in Different Microbes of the DNA Encoding *Pseudomonas putida* U Phenylacetyl-CoA Ligase

USE OF THIS GENE TO IMPROVE THE RATE OF BENZYLPENICILLIN BIOSYNTHESIS IN *PENICILLIUM CHRYSOGENUM* 

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The gene encoding phenylacetyl-CoA ligase (*pcl*), the first enzyme of the pathway involved in the aerobic catabolism of phenylacetic acid in *Pseudomonas putida* U, has been cloned, sequenced, and expressed in two different microbes. In both, the primary structure of the protein was studied, and after genetic manipulation, different recombinant proteins were analyzed. The *pcl* gene, which was isolated from *P. putida* U by mutagenesis with the transposon Tn5, encodes a 48-kDa protein corresponding to the phenylacetyl-CoA ligase previously purified by us (Martínez-Blanco, H., Reglero, A. Rodriguez-Aparicio, L. B., and Luengo, J. M. (1990) *J. Biol. Chem.* 265, 7084–7090). Expression of the *pcl* gene in *Escherichia coli* leads to the appearance of this enzymatic activity, and cloning and expression of a 10.5-kb DNA fragment containing this gene confer this bacterium with the ability to grow in chemically defined medium containing phenylacetic acid as the sole carbon source. The appearance of phenylacetyl-CoA ligase activity in all of the strains of the fungus *Penicillium chrysogenum* transformed with a construction bearing this gene was directly related to a significant increase in the quantities of benzylpenicillin accumulated in the broth (between 1.8- and 2.2-fold higher), indicating that expression of this bacterial gene (*pcl*) helps to increase the pool of a direct biosynthetic precursor, phenylacetyl-CoA. This report describes the sequence of a phenylacetyl-CoA ligase for the first time and provides direct evidence that the expression in *P. chrysogenum* of a heterologous protein (involved in the catabolism of a penicillin precursor) is a useful strategy for improving the biosynthetic machinery of this fungus.

Bacteria of the genus *Pseudomonas* are able to grow in many different chemically defined media containing a wide number of carbon sources (1, 2). This catabolic versatility is specially interesting in strains belonging to the species *Pseudomonas putida*, which can grow in media which are, or may be, toxic for other microbes (2, 3). Some years ago, we showed that *P. putida* U could be cultured in minimal medium containing phenylacetic acid (PA) as the sole carbon source (4) and that the degradation of this compound was carried out through a newly discovered catabolic pathway involving the participation of a phenylacetyl-CoA ligase enzyme, which under aerobic conditions, catalyzes the activation of PA to PA-CoA. This enzyme, which had never been reported to be involved in the catabolism of aromatic compounds, seemed to be functionally similar to a protein that participates in a very different route, the biosynthetic pathway of benzylpenicillin (penicillin G), in several fungi (see Fig. 1) (5–7).

Biochemical and genetic studies carried out with *Penicillium chrysogenum* and other related fungi have allowed the purification and characterization of some biosynthetic enzymes belonging to the penicillin pathway: δ-(L-α-aminoacidipil)-L-cysteylnyl-D-valine synthetase (ACVS), isopenicillin N synthase and acyl-CoA:6-aminopenicillanic acid/isopenicillin N acyltransferase, as well as the isolation of the genes encoding these proteins (8–13) (Fig. 1). It has been reported that neither the number of copies of these genes nor the quantities of these enzymes are rate-limiting for penicillin biosynthesis in the different strains studied (14, 15). We were therefore prompted to characterize the enzyme phenylacetetyl-CoA ligase (PA-CoA ligase, PCL) which activates phenylacetic acid (the side chain precursor of benzylpenicillin) to phenylacetyl-CoA in both low producer and in industrial strains of *P. chrysogenum*. This protein has never been purified, and its gene sequence remains unknown. The absence (or undetectable levels) of PCL activity in cell-free extracts of *P. chrysogenum* suggested that this enzyme activity could be a true limiting step in the biosynthetic pathway of benzylpenicillin. To confirm this hypothesis, we changed our research strategy; we now selected different microbes as a function of their capacity to grow in minimal medium containing PA as the sole carbon source (4). In this way, we identified a bacterial strain (*P. putida* U) which, as indicated above, catalyzes the first step of the PA catabolic pathway by activation of PA to PA-CoA (4–16). This enzyme (PCL) was purified (4), characterized (17), and coupled with isopenicillin N synthase and acyltransferase from *P. chrysogenum*, leading to the synthesis of many different penicillins (18) and showing, for the first time, that reproduction of the last three text attributes.

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$ The abbreviations used are: PA, phenylacetic acid; ACVS, δ-(L-α-aminoacidipil)-L-cysteylnyl-D-valine synthetase; PCL, phenylacetyl-CoA ligase; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s).
FIG. 1. Biosynthetic pathway of penicillins in *P. chrysogenum*. ACV, L-\(\alpha\)-aminoadipoyl-L-cysteinyl-D-valine; \(\alpha\)-AAA, \(\alpha\)-aminoadipic acid; 6-APA, 6-aminopenicillanic acid; PTS, phenylacetic acid transport system; PA-CoA, phenylacetyl-CoA.

FIG. 2. Schematic representation of the *P. putida* DNA fragment containing the *pec* gene (cloned in pUC18) indicating the restriction sites.
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Computer analysis of the sequences obtained were performed with the PCGENE software package (IntelliGenetics, Inc.).

Strains, Vectors, Media, and Culture Conditions—The P. putida strain U used in the experimental work was from our collection. The strain was originally obtained from R. A. Cooper (Department of Biochemistry, University of Leicester, Leicester, UK). P. chrysogenum Ws 54-1255 (ATCC 28069) and Micrococcus luteus ATCC 9341 (used for determination of penicillins by bioassay) (18) were obtained from the American Type Culture Collection. E. coli DH5α, obtained from Life Technologies, Inc., was used for plasmid propagation. E. coli strain NM538 (20) was used for generation and amplification of a P. putida genomic library. E. coli HB101 carrying the plasmid pGS9, which includes the transposon Tn5 (21), was kindly supplied by Dr. J. L. Ramos (Estacion Experimental del Zaidín, CSIC, Granada, Spain). All strains were kept lyophilized.

The plasmid pUC18 was used for subcloning genomic fragments. The replacement vector EMBL4 (22) was used to obtain a P. putida genomic library. The different strains used in the experiments described below were cultured in the media and conditions reported elsewhere (4, 23).

Isolation of Mutants Unable to Catabolize PA—Mutants handicapped in the aerobic degradation of PA (PA strain) were selected by insertion of the transposon Tn5 (21) in some of the genes involved in the catabolism of PA in P. putida. Mutagenic procedures and isolation of the different mutants were carried out as reported earlier (24).

DNA Manipulations—Standard recombinant DNA techniques (25) were routinely used for cloning and analyzing the P. putida pel gene in E. coli DH5α. Genetic engineering in P. chrysogenum Ws 54-1255 was carried out according to the protocols reported by Sánchez et al. (26, 27).

Nucleotide sequence analysis was performed using the method described by Sanger et al. (28).

DNA Amplification—When required, DNA amplification was carried out by the polymerase chain reaction (PCR). In these experiments, the reaction mixture contained DNA primers, 0.4 mM MgCl2, 0.2 mM; 5 μl of DNA; 100 ng of Taq DNA polymerase buffer; 100 ng of Taq DNA polymerase in a final reaction volume of 50 μl. The first cycle of the PCR was carried out as follows: 94°C for 90 s, 53°C for 30 s, raised to 72°C in the next 60 s. The subsequent 34 cycles were performed in a similar way, except that the denaturation time at 94°C was 30 s and the extension time underwent an increment of 3 s/cycle. The reactions were carried out in a Perkin-Elmer Thermocycler.

Purification, Partial Sequencing, and Characterization of the Recombinant PCLs—PCLs obtained from the different recombinant strains were purified from cell-free extracts following the procedure previously reported (4). Enzymatic activity was assayed either in cell-free extracts or in purified preparations by HPLC (4, 16) or spectrophotometrically (29).

PCLs purified to homogeneity were hydrolyzed in twice-distilled HCl (6 M) at 110°C for 24, 48, and 72 h in evacuated sealed tubes. Hydrolysis was analyzed on a Durrum D-500 amino acid analyzer. In some experiments, PCLs (250–500 μg) were digested with trypsin (5–10 μg), and the peptides released were purified by HPLC (30) and sequenced using an Applied Biosystems model 473A protein sequencer.

Isoelectric gel focusing was carried out as indicated elsewhere (31). The presence of metal ions in PCL was evaluated by Graphite Furnace Atomic Absorption Spectrophotometry using a Thermo Jarrel SH/11. Substrate specificity was analyzed by the native protein or in purified preparations by HPLC (4, 16) or spectrophotometrically (29).

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Results and Discussion

Isolation and Characterization of the Gene Encoding PCL in P. putida—Study of the pel gene in P. putida U was approached by selecting mutants unable to assimilate PA as the sole source of carbon. These strains were selected using the transposon Tn5 mutagenesis procedure (see “Experimental Procedures”). These random mutants isolated were cultured in minimal medium containing as carbon sources 4-hydroxyphenylacetic acid (which does not induce PCL but does support bacterial growth) and PA (which, although it cannot be catabolized, does serve as

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an inducer) (17). Analysis of these mutants revealed that they can be included in two different groups: (i) those lacking PCL activity (PCL\(^-\)) and (ii) those containing functional PCL (PCL\(^+\)).

In one of these PCL\(^-\) mutants, the site of insertion of the transposon was located using a labeled oligonucleotidic probe identical to the repeated sequence present at the end of Tn5 (5' → 3': ACCTTGTTGATAAGACTCAG). The DNA fragment linked to the transposon was cloned in the plasmid pUC18, and this construction was used to transform E. coli DH5\(\alpha\) using well known procedures (25–29). Later, the genomic insert (0.5 kb) cloned in pUC18 was labeled with \(^{32}\)P and used for screening a P. putida gene library constructed in phage \(\alpha\)EMBL 4 (22). The DNA of one of the phages showing positive hybridization was digested with EcoRI, and a 1.0-kb fragment (Fig. 2) was cloned in pUC18. This plasmid was later used to transform E. coli DH5\(\alpha\)'s. Expression of this piece of DNA confers E. coli with the capacity to grow in minimal medium + PA, whereas E. coli DH5\(\alpha\)'s and the same strain containing pUC18 without the 10.5-kb insert were unable to grow in this minimal medium. These data unequivocally show that the genetic information required for the aerobic catabolism of PA is included in the 10.5-kb fragment. Furthermore, as expected, this fragment contained the pcl gene, since a high degree of PCL activity was detected in the cell-free extracts of E. coli DH5\(\alpha\) transformed with pUC18 containing the 10.5-kb insert (8 μg of PA-CoA/ ml/mg of protein). However, it was not detected in the same bacterium bearing the pUC18 without the insert.

Restriction analysis of this 10.5-kb fragment allowed us to isolate an 8-kb EcoRI generating a 4-kDa protein (representing 25% of the total bacterial protein) showing PCL activity. However, when the same fragment was cloned in pUC19, PCL activity was not detected in cell-free extracts, suggesting that either (i) E. coli DH5\(\alpha\)'s does not recognize the promoter of P. putida, or (ii) no promoter exists in this piece of DNA. In any case, these data do indicate that expression of the pcl gene is under the control of the \(\beta\)-galactosidase promoter present in pUC18.

The sequences of the gene (pcl) and protein (PCL) are shown in Fig. 3. In this sequence we found (i) the same NH\(_2\)-terminus reported for the PCL purified from P. putida U (MMYH- DADRI), (ii) different peptides obtained by trypptic digestion of the protein (underlined sequences), and (iii) an AMP-binding site consensus sequence (SSGGTGGKP) (35) (doubly underlined) very similar to those reported in other acyl-CoA-activating enzymes (see Table I) (36–63). However, whereas in all of the other cases the COOH-terminal end of this consensus core is always PKG, in PCL the COOH-terminal end is PTV, suggesting that at least in this protein the lysine involved in the phospho-loop binding site must be a different lysine (64). This was the only similarity found when PCL was compared with other acyl-CoA-activating enzymes.

**Analysis of the Recombinant PCL Protein**—In order to isolate a shorter piece of DNA containing the pcl gene, the construction indicated as pLSTu (Fig. 2) was digested by using an Erase-a-base system (Promega). Analysis of the different clones obtained revealed that whereas (i) in some cases the NH\(_2\) terminus sequence of the native protein (see Fig. 3 and bal116, Table II) could be elongated, the original NH\(_2\) terminus remained constant (see bal112, MTMTNNSNSSEAMMN... or elongated and modified (see bal101, MTMTNNSSDA... and bal142, MTMTN5RYH...), with no loss of activity (in all these cases functional PCLs were obtained), in one case (ii) elongation of the amino-terminal sequence involved a loss of the function. Thus, in bal110 the presence of an internal extra sequence (WRAAYKN), which does not exist in bal112, caused a loss of PCL activity. This result is particularly interesting.

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**TABLE I**

| Enzyme | Position* | Sequence | Reference |
|--------|-----------|----------|----------|
| Phenylacetyl CoA ligase from Pseudomonas putida U | 94 | rha SSSTGTKPTV | This paper |
| Acetyl-CoA synthetase from Emericella nidulans (Aspergillus nidulans) | 278 | fliy TSSGTGKPFG | vmlt 36 |
| Acetyl-CoA synthetase from Neurospora crassa | 239 | fliy TSSGTGKPFG | vhs 36 |
| Acetyl-CoA synthetase from Penicillium chrysogenum | 278 | fliy TSSGTGKPFG | vmlt 37 |
| Acetyl-CoA synthetase from Phanerochaete chrysosporium | 275 | fliy TSSGTGKPFG | liht 38 |
| Acetyl-CoA synthetase from Methanobrevibacter smithii | 258 | yily TSSGTGKPFG | leha 39 |
| Acetyl-CoA synthetase from Saccharomyces cerevisiae | 316 | fliy TSSGTGKPFG | vqhs 40 |
| Acetyl-CoA synthetase from Escherichia coli | 260 | fliy TSSGTGKPFG | vnlh 41 |
| Acetyl-CoA synthetase from Alcaligenes eutrophus | 286 | slyc TSSGTGKPFG | amvt 42 |
| Acetyl-CoA synthetase from Phanerochaete chrysosporium | 230 | fliy TSSGTGKPFG | vnh 43 |
| Acetyl-CoA synthetase from Bacillus subtilis | 210 | lhly TSSGTGKPFG | vnh 44 |
| Medium chain fatty acid CoA ligase from Pseudomonas oleovorans | 181 | slcy TSSGTGKPFG | vlys 45 |
| Long chain fatty acid CoA ligase from Pseudomonas oleovorans | 266 | cimy TSSGTGKPFG | vnlk 46 |
| Long chain fatty acid CoA ligase 2 from S. cerevisiae | 296 | tisf TSSGTGKPFG | vems 47 |
| Long chain fatty acid CoA ligase 3 from S. cerevisiae | 266 | cimy TSSSISAKPFG | vnlk 47 |
| Long chain fatty acid CoA ligase from Rattus norvegicus (liver isoenzyme) | 273 | llicf TSSGTGKPFG | amvt 48 |
| Long chain fatty acid CoA ligase from R. norvegicus (brain isoenzyme) | 272 | lvcf TSSGTGKPFG | amlt 49 |
| Long chain fatty acid CoA ligase from Mus musculus | 273 | llicf TSSGTGKPFG | amlt 50 |
| Long chain fatty acid CoA ligase 1 from Homo sapiens | 272 | vicf TSSGTGKPFG | amvt 51 |
| Long chain fatty acid CoA ligase 2 from H. sapiens | 272 | vicf TSSGTGKPFG | amvt 52 |
| Long chain fatty acid CoA ligase from E. coli | 210 | flsq TSSGTGKPFG | vnlk 53 |
| Crotonobetaine/carnitine CoA ligase from E. coli | 182 | elfi TSSGTGKPFG | vnlk 54 |
| 4-Coumarate CoA ligase from Oryza sativa | 205 | alpy TSSGTGKPFG | vnlk 55 |
| 4-Coumarate CoA ligase 1 from Petroselinum crispum | 186 | alpy TSSGTGKPFG | vnlk 56 |
| 4-Coumarate CoA ligase 2 from P. crispum | 186 | alpy TSSGTGKPFG | vnlk 56 |
| 4-Coumarate CoA ligase 1 from Solanum tuberosum | 188 | alpy TSSGTGKPFG | vnlk 57 |
| 4-Coumarate CoA ligase 2 from S. tuberosum | 188 | alpy TSSGTGKPFG | vnlk 57 |
| 4-Coumarate CoA ligase from Glycine max | 64 | ampt TSSGTGKPFG | vnlk 58 |
| 4-Chlorobenzoate dehalogenase from Pseudomonas sp. CBS-3 | 157 | vfvy TSSGTGKPFG | avip 59 |
| 4-Chlorobenzoate CoA ligase from Arthrobacter sp. | 157 | vfvy TSSGTGKPFG | avip 60 |
| 4-Hydroxybenzoate CoA ligase from Rhodopseudomonas palustris | 208 | ywvy TSSGTGKPFG | vnh 61 |
| O-Succinylbenzoic acid CoA ligase from B. subtilis | 139 | tlmv TSSGTGKPFG | vqqt 62 |
| O-Succinylbenzoic acid CoA ligase from E. coli | 138 | smtl TSSGTGKPFA | avht 63 |

* Position indicates the starting number of the sequence from the NH\(_2\)-terminus.
since it indicates that, as long as the pcl open reading frame is in phase with the two ATGs present in the polylinker of the plasmid pUC18, PCL will begin to be translated from one of the two AUGs of the mRNA corresponding to these ATGs (see MTM in bal110 and bal112) but never from its own initiation translation signals, unless, as occurred in bal106, bal116, and bal117, STOP signals are present in the three different frames. To clarify how these two methionines (MTM) are responsible for maintaining the function of the protein, we used a variant of pUC18 in which a deletion of one of the two cytosines located between the two ATGs had been produced, thus generating a STOP signal. The nucleotide sequence of the plasmid then became ATG ACA TGA TTA... (see Table II). Using this vector we studied the expression of a construction containing a shorter pcl gene in which the sequence corresponding to the MNM present in the native PCL had been eliminated (see Table II).

### Table II

| Sequence | PCL activity |
|----------|--------------|
| bal101. | YES |
| bal106. | YES |
| bal107. | YES |
| bal110. | NO |
| bal112. | YES |
| bal116. | YES |
| bal117. | YES |
| bal112 (mutant pUC18). | YES |

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|----------|--------------|
| bal101. | YES |
| bal106. | YES |
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NH₂-terminal sequences of the different PCLs encoded by the clones obtained after Erase-a-base treatment of the construction indicated as plStu (Fig. 2)

Nucleotide sequences in normal text are genomic DNA of P. putida U. Nucleotide sequences in bold cursive script correspond to pUC18 polylinker sequence. Amino acid sequences underlined correspond to the NH₂ terminus of the protein encoded generated as fusion protein between the pcl gene and the gal gene. Amino acid sequences in double “underlining” are the beginning of the protein encoded by the pcl gene in each clone. Amino acids in crossed-out text correspond to amino acids encoded by the DNA but that are not transcribed.

| Sequence | PCL activity |
|----------|--------------|
| bal101. | YES |
| bal106. | YES |
| bal107. | YES |
| bal110. | NO |
| bal112. | YES |
| bal116. | YES |
| bal117. | YES |
| bal112 (mutant pUC18). | YES |
ba1142 in the pUC18 mutant, Table II). Analysis of the PCL expressed revealed that it was a functional protein even though the first methionine and the threonine (MT) present in ba1110 and ba1112 had been removed from the NH2-terminal sequence. All these data allow us to conclude that (a) the gene cloned (pcl) corresponds to the one responsible for encoding PCL in P. putida U; (b) discrete modifications in the amino-terminal lead to functional PCL, whereas excessive elongation causes a total loss of PCL activity; and (c) this gene is expressed in E. coli DH5α under the control of the β-galactosidase promoter present in the plasmid pUC18.

The PCL expressed in E. coli DH5α containing the construction corresponding to ba1101 (Table II) was purified following the same procedure reported for the enzyme isolated from P. putida (4). This protein showed similar properties to the native enzyme (optimal pH value for the in vitro assay, optimal temperature, stability, substrate specificity, metal ion requirements, molecular weight, and so forth) and could also be coupled to aeryltrasferase from P. chrysogenum, leading to the formation of different hydrophobic penicillins (F, D, K, G, and V) in vitro. These data indicate that the enzyme expressed in E. coli is identical to that previously purified from P. putida U (4).

Cloning and Expression of the pcl Gene in P. chrysogenum and Study of Its Effect on the Rate of Benzylpenicillin Biosynthesis—After characterization of the pcl gene, the subsequent objective was to achieve its expression in P. chrysogenum in order to determine its effect on the production of benzylpenicillin. In these experiments, the strain selected was P. chrysogenum Wis 54-1255. This fungus was cultured in minimal medium as previously reported (26, 27), and mycelia were used to obtain fungal protoplasts as described by Sanchez et al. (26). Protoplasts were transformed, following standard procedures (26, 27), with a plasmid derived from pBC (Stratagene) in which the following genetic information had been included: (i) a gene that confers on the fungus resistance to the antibiotic bleomycin (ble') (65) and that is under the control of the promoter of P. chrysogenum glutamate dehydrogenase (Pgdh; (ii) the promoter of the pclAB gene which encodes ACVS in P. chrysogenum (PpclAB) (65); (iii) the pcl gene isolated from P. putida U and obtained from the clone indicated as ba1101 in the promoter of the pcl gene isolated from P. chrysogenum (Ppcl); (iv) terminator of the trpC gene of P. chrysogenum (TtrpC); (v) sequence located between the oligonucleotides: (5'-AGC ACT AGG GAC ACC ATC AAT TTC GAT GCG GCT-3') (positions 458–473, Fig. 3) and (65) and that is under the control of the promoter of P. chrysogenum glutamate dehydrogenase; ble', bleomycin resistance gene; cm', chloramphenicol resistance gene.

Table II; and (iv) the terminator of the trpC gene (TtrpC) of P. chrysogenum. The construction designated pALPs9 is represented schematically in Fig. 4. It may be seen that pcl is under the control of the promoter of the pclAB gene of P. chrysogenum.

Fungal transformants expressing the ble' gene were selected (27) and analyzed. All of these strains contained the pcl gene, as demonstrated after PCR amplification of an internal pcl sequence located between the oligonucleotides: (5'-AGC ACT AGG GAC ACC ATC AAT TTC GAT GCG GCT-3') (positions 458–473, Fig. 3) and (65) and that is under the control of the promoter of P. chrysogenum glutamate dehydrogenase; ble', bleomycin resistance gene; cm', chloramphenicol resistance gene.
randomly selected different transformants, controls, and fungal strains transformed with a construction similar to pALPs9 but not containing the pcl gene; in all these, the presence of PCL activity was analyzed in cell-free extracts (4, 29). Table III shows that in all the transformants containing the pcl gene, good PCL activity was found, whereas in our assay conditions such activity was not detected either in the controls or in the transformants lacking this gene (see above). These data allow us to conclude that the pcl gene from P. putida was being efficiently expressed in P. chrysogenum.

Finally, the quantities of penicillin G accumulated in the culture broths by these strains were measured by HPLC (33, 34). As indicated in Table III, all the transformants containing pcl from P. putida U produced between 84 and 121% more benzylpenicillin than the controls, showing unequivocally that the expression of this gene in P. chrysogenum is responsible for a strong increase in the rate of synthesis of penicillin G in this fungus. Furthermore, this effect specifically concerns benzylpenicillin biosynthesis since the rates of synthesis of 6-aminopenicillanic acid (the nucleus of all the penicillins lacking side chains) and natural penicillins (DF, F, K, in which the phenylacetic acid moiety present in the penicillin G has been replaced by several aliphatic acids whose carbon length ranges between C₆ and C₈ carbon atoms) were not affected when P. chrysogenum transformants were cultured in fermentation broths lacking phenylacetic acid (data not shown).

The results reported here are interesting for several reasons. First, we have analyzed the pcl gene, as well as the protein encoded in it (PCL), which catalyzes the first step of a newly identified catabolic pathway (which seems to be encoded in a single transcription unit) involved in the aerobic degradation of phenylacetic acid, this being the first description of the sequence of a PCL. Second, this enzyme is of biotechnological interest since its expression in P. chrysogenum contributes to increasing the biosynthetic rate of the important antibiotic benzylpenicillin. Moreover, these findings open new possibilities for the isolation of high producer strains. Thus, as shown above, the cloning and expression in this fungus (or in other related microorganisms) of different acyl-CoA-activating enzymes (3) or other heterologous proteins with similar functions to those involved in the penicillin biosynthetic pathway could help to increase the pool of intermediates (such as PA-CoA) that are synthesized at very low rates in the original strain and that are essential to ensure an efficient synthesis of benzylpenicillin. Finally, as indicated in Table III, all the transformants containing the pcl gene; in all these, the presence of PCL activity was analyzed in cell-free extracts (4, 29). Table III shows that in all the transformants containing the pcl gene, good PCL activity was found, whereas in our assay conditions such activity was not detected either in the controls or in the transformants lacking this gene (see above). These data allow us to conclude that the pcl gene from P. putida was being efficiently expressed in P. chrysogenum.

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