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Molecular Characterization of Three Loss-of-Function Mutations in the Isopenicillin *N*-Acyltransferase Gene (*penDE*) of *Penicillium chrysogenum*

FRANCISCO J. FERNÁNDEZ, SANTIAGO GUTIERREZ, JAVIER VELASCO, EDUARDO MONTENEGRO, ANA T. MARCOS, AND JUAN F. MARTÍN*

Section of Microbiology, Department of Ecology, Genetics and Microbiology, Faculty of Biology, University of León, 24071 León, Spain

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Five mutants of *Penicillium chrysogenum* blocked in penicillin biosynthesis (*npe*) which are deficient in isopenicillin *N*-acyltransferase were isolated previously. Three of these mutants, *npe6*, *npe7*, and *npe8*, have been characterized at the molecular level and compared with *npe10*, a deletion mutant. Transcripts of normal size (1.15 kb) of the *penDE* genes, which encode isopenicillin *N*-acyltransferase, and also of the *pcbAB* (11.5 kb) and *pcbC* (1.1 kb) genes were observed in all mutants except for the *npe10* mutant. Immunoblotting studies using antibodies against isopenicillin *N*-acyltransferase showed that all mutants (except *npe10*) formed the 40-kDa (unprocessed) protein and the 29-kDa subunit of the isopenicillin *N*-acyltransferase. The 11-kDa subunit could not be observed in the immunoblots. The mutant *penDE* genes of strains *npe6*, *npe7*, and *npe8* were cloned and sequenced. These three strains showed a mutation in the *penDE* genes which results in a single amino acid change in each modified isopenicillin *N*-acyltransferase. The mutation in *npe6* resulted in a change of Gly-150 to Val, whereas the mutation in both *npe7* and *npe8* introduced a change of Glu-258 to Lys. Replacement of the Val-150 and Lys-258 mutations by constructing hybrid isopenicillin *N*-acyltransferase molecules led to the recovery of the isopenicillin *N*-acyltransferase activity. The mutations in *npe6*, *npe7*, and *npe8* do not affect the ability of the 40-kDa isopenicillin *N*-acyltransferase to be processed into the component subunits.

Penicillin biosynthesis in filamentous fungi is carried out in three reactions (1, 5, 9, 11, 19). The last step involves the exchange of the α-aminoadipic acid side chain of isopenicillin N (IPN) by phenylacetic acid (activated as phenylacetyl-coenzyme A [CoA] or phenylacetylglutathione) (2); this reaction is catalyzed by the enzyme acyl CoA:isopenicillin *N*-acyltransferase (IAT), encoded by the *penDE* gene, an enzyme that shows four other related activities (2). The IAT is a heterodimer (3, 28, 32) formed of two subunits of 29 and 11 kDa which are encoded by a single gene, *penDE*, both in *Penicillium chrysogenum* (6, 29) and in *Aspergillus nidulans* (21, 29). Initial studies indicated that a single transcript of 1.15 kb encoding both subunits was formed, and a 40-kDa form of the IAT was observed in addition to the 29- and 11-kDa subunits, suggesting that a 40-kDa precursor protein is posttranslationally cleaved to form the two subunits (6, 32). The acyl-CoA:6-aminopenicillanic acid acyltransferase activity of IAT has been shown to be associated with the heterodimer (29 + 11 kDa) (28). However, the role of each of the two subunits in catalyzing the five related activities shown by the enzyme remains unclear.

We have described previously the isolation (18) and characterization of nine mutants of *P. chrysogenum* impaired in penicillin biosynthesis (8). Surprisingly, five of the nine mutants (*npe1*, *npe4*, *npe6*, *npe7*, and *npe8*) were blocked in the last step of penicillin biosynthesis, whereas only one (*npe5*) was mutated in the unusually large gene *pcbAB*, encoding the α-aminoadipyl-cysteinyl-valine synthetase. Three of them (*npe6*, *npe7*, and *npe8*) completely lacked acyltransferase activity.

The mutation(s) existing in the *penDE* genes of these mutants may affect transcription or translation of the genes or it may alter some of the amino acids in the active center(s) of the protein. The mutation(s) may also affect the posttranslational processing of the 40-kDa protein or the proper location of the protein in microbodies (22, 23). It was, therefore, of interest to characterize those three mutants at the molecular level.

We describe in this article how these three *npe* mutants form transcripts of normal size (1.15 kb) which are translated into 40-kDa IAT proteins of the same size as that of the parental strain; the mutant IAT protein gave a positive reaction with antibodies against the acyltransferase, and they were processed to the 29- and 11-kDa subunits. Sequence analysis of these genes has identified point mutations in each of the mutant *penDE* genes. Construction of hybrid genes to replace the mutations has confirmed that these mutations produce loss of enzyme activity.

MATERIALS AND METHODS

Microorganisms. *P. chrysogenum npe5* (altered in the *pcbAB* gene), *npe6*, *npe7*, *npe8* (all blocked in the last step of the penicillin biosynthesis), and *npe10* (a deletion mutant that lacks the penicillin gene cluster) are derivatives of *P. chrysogenum* Wis 54-1255, a low penicillin producer (8).

Micrococcus luteus ATCC 9341, a strain highly sensitive to penicillin, was used for penicillin bioassays (17).

Escherichia coli DH5α (15) was used as the recipient strain for high-frequency plasmid transformation (10^6 to 10^8 transformants per μg of DNA); single-stranded DNA from the pBluescript plasmids was obtained from *E. coli* WK6 (14). Phage M13K07 was used as helper in the infection of strain WK6 with the pBluescript plasmids (20). *E. coli* LE392 and

* Corresponding author. Phone: 34-87-291505. Fax: 34-87-291506.

NM539 (26) were used for the preparation and amplification of *P. chrysogenum npe6*, *npe7*, and *npe8* genomic libraries.

Lambda-GEM-12 (Promega, Madison, Wis.) was used as a vector for cloning large DNA fragments (12 to 18 kb) of *P. chrysogenum*. DNA fragments were subcloned in pBluescript plasmids KS(+) and KS(−) (Stratagene, La Jolla, Calif.).

Media and culture conditions. *P. chrysogenum* spores were obtained from Power medium plates (a 1:1 mixture of PM1 medium [17]) and modified Czapeck medium (30 g of sucrose per liter, 25 mM $NaNO_3$, 3 mM K_2HPO_4, 2 mM $MgSO_4$, 0.07 mM $FeSO_4$, 0.7 M KCl, and 2.5% [wt/vol] agar at a final pH of 6.5) after 5 days at 25°C. Liquid cultures were developed as described previously (8). Mycelia were collected by filtration through Nytal filters (Nytal, Nytal, Switzerland), washed with 0.9% NaCl, and immediately frozen at −80°C until they were used for RNA extraction or protein analysis.

RNA isolation and Northern (RNA) blotting. Total RNA was obtained by the phenol-sodium dodecyl sulfate (SDS) method (4). Northern blotting and hybridization with probes D, C, E, and G (see Fig. 1), corresponding to the *pcbAB*, *pcbC*, *penDE*, and *pyrG* genes, were carried out as described previously (11, 14).

Cell extracts and IPN acyltransferase assays. Frozen mycelia were ground to powder in liquid nitrogen in a mortar. The disrupted cells were then resuspended in 2 ml of TD buffer (50 mM Tris-HCl [pH 8.0] and 5 mM dithiothreitol). The mixture was centrifuged at 20,000 × *g* for 30 min at 4°C, and the supernatant was recovered. The acyl-CoA:6-APA acyltransferase activity of the IAT was measured by monitoring the formation of benzylpenicillin from 6-APA as described by Alvarez et al. (1, 2).

Polyacrylamide gel electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was carried out as described by Laemmli (16), by using duplicated 12% polyacrylamide gels in a Slab Gel Electrophoresis Unit SE 600 (Hoefer, San Francisco, Calif.). One gel was stained with Coomassie blue (26), and the other was blotted onto polyvinylidene difluoride membrane (Immobilon-P, 0.45-μm pore size; Millipore, Bedford, Mass.), previously saturated in methanol for 5 s, washed in distilled H_2O for 4 min, and incubated for 10 min in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, and 20% methanol). The protein transfer was carried out with an LKB 2117 Multiphor II Electrophoresis Unit (Pharmacia-LKB, Uppsala, Sweden) for 40 min, with a constant intensity of 0.8 mA per cm^2. The membrane was incubated for 30 min in saturation buffer (50 mM Tris HCl [pH 7.5], 0.9% NaCl, 1% bovine serum albumin (BSA) and 0.02% NaN_3) and then overnight in a 1:1,000 dilution in saturation buffer of an antibody against the 29-kDa active IAT subunit (23) with gentle agitation. Later, the membrane was washed three times for 10 min with washing buffer (50 mM Tris HCl [pH 7.5], 0.9% NaCl, 0.05% Tween 20) and incubated for 2 h in incubation buffer (30 mM Tris HCl [pH 8.0], 0.25 M NaCl, 0.5% BSA, 0.02% NaN_3) containing the second antibody with conjugated peroxidase activity (Promega) at a dilution of 1:7,500. Finally, the filter was washed three times for 5 min each in washing buffer and was incubated for 5 min in reaction buffer (100 mM Tris HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). This buffer was replaced by 10 ml of fresh reaction buffer containing 66.6 μl of nitroblue tetrazolium (50 mg/ml) and 33.3 μl of 5-bromo-4-chloro-3-indolylphosphate (BCIP) (50 mg/ml; Promega). The reaction was stopped with distilled water. Antibodies against the 29-kDa subunit of the IAT were provided by R. Bovenberg (Delft, The Netherlands).

DNA isolation, cloning, and sequencing. Total DNA was obtained from lyophilized mycelia as described before (27). Southern blotting and hybridizations were carried out according to the method of Sambrook et al. (26).

Total DNA from *P. chrysogenum npe6*, *npe7*, and *npe8* was partially digested with *Sau3A*, and 12- to 18-kb fragments were selected from sucrose gradients. Purified fragments were ligated with vector lambda-GEM-12 DNA digested with *Bam*HI and *Eco*RI. Ligations were packaged by using the Gigapack II Plus packaging system (Stratagene, La Jolla, Calif.). Genomic libraries from each mutant were amplified and stored at 4 and −70°C, according to the instructions of the manufacturers.

About 40,000 PFU from each amplified genomic library was hybridized with probes A (a 677-bp *Sal*I-*Xba*I fragment upstream from the *pcbC* gene) and B (a 350-bp *Xba*I-*Sal*I fragment downstream from the *penDE* gene) (Fig. 1) labeled by nick translation. The 5.1-kb *Sal*I fragment containing the *pcbC* and *penDE* genes was cloned from each of the three mutants.

A 3.1-kb *Bam*HI-*Xba*I fragment carrying the *penDE* genes from each mutant and their promoter regions were subcloned into pBluescript KS(+) and KS(−), and these plasmids were purified by CsCl-gradient ultracentrifugation. Clones used in DNA sequencing were generated by the erase-a-base method (Promega). DNA fragments were sequenced (both strands) by the dideoxynucleotide method by using the Sequenase 2.0 system (U.S. Biochemicals, Cleveland, Ohio).

Transformation of *P. chrysogenum*. Protoplasts were obtained from *P. chrysogenum npe10*, and transformation was performed as described previously (7, 10) by using plasmid pULC43, which carries the phleomycin resistance gene from *Streptoalloteichus hindustanus* as a selective marker (expressed under the *pcbC* gene promoter of *Cephalosporium acremonium*). Derivatives carrying either mutant or hybrid *penDE* genes were constructed in this vector. Transformants were selected in modified Czapeck medium as described above, supplemented with 30 μg of phleomycin per ml and 1 M sorbitol, after incubation at 28°C for 5 days.

RESULTS

Transcripts of penicillin biosynthetic genes in the *npe* mutants. Total RNA corresponding to *P. chrysogenum npe5*, *npe6*, *npe7*, *npe8*, and *npe10* (a deletion mutant lacking the entire cluster) and the parental strain Wis 54-1255 as a control, were hybridized with a probe carrying the entire *penDE* gene (1.6-kb *Xho*I-*Xba*I fragment; probe C in Fig. 1). A single transcript of the same size as that of the parental strain (1.15 kb) was found in all *npe* mutants except *npe10* (Fig. 2C).

Transcripts of the expected sizes corresponding to the *pcbAB* and *pcbC* genes (11.5 kb and 1.1 kb, respectively) were observed in all mutants except *npe10* (Fig. 2A and 2B). All strains, including *npe10*, showed the 1.1-kb transcript of the *pyrG* gene used as a control (Fig. 2D). The *pyrG* gene encodes one enzyme of primary metabolism for pyrimidine biosynthesis (orotidine monophosphate decarboxylase) and is located in a chromosome of *P. chrysogenum* (chromosome II) different from that for the penicillin gene cluster (chromosome I) (12). This gene is poorly transcribed, and the film needed to be exposed for a longer time to visualize the hybridizing band.

Immunodetection of IPN acyltransferase in the blocked mutants. Proteins in crude extracts of *npe6*, *npe7*, and *npe8* and the parental Wis 54-1255 mutant were separated by SDS-PAGE as indicated in Materials and Methods and blotted onto Immobilon-P membranes, and the IPN acyltransferase was detected with antibodies against the 29-kDa IAT that also react with the 40-kDa precursor (6a). As shown in Fig. 3,

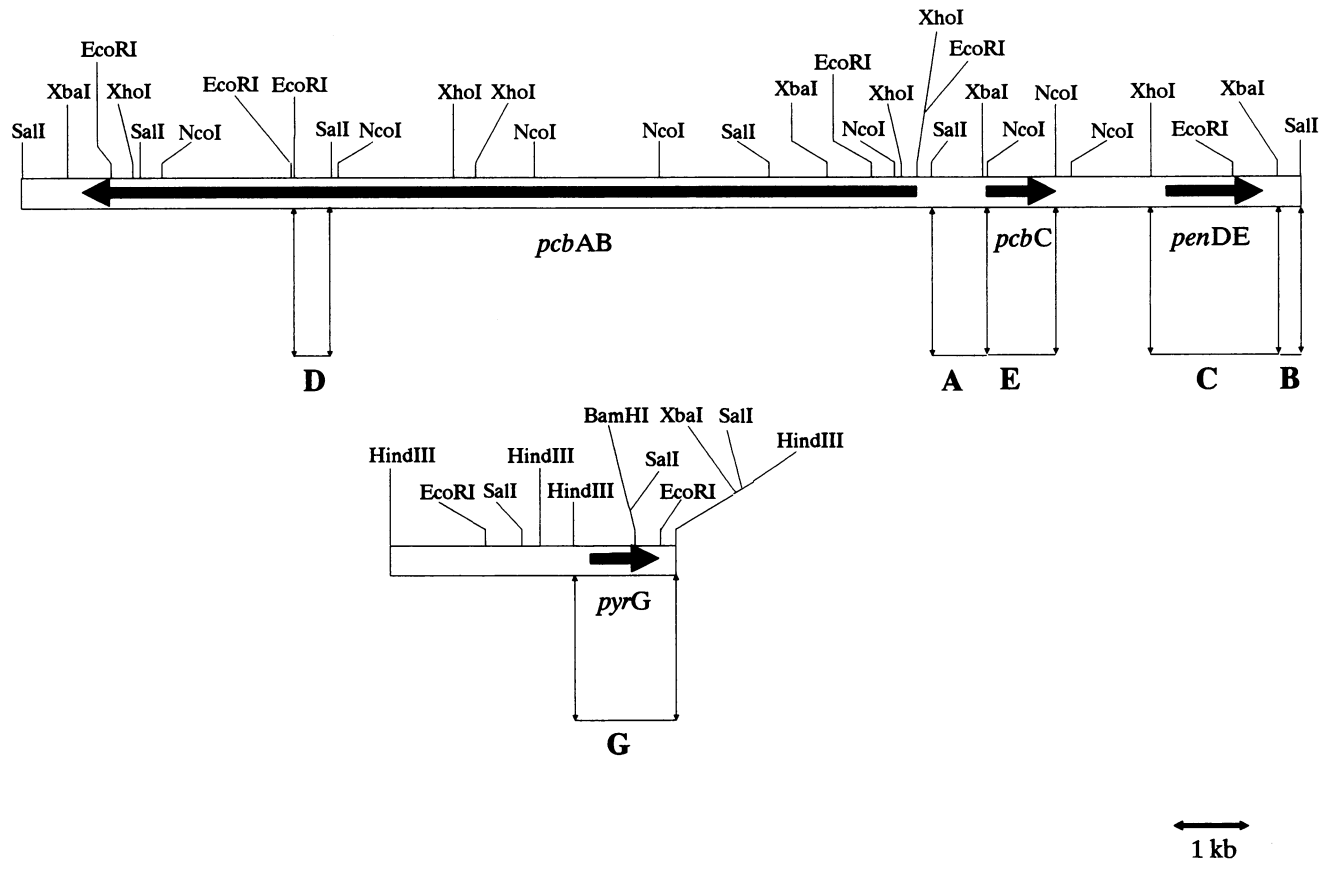

FIG. 1. *P. chrysogenum* DNA fragments carrying the penicillin gene cluster (upper portion) and the *pyrG* gene (lower portion). Probes A and B were used for screening phage libraries, and probes C, D, E, and G were used for transcription analysis by Northern hybridization. The *pcbAB*, *pcbC*, and *penDE* genes are shown by thick arrows. Arrowheads indicate the direction of transcription.

extracts of all mutants (excluding *npe10*) contained the 40-kDa precursor protein (which is not split in SDS-containing buffers) and an intense 29-kDa subunit. All *npe* mutants showed the ability to process the 40-kDa IAT to the 29-kDa (and 11-kDa) subunits. Since previous evidence showed that mutants *npe6*, *npe7*, and *npe8* lacked IAT activity, these results suggest that both the 40-kDa protein and the heterodimer (29 + 11 kDa) formed in the mutants are enzymatically inactive.

Mutant *npe10* showed no formation of either the 40- or the 29-kDa forms of the IAT. This mutant has suffered a long deletion of the penicillin region in chromosome I, including all the penicillin biosynthetic genes (13), resulting in its inability to

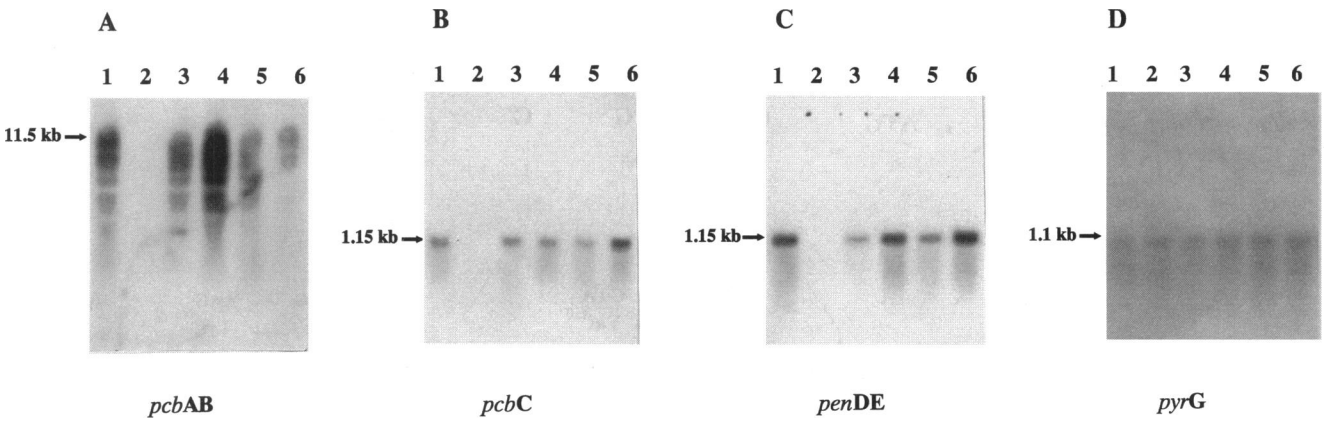

FIG. 2. Transcripts of the genes *pcbAB* (A), *pcbC* (B), *penDE* (C), and *pyrG* (G) shown by hybridization with probes D, E, C, and G, respectively (Fig. 1). Lanes: 1, parental strain *P. chrysogenum* Wis 54-1255; 2, *npe10*; 3, *npe8*; 4, *npe7*; 5, *npe6*; and 6, *npe5*. Note that the *pcbAB* gene transcript (11.4 kb) is partially degraded because of its large size. The *pyrG* hybridizations were exposed for a longer time (14 days) than the others (2 days) because of the low transcription levels of this gene. Five micrograms of total RNA from the different strains was applied to each well.

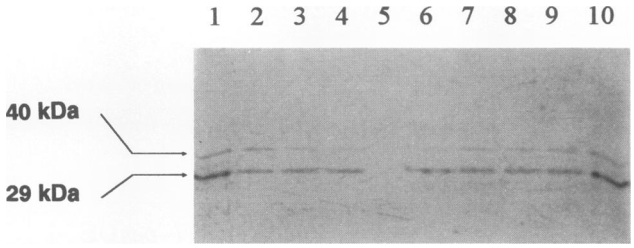

FIG. 3. Immunodetection of the 40-kDa form (unprocessed) and 29-kDa subunit of the acyltransferase of *P. chrysogenum* Wis 54-1255 (lanes 1 and 10); the mutant acyltransferases of *npe6* (lane 2), *npe7* (lane 3), *npe8* (lane 4), and *npe10* (lane 5); and the acyltransferases resulting from the constructions pULPP6M and pULPP6H (lanes 6 and 7) or pULPP7M and pULPP7H (lanes 8 and 9) expressed in *P. chrysogenum npe10*. The hybrid constructions pULPP6H and pULPP7H for mutation replacement are shown in Fig. 5.

form any of the penicillin biosynthetic enzymes (8). The lack of immunodetection of proteins in the *npe10* mutant serves as a good negative control, confirming the specificity of the anti-IAT antibodies used in this work.

Characterization of the mutations in the mutant *penDE* genes. Since the mutations appear to affect the activity but not the processing of the IPN acyltransferase, the *penDE* genes of the three mutants were cloned and sequenced. A 3.1-kb *Bam*HI-*Xba*I fragment containing the complete intergenic region between *pcbC* and *penDE* and the entire *penDE* gene was cloned from *P. chrysogenum npe6*, *npe7*, and *npe8*, and both strands of the corresponding fragments were sequenced; the nucleotide sequences obtained were compared with those of the previously published genes from the high penicillin producer *P. chrysogenum* AS-P-78 (6). As shown in Fig. 4, all three mutants showed three nucleotide changes but showed only one amino acid change with respect to the AS-P-78 strain. Two of the nucleotide changes are common to all three mutants and did not affect the IAT amino acid sequence. The first change (A to C in position 530) is located in the third intron of the gene within a region nonessential for processing of the transcript. The second nucleotide change (C to G at position 684) affects a nucleotide at the third position of Gly-161, encoding codons (GGC to GGG) without alteration of the amino acid. The third mutation resulted in amino acid changes in the protein in each mutant; these appeared to be the reason for the lack of IPN acyltransferase activity. The mutation in *npe6* (G to T at position 650) resulted in a change of Gly-150 to Val, whereas the mutations in both *npe7* and *npe8* (G to A at position 973) resulted in a change of Glu-258 to Lys; i.e., mutants *npe7* and *npe8* were found to be identical. This mutation in *npe7* and *npe8* was shown to modify a *Sst*I restriction site in the gene. The presence of the *Sst*I restriction site in the *penDE* gene of the parental Wis 54-1255 and the lack of the recognition sequence in mutants *npe7* and *npe8* were confirmed by hybridization of *Sst*I-digested DNA with probe C (data not shown).

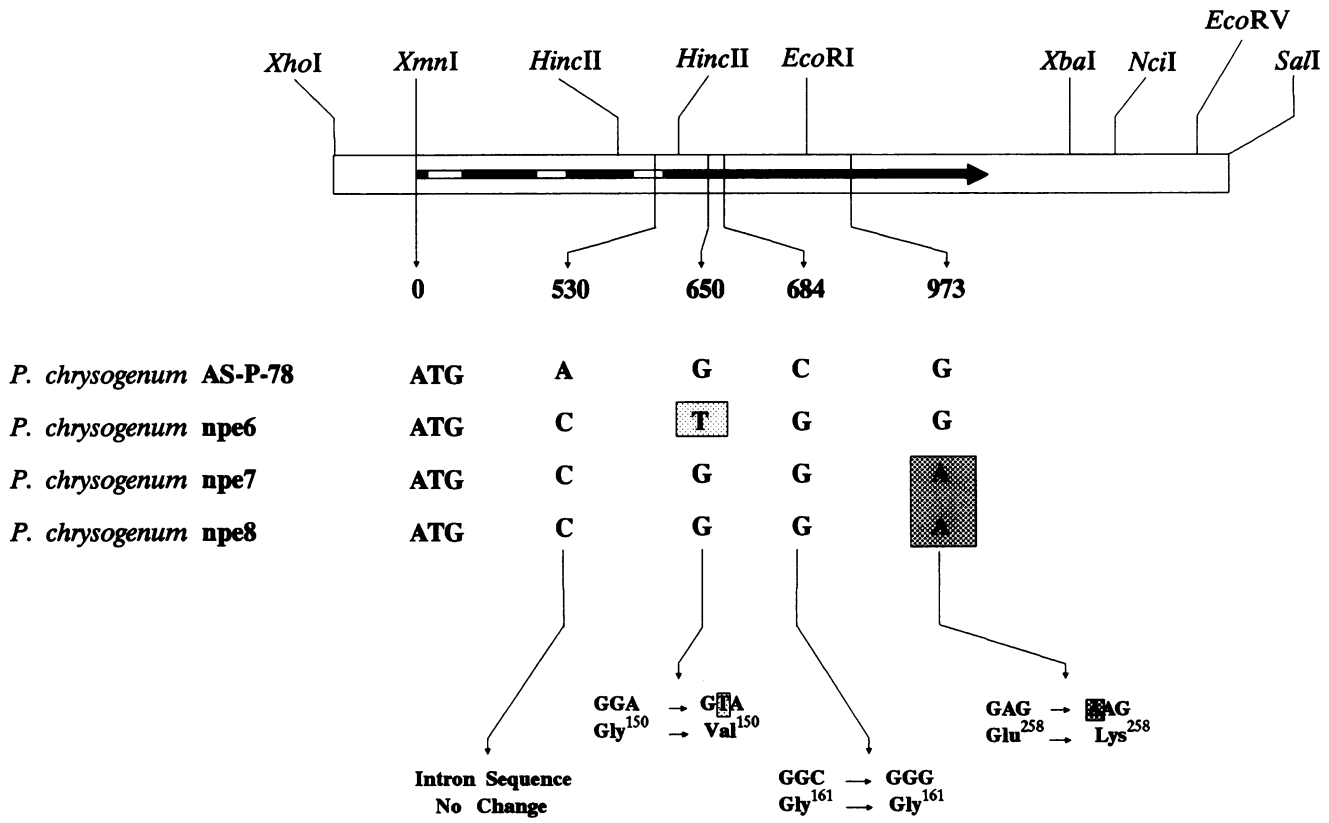

FIG. 4. Summary of the mutations in strains *npe6*, *npe7*, and *npe8*, compared with the nucleotide sequence of *P. chrysogenum* AS-P-78 (GenBank accession number M31454). The thick arrow indicates the position of the *penDE* gene, and the three open boxes inside the arrow correspond to the three introns of the gene. The mutations at nucleotide positions 650 and 973, which originate amino acid changes (Gly-150 to Val and Glu-258 to Lys, respectively), are boxed and shadowed.

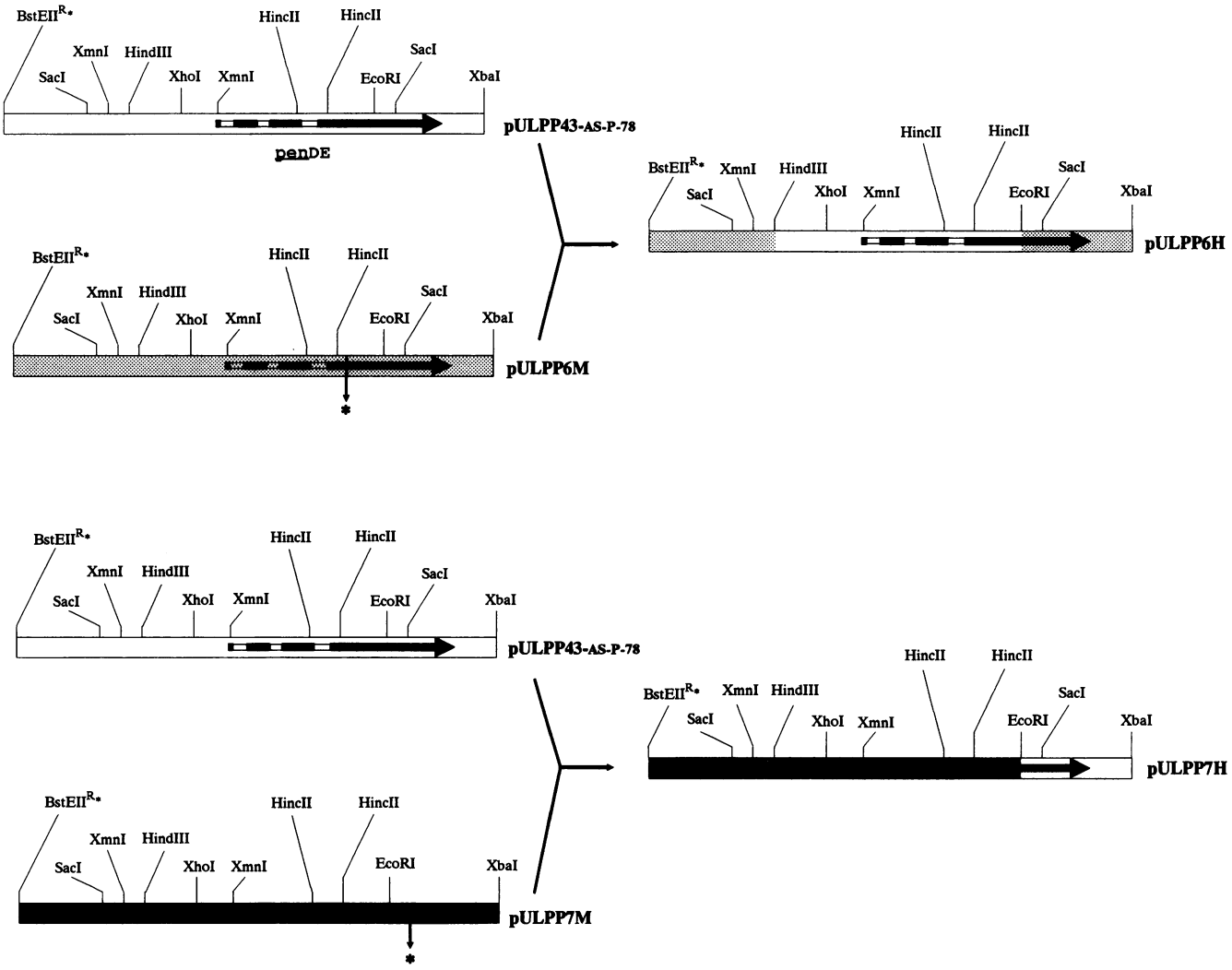

FIG. 5. Replacement of the mutations in *npe6* (asterisk in plasmid pULPP6M) and *npe7* (asterisk in plasmid pULPP7M) by fusing fragments of the mutant genes (shaded boxes) with fragments of the *penDE* gene of *P. chrysogenum* AS-P-78 (white boxes). Hybrid pULPP6H was obtained by replacement of the *Hin*dIII-*Eco*RI fragment (1347 bp) of pUL6PPM by the homologous region of pULPP43-AS-P-78. pULPP7H was obtained by replacing the *Eco*RI-*Xba*I fragment of pULPP7M by the homologous region of pULPP43-AS-P-78.

The two nucleotide changes which are common to all sequenced *npe* mutants (nucleotide positions 530 and 684) are most likely due to differences between the parental strain Wis 54-1255 (from which all *npe* mutants derived) and strain AS-P-78, both of which form active IPN acyltransferase. Unfortunately, the *penDE* genes have been cloned only from high-producing AS-P-78 (6) or OM6.232.12 strains (29), and the corresponding gene of strain Wis 54-1255 has not been cloned. The IATs of the high-producing AS-P-78 and OM6.232.12 have 100%-identical amino acid sequences. To confirm that the nucleotide differences at positions 533 and 684 in AS-P-78 and the Wis 54-1255-derived *npe* mutants do not affect enzyme activity and that the lack of IAT activity is due to the mutations at nucleotide positions 650 or 973, hybrid genes were constructed to replace the Gly-150 and Glu-258 mutations.

Replacement of the Gly-150 and Glu-258 mutations leads to active IAT. Hybrid proteins were synthesized by constructing fused *penDE* genes in which the 1347-bp *Hin*dIII-*Eco*RI fragment carrying the *npe6* mutation was replaced by the homologous fragment of the *penDE* gene of AS-P-78, as indicated in Fig. 5 (constructions pULPP6H [H for hybrid]). Similarly, the 588-bp *Eco*RI-*Xba*I fragment carrying the *npe7* (or *npe8*) mutation was replaced by the homologous fragment of the *penDE* gene of AS-P-78 (construction pULPP7H). Constructions with the hybrid genes were introduced in the deletion mutant *npe10*, which showed no traces of the 40- or 29-kDa proteins or IAT activity. The fused genes were expressed and synthesized active IAT (Table 1), confirming that the lack of IAT was due to the mutations in Gly-150 (*npe6*) and Glu-258 (*npe7* and *npe8*). No activity was observed in clones transformed with the vector without an insert (pULC43), and a normal activity was found in clones transformed with the *penDE* gene of AS-P-78.

As expected, different transformants with the same construction showed different IAT activity, probably because of integration of one or more copies of the gene by nonhomologous recombination, since the *npe10* strain used as a host in these

TABLE 1. IAT activities of several clones of *P. chrysogenum npe10* transformed with a mutant or hybrid *penDE* gene

Transformant	Plasmid used for transformation	Isopenicillin *N*-acytransferase activity in:	
		mU/ml	mU/mg of protein
43.6 (Control)[a]	pULC43	9	1.8
43.7 (Control)[a]	pULC43	0	0
43-AS-P-78[b]	pULPP43-AS-P-78	150.6	33
4-6M[c]	pULPP6M	0	0
1-6H[d]	pULPP6H	549	121.8
2-6H[d]	pULPP6H	554	123
3-6H[d]	pULPP6H	429	94.8
4-6H[d]	pULPP6H	151.2	33.6
6-6H[d]	pULPP6H	928	206.4
1-7M[e]	pULPP7M	0	0
1-7H[f]	pULPP7H	148	33

[a] With pULC43 without insert.
[b] With the *penDE* gene of *P. chrysogenum* AS-P-78.
[c] With the mutant *npe6 penDE* gene.
[d] With hybrid *penDE* gene derived from the *npe6* mutant.
[e] With the mutant *npe7 penDE* gene.
[f] With hybrid *penDE* gene derived from the *npe7* mutant.

transformation experiments lacks the entire penicillin cluster. Transformation of *P. chrysogenum* is known to produce a variety of transformants with one to several copies of the plasmid in the integrated form (7).

DISCUSSION

Mutants blocked in antibiotic biosynthesis are very useful instruments for understanding in vivo gene-enzyme relationships, since they allow complementation studies (24). Alteration of specific nucleotide sequences in a gene encoding an enzyme for the biosynthesis of penicillin may provide information on the active center(s) or regulatory domains of that enzyme. There are many known *P. chrysogenum* or *C. acremonium* (synonymous with *Acremonium chrysogenum*) mutants with decreased or enhanced ability to synthesize penicillin or cephalosporin. However, in only one case has a mutation (*C. acremonium* N2) been fully characterized at the molecular level (25).

We isolated several mutants that were deficient in IAT activity (8, 18). These mutants lacked not only IAT but also four other related activities which are encoded by the *penDE* gene (2). These mutants were used to clone by complementation the *penDE* genes of *P. chrysogenum* AS-P-78 (6, 30) and *A.*

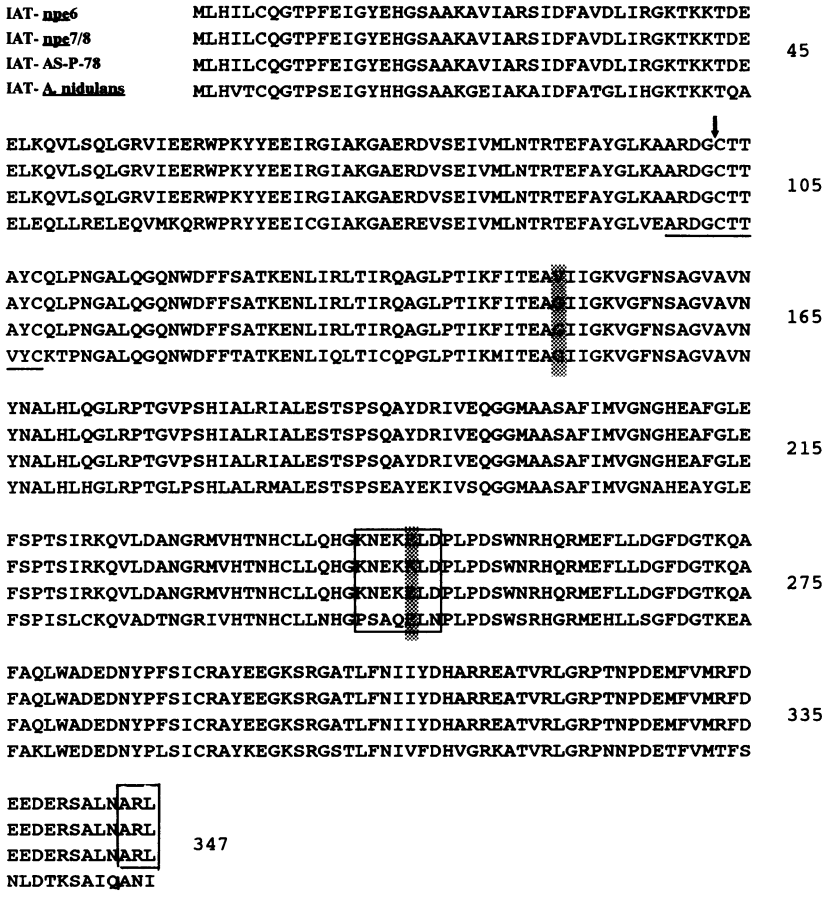

FIG. 6. Deduced amino acid sequences of the IATs of *P. chrysogenum npe6*, *npe7*, or *npe8*, AS-P-78 and *A. nidulans* showing the processing site (vertical arrow) and the amino acid changes (Gly-150 to Val or Glu-258 to Lys) in the *npe* mutants (shaded). Note that Gly-150 and Glu-258 are conserved in *A. nidulans*. Gly-150 is nested in a highly conserved region in *P. chrysogenum* and *A. nidulans*, whereas Glu-258 is the first conserved residue following a region of poorly conserved amino acids in *P. chrysogenum* and *A. nidulans* (boxed). The mutations do not affect the conserved region around the processing site (underlined) or the microbody targeting signal (alanine-arginine-leucine [ARL]) at the C-terminal end of the protein (boxed).

nidulans (21). Three of the mutants that showed a complete lack of IAT activity, *npe6*, *npe7*, and *npe8*, synthesized a transcript of the expected size (1.15 kb) and of an intensity similar to that of the parental strain, indicating that there is no significant difference between the transcription initiation activity of the *penDE* gene in the mutants and that in the parental strain. In all strains except *npe10*, the *penDE* gene was transcribed more intensively than was the *pyrG* gene; the latter gene, which encodes orotidine monophosphate decarboxylase, an enzyme required for pyrimidine biosynthesis, is also poorly expressed in *C. acremonium* (31).

All *npe* mutants except *npe10* also showed transcripts of normal size of the *pcbAB* and *pcbC* genes, excluding an indirect effect of the products of these early genes on transcription of the *penDE* gene. Indeed, *npe6*, *npe7*, and *npe8* showed normal α-aminoadipyl-cysteinyl-valine synthetase and IPN synthase activities (8).

These three mutants showed point mutations in the 3′ half of the *penDE* gene that corresponds to the 29-kDa subunit of the IAT. The *npe6* Gly-150 to Val change is located in the amino acid residue at position 47 from the amino-terminal end of the 29-kDa subunit in a highly conserved region in both *P. chrysogenum* and *A. nidulans* acyltransferases. The mutation in *npe7* and *npe8* (Glu-258 to Lys) produces a significant change in the charge of the polypeptide in that region that may explain the lack of enzymatic activity. Indeed, mutants *npe7* and *npe8* show two consecutive lysine residues (Lys-257 and Lys-258) in this region. Glu-258 and Leu-259 are conserved amino acids in the IATs of *P. chrysogenum* and *A. nidulans* in a region of nonconserved amino acids, suggesting that they are essential for IAT activity. It is interesting that these mutations resulted also in the loss of the isopenicillin *N*-amidohydrolase, penicillin transacylase, and penicillin amidase activities (2), supporting the hypothesis that the different activities of IAT share the same active center(s) (19).

Immunoblotting studies provide useful information about the formation of the 40- and 29-kDa subunits of the IAT in the mutants. The great specificity of the antibodies, as shown by the lack of immunoreaction with extracts of the deletion mutant *npe10*, strongly supports the conclusions that all mutants are able to form the 40-kDa IAT and that all of them are able to process the 40-kDa polypeptide into the 29-kDa subunit. The processing of the 40-kDa IAT into the 29- and 11-kDa subunits is now well established (3, 6, 28). Tobin and coworkers (28) proposed that the IAT is processed autocatalytically into its subunits. The mutations in *npe6*, *npe7*, and *npe8* do not affect the ability of the IAT to be processed. The amino acid sequence around the known cleavage site (Fig. 6) RDG ↓ CTT (where the arrow indicates the cleavage position) has not been altered in any of the three mutants sequenced.

The IAT of *P. chrysogenum* is normally located in microbodies (22, 23). The *npe* mutations may also result in an abnormal location of the mutant protein because of the lack of the proper configuration. Using our mutant *npe6*, Müller and coworkers (22) failed to observe by immunoelectron microscopy accumulation of the mutant IAT protein in the microbodies of *P. chrysogenum npe6*. Microbodies seem to play a crucial role in the biosynthesis of penicillin (22). The mutation Gly-150 to Val of *npe6* affects, therefore, proper deployment of IAT in microbodies. The mutant IAT from *npe6* retains the putative microbody targeting signal, a C-terminal alanine-arginine-leucine sequence (Fig. 6) (22). The lack of accumulation of *npe6* IAT in the microbodies suggests that in addition to the C-terminal alanine-arginine-leucine motif, Gly-150 plays an important role in the allocation of IAT to those organelles.

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