Cloning and Characterization of the Isopenicillin N Synthase Gene of *Streptomyces griseus* NRRL 3851 and Studies of Expression and Complementation of the Cephamycin Pathway in *Streptomyces clavuligerus*

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Received 7 May 1990/Accepted 14 September 1990

A gene, pcbC, encoding the isopenicillin N synthase of *Streptomyces griseus* NRRL 3851, has been cloned in a 6.4-kb *BglII* DNA fragment and located in an internal 1.55-kb *PvuII* segment by hybridization with the *Penicillium chrysogenum* pcbC gene. Hybridization studies revealed the presence of homologous sequences in the DNAs of several *Streptomyces* strains and *Nocardia lactamdurans*. The *S. griseus* pcbC gene was not expressed in *Streptomyces lividans* but was expressed in *Streptomyces clavuligerus* and complemented a mutation, *nce2*, that impaired isopenicillin N synthase and cephamycin biosynthesis. The pcbC gene contained an open reading frame of 990 nucleotides that encodes a protein of 329 amino acids with a deduced Mr of 37,371. The isopenicillin N synthase formed after expression of the *pcbC* gene in the *S. clavuligerus* *nce2* mutant strain was found to have an Mr of 38,000 by gel filtration. A protein of about 38 kDa was observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of extracts of a transformant of the *nce2* mutant strain; this protein was absent from the untransformed mutant strain. The G+C content of the *pcbC* gene was 63.6%, and the strongly biased codon usage was typical of that of *Streptomyces* strains. A transcription initiation site was found 44 nucleotides upstream of the ATG translation initiation triplet. A transcript of 1.1 kb was observed in the donor *S. griseus* strain and also in the *S. clavuligerus* *nce2* mutant strain transformed with the *pcbC* gene, suggesting that it is transcribed as a monocistronic mRNA.

β-Lactam antibiotics are microbial metabolites that include the classical penicillins and cephalosporins and the more recently discovered cephamycins, nocardicins, carbapenems, clavams, and monobactams. The biosynthetic pathways of penicillins and cephalosporins are rather well understood, since most of the enzymes involved have been characterized (24), whereas less attention has been paid to the biosynthesis of cephamycins and the newer β-lactams. Penicillins, cephalosporins, and cephamycins have in common the initial steps forming the tripeptide 8-(L-α-aminoacidyl)-L-cysteinyl-D-valine (ACV), mediated by the enzyme ACV synthetase, and cyclizing ACV to isopenicillin N, mediated by isopenicillin N synthase (IPNS). The latter enzyme is encoded by the gene *pcbC* (23). After isopenicillin N the pathway diverges in penicillin G producers (1) and in cephalosporin and cephamycin producers (23, 26).

A gene (*pcbC*) encoding IPNS has been cloned from *Penicillium chrysogenum* (2, 4), *Cephalosporium acremonium* (*Acremonium chrysogenum*) (32), *Aspergillus nidulans* (28), and the cephamycin producers Streptomyces clavuligerus (20), *Streptomyces lipmamii* (38), and *Streptomyces jumonjinensis* (35). Cloning and characterization of *pcbC* genes from eucaryotic and procaryotic β-lactam producers are of great interest as a means of understanding the divergent evolution of similar genes. In addition, knowledge of the conserved nucleotide sequences in *pcbC* genes from different microorganisms will be helpful in identifying amino acids associated with cofactor and substrate binding at the active center of IPNSs.

The production by *Streptomyces griseus* NRRL 3851 of cephamycins A and B (a class of cephamycins with aromatic substituents at position 3', differing in this respect from the better-known cephamycin C produced by *S. clavuligerus*) might suggest a different organization of the genes encoding the biosynthetic pathway in *S. griseus* as compared with that in other cephamycin-producing actinomycetes. For this reason also, it was of interest to characterize the genes of the cephamycin biosynthetic pathway of *S. griseus* NRRL 3851. This is a fast-growing strain with good sporulation; moreover, initial characterization of its genetic system has been reported by Parag (27).

In this paper we report the presence of DNA sequences hybridizing with fragments of the *P. chrysogenum* *pcbC* gene in several actinomycetes, including some strains which are not known to produce β-lactams, and we describe the cloning, characterization, and expression of the *pcbC* gene of *S. griseus*, a producer of cephamycins A and B. The promoter region has been identified, and the size of the transcript has been established.

**MATERIALS AND METHODS**

Microorganisms. *S. lividans* J11326, *S. griseus* NRRL 3851, and a mutant of *S. clavuligerus* blocked in cephamycin biosynthesis and deficient in IPNS (*nce2* mutant strain) (30) were used for cloning experiments. *Streptomyces fradiae* ATCC 10475, *Nocardia lactamdurans* JC1843 (5), *S. griseus* ATCC 10137, *Streptomyces coelicolor* A3 (2) J12280, *Streptomyces cattleya* NRRL 8057, *S. clavuligerus* NRRL 3585, and *S. lipmamii* NRRL 3584 were used for hybridization studies.

Culture conditions. The *Streptomyces* strains and *N. lac-
tamduran were grown routinely in YEME medium or YEME medium supplemented with 1% glycerol to obtain cells for DNA preparation (14). To determine the production of cephamycin C, we grew S. clavuligerus, S. lividans, and the transformant strains in GSPG medium (30), which supports good cephamycin production. Cultures of S. griseus for the production of cephamycins A and B were grown in medium C (37). Cephamycin and clavulanic acid concentrations were determined by bioassay or high-pressure liquid chromatography as described previously (30).

IPNS assays. In experiments to determine IPNS activity in S. clavuligerus, in the nec2 mutant, and in transformed clones, an inoculum of each strain was grown for 48 h in a medium containing 1% malt extract, 1% Bacto-Peptone, 2% glycerol, and 1,000 ml of distilled water (pH 7.0); 10 ml was used to inoculate 100 ml of GSPG medium in 500-ml baffled flasks. Cells grown for 52 h were centrifuged and disrupted by sonication; IPNS was assayed as described previously (5, 16).

Genomic and plasmid DNA preparations. Plasmids from Streptomyces strains were obtained by the alkaline lysis method (14). Total DNA was obtained with the Kirby lytic mixture (14). Fragments of S. griseus genomic DNA were separated by ultracentrifugation (SW41 rotor, L8-70 Ti Beckman ultracentrifuge) in a sucrose gradient (10 to 40%). Plasmid DNA fragments were isolated from low-melting-temperature agarose by the procedure of Langridge et al. (19). Escherichia coli plasmid DNA minipreparations were obtained by the procedure of Holmes and Quigley (12).

DNA labeling and Southern hybridization. Labeling of DNA was done by nick translation with [α-32P]dCTP and the Klenow fragment of DNA polymerase (22). DNA fragments were blotted as described by Southern (36) with 0.45-μm-pore-size nitrocellulose BA85 filters (Schleicher & Schuell, Keene, N.H.).

The nitrocellulose filters were incubated in prehybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate], 1× Denhardt solution, 30% formamide, 0.1% sodium dodecyl sulfate [SDS], 50 μg of denatured salmon sperm DNA per ml) at 42°C for 3 h. After the addition of the denatured, labeled probe, hybridization was carried out at 42°C for 24 h. The filters were washed twice for 30 min in 2× SSC-0.1% SDS and twice for 30 min in 0.2× SSC-0.1% SDS at 70°C.

Sequencing procedure. DNA fragments were subcloned in pBLUESCRIPT KS(+) and KS(-) vectors and sequenced by the dideoxy chain termination method (34) with [α-32P]dATP (Amersham Inc., Amersham, United Kingdom) and the Sequenase system (US Biochemical Corp., Cleveland, Ohio).

S1 nuclease mapping. The pcBC gene transcription initiation site was determined by S1 nuclease mapping as described by Favaloro et al. (7). A 401-nucleotide PvuII fragment was labeled at both 5' ends with polynucleotide kinase by use of [γ-32P]ATP, and the radioactive PvuII fragment (1.4×10⁵ cpm) was used to hybridize with 100 μg of RNA by the method of Hopwood et al. (14). The prehybridization solution contained 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer, 400 mM NaCl, 1 μM EDTA, and 0.4% formamide. The DNA was melted by being heated at 85°C for 10 min, and hybridization was carried out for 2 h at 60°C in 50 μl of hybridization buffer (1× SSC, 0.1% SDS). The mixture was then passed through a 0.45-μm-pore-size nitrocellulose filter (Schleicher & Schuell, Keene, N.H.), and the nitrocellulose filter was washed twice for 10 min in 1× SSC-0.1% SDS and once in 0.2× SSC-0.1% SDS at 70°C.

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RNA isolation and Northern (RNA) blotting. Northern analyses was carried out essentially as described by Maniatis et al. (22). RNA for S1 nuclease mapping experiments and Northern blots was isolated from cells grown for 52 h at 30°C in cephamycin production medium. The mycelium was collected by centrifugation and resuspended in a buffer containing 20 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, 100 mM NaCl, and 1% SDS. Cells were disrupted with a French press at 50 lb/in² (21). The lysate was loaded in CsCl and ultracentrifuged as described by Glisin et al. (11). The RNA was resuspended in TE buffer and quantified by spectrophotometry (22).

RNAs were resolved in denaturing formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized at 42°C with a 0.39-kb AvaI internal fragment of the pcBC gene labeled by nick translation with [α-32P]dCTP.

Restriction endonucleases and nucleic acids. Restriction enzymes, T4 ligase, the Klenow fragment of E. coli DNA polymerase, yeast RNA, and lambda phage DNA restricted with HindIII or with HindIII and EcoRI were obtained from Boehringer (Mannheim, Federal Republic of Germany) or from Amersham. Salmon sperm RNA was made from Sigma Chemical Co. (St. Louis, Mo.).

Nucleotide sequence accession number. The EMBL Data Library nucleotide sequence accession number for the pcBC gene of S. griseus is X54609.

RESULTS

Sequences homologous to pcBC in the genomes of actinomycetes. Since it has been established that there is sequence similarity between pcBC of P. chrysogenum and other known pcBC genes (23, 38), a 258-bp PstI fragment (probe B) internal (nucleotides 692 to 949) to the pcBC gene of P. chrysogenum (2) was hybridized with the DNAs of several actinomycetes.

A positive hybridization band was observed with BglIIdigested total DNAs of several actinomycetes (Fig. 1), including the β-lactam-producing strains S. griseus NRRL 3851 (which produces cephamycins A and B), S. atrovermex (producer of cephamycin C and thienamycin), and N. lactamurans (producer of cephamycin C). Surprisingly, clear hybridization bands were observed in the DNA of S. coelicolor A3 (2) J12280 and, to a lesser extent, in the DNA of S. lividans J1326, two strains which are known to produce β-lactam antibiotics. Cultures of S. lividans grown under cephamycin-producing conditions failed to synthesize any cephamycin in a very sensitive bioassay with the supersensitive E. coli strain 3242 (see Discussion). No hybridization was observed with the DNAs of S. fradiae ATCC 10475 and S. griseus ATCC 10137, and poor hybridization was obtained with the DNAs of S. clavuligerus NRRL 3585 and S. lipmanii NRRL 3584.

Cloning of the S. griseus pcBC gene. Results from total DNA hybridization experiments indicated that the pcBC gene was located in a 6.4-kb BglII DNA fragment. Total DNA of S. griseus was digested to completion with BglII and fractionated by centrifugation in a 10 to 40% sucrose gradient. Samples of each fraction were blotted on nitrocellulose paper and hybridized with the 258-bp internal fragment of the P. chrysogenum pcBC gene (probe B in Fig. 2). Fractions containing BglII fragments in the range of 6 to 7 kb gave a strong hybridization signal with the probe. These fragments were ligated to BamHI-digested dephosphorylated pUC13 and used to transform E. coli DH5α. A total of 894 white, ampicillin-resistant clones containing recombinant plasmids
were obtained. Plasmids were isolated from 96 clones, blotted to nitrocellulose filters, and hybridized with probe B. Three of the plasmids that gave a very strong hybridization signal were purified and mapped with restriction enzymes; the three contained identical 6.4-kb BglII DNA inserts, and one of them was designated pULMG2. The 6.4-kb fragment was subcloned into pIJ2921 (modified pUC18 with two BglII sites in the polylinker), creating pULNF1. From this plasmid the insert could be rescued with BglII cohesive ends.

Restriction endonuclease analysis of the 6.4-kb BglII insert and hybridization with probe B indicated that the hybridizing region mapped in a 0.42-kb BglII fragment and in a 1.1-kb PvuII fragment internal to a larger 1.55-kb PvuII DNA fragment (Fig. 2).

**Orientation of the pcbC gene.** To determine whether the whole gene was present in the 1.55-kb PvuII fragment and to establish the orientation of the gene, we carried out additional Southern hybridizations of the restriction fragments derived from the original 6.4-kb insert by using probe A (750 bp), which carried the 5' and central regions of the *P. chrysogenum* pcbC gene (Fig. 2). The results were compared with those from the hybridizations with probe B, which carried the 3' region of the gene. They indicated that the gene was read from left to right in Fig. 2. Positive hybridizations of different fragments of the cloned DNA with probe A suggested that a gene of the expected size of the *pcbC* gene could be contained within the 1.55 (1.1 + 0.45)-kb PvuII fragment.

**Expression in the S. clavuligerus nce2 mutant and complementation of the deficiency in cephapycin biosynthesis.** Mutants of *S. clavuligerus* blocked in different steps of cephapycin biosynthesis were obtained previously (30). Since *S. clavuligerus* is difficult to transform (particularly with large DNA fragments) and since there are few available vectors (10), the 6.4-kb BglII fragment of *S. griseus* was subcloned into the positive selection plasmid pIJ699, giving rise to plasmid pULMG13 (Fig. 3). This plasmid was introduced into and amplified in *S. lividans* and used to transform the *S. clavuligerus* nce2 mutant for complementation studies. Figure 4 shows the production of cephapycin C and the unrelated β-lactamase inhibitor clavulanic acid by the *S. clavuligerus* nce2 mutant transformed with pULMG13. Control cultures were grown in parallel with the wild-type strain *S. clavuligerus* NRRL 3585, the nce2 mutant, and the wild-type strain transformed with pIJ702; control cultures of *S. clavuligerus* transformed with pIJ699 without inserts do not accept the long *E. coli* DNA stuffer present in pIJ699, and the plasmid is lost.

The addition of thiostrepton (2 μg/ml) (a positive selection agent required to stabilize the plasmids) to the cultures produced a delay in the growth phase and, therefore, reduced by 20 to 30% the levels of cephapycin and clavulanic acid produced by the wild type. Clones of the *S. clavuligerus* nce2 mutant transformed with pULMG13 regained the ability to produce cephapycin C, although the level of cephapycin produced was about one-third that produced in the wild type grown with thiostrepton (Fig. 4B) (see Discussion).

The production of clavulanic acid in the nce2 mutant was not affected by the introduction of the 6.4-kb DNA fragment carrying the *pcbC* gene. The production of clavulanic acid was significantly higher in both strains [nce2 and nce2(pULMG13)] than in the wild type, confirming previous results (30).

**Characterization of IPNS in the nce2 mutant transformed with pULMG13.** IPNS activity was found in the ammonium sulfate precipitate (40 to 80% saturation) of extracts of the *S.
clavuligerus nce2(pULMG13) transformant. After Sephadex G-75 gel filtration, a single peak of enzyme activity was observed (1.79 pkat/mg of protein). Cell extracts of the nce2 mutant did not show IPNS activity, confirming previous results (30). The $M_r$ of the native form of the enzyme as calculated from gel filtration was 38,000 ± 1,000.

To confirm the formation of IPNS protein following expression of the pcbC gene in the transformants, we obtained ammonium sulfate fractions (40 to 80% saturation) of control (S. clavuligerus NRRL 3585 and nce2 mutant) and transformed [S. clavuligerus nce2(pULMG13)] strains after 52 h of incubation and compared them by SDS-polyacrylamide gel electrophoresis. A protein ($M_r$ 38,000) that was present in the wild type and lacking in the nce2 mutant was formed in the S. clavuligerus nce2(pULMG13) transformant (Fig. 5). The $M_r$ of this protein agrees with the $M_r$ of IPNS established by gel filtration (see above) and with the $M_r$ established for other cyclases (see Discussion).

Nucleotide sequence of the pcbC gene. With the strategy indicated in Fig. 2, the nucleotide sequence of a 1.36-kb PvuII-BglII DNA fragment was established (Fig. 6). This region contained an open reading frame of 990 nucleotides encoding a protein of 329 amino acids with a deduced molecular weight of 37,371 and a pI of 5.67. Two in-frame ATG triplets were present in the region of the expected initiation of the open reading frame. Computer analysis of the nucleotide sequence revealed the presence of a putative Shine-Dalgarno sequence, GGAGG, 6 to 11 nucleotides upstream of the first ATG initiation triplet. This location agrees with the location of the Shine-Dalgarno sequences for most Streptomyces genes involved in antibiotic biosynthesis (13, 25). The $M_r$ of the protein encoded by the open reading frame defined by this initiation triplet correlated closely with the $M_r$ (38,000) determined by gel filtration and SDS-polyacrylamide gel electrophoresis.

A 12-bp inverted repeat sequence 52 nucleotides downstream from the TGA termination codon may form two alternative stem and loop structures (Fig. 6) with free energy charges of −22.8 and −20.2 kcal/mol (ca. −95.3 and −84.5 kJ/mol, respectively). This structure may play a role in transcription termination.

The G+C content of the open reading frame was 63.6%. The codon usage was typical of that of Streptomyces strains, and the G+C contents in the first, second, and third codon positions were 58, 38, and 94%, respectively; i.e., almost all

FIG. 3. Construction of pULMG13 by subcloning of the 6.4-kb BglII fragment of S. griseus DNA into the positive selection vector pIJ699. Thick line, S. griseus DNA; thin line, pIJ699 DNA fragment carrying the origin of replication and the thioestrepton resistance gene (tar). The PvuII fragment carrying pcbC is indicated by the black box.

FIG. 4. Complementation of the S. clavuligerus nce2 mutation by the pcbC gene of S. griseus. Shown are growth kinetics (A), cephamycin C production (B), and clavulanic acid production (C) by S. clavuligerus NRRL 3585 (○), S. clavuligerus NRRL 3585 transformed with pIJ702 (○), the S. clavuligerus nce2 mutant (▲), and the S. clavuligerus nce2 mutant transformed with pULMG13 (△). Cultures transformed with pIJ702 or pULMG13 were grown in the presence of 2 μg of thioestrepton per ml. CDW, Cell dry weight.
triplets in the gene contained a G or a C in the third position in the codon, resulting in a strongly biased codon utilization (13).

The nucleotide sequence of the S. griseus pcbC gene and the deduced amino acid sequence of the gene product were very similar to those of other fungal and Streptomyces pcbC genes and their gene products (Fig. 7); amino acid sequence homologies ranged from 52 to 57% with the fungal enzymes to 72 to 81% with the Streptomyces enzymes. Four cysteine residues that were found in the S. griseus IPNS were located in the same positions as in the IPNS of S. clavuligerus (see Discussion).

Analysis of the pcbC transcript. Since genes for cephambiosynthesis are clustered together in S. clavuligerus and N. lactamadurans (31), it was of interest to establish whether pcbC of S. griseus was expressed as a single transcript or whether it formed part of a larger polycistronic mRNA.

RNAs from cephamycin-producing cultures of the donor S. griseus NRRL 3851, of S. clavuligerus NRRL 3585, of the S. clavuligerus nce2 mutant, and of the S. clavuligerus nce2 (pULMG13) transformant were prepared as described in Materials and Methods and hybridized with a 0.39-kb AvaI probe internal to the pcbC gene. As size controls, E. coli rRNAs were used. Although the RNA of Streptomyces strains is notoriously unstable and degrades easily, giving diffuse bands (15), a strongly hybridizing band of about 1.1 kb was observed in wild-type S. griseus (Fig. 8). The S. clavuligerus nce2 (pULMG13) transformant also formed a transcript of the same size, although with a lower hybridization intensity than in S. griseus. This result correlates with the low level of cephamycin formation described above. No significant hybridization was observed with the RNA of S. clavuligerus or the untransformed nce2 mutant because of the use of an S. griseus probe under stringent conditions. A minor band of about 1.5 kb hybridized with the same probe in the total RNA from S. griseus or S. clavuligerus transformed with pULMG13, suggesting the formation of two transcripts, probably from different transcription start sites (see below and Discussion).

Transcription initiation. To confirm the presence of a specific promoter for the pcbC gene and to characterize the initiation of transcription, we carried out S1 nuclease mapping protection studies by using a 401-bp PvuII fragment that carries the ATG initiation triplet at position 246 (Fig. 9). One major and two very minor mRNA protection bands were found in the upstream region of the open reading frame of the pcbC gene. The major protection band corresponded to a transcript starting at a G 44 nucleotides upstream of the ATG translation initiation triplet. A putative −10 consensus sequence (GCATGT) was found 6 nucleotides upstream of the first transcribed nucleotide, but no standard −35 region could be identified (see Discussion). The two very minor protection bands (corresponding to transcripts starting at nucleotides 47 and 16 upstream of the ATG) were barely detectable in the autoradiogram. It is unclear whether they represent true transcription initiation sequences (see Discussion).

DISCUSSION

The genetic information for the production of penicillins, cephalosporins, and cephamycins is present in many filamentous fungi, actinomycetes, and some gram-negative bacteria. An interesting question is whether silent β-lactam biosynthesis genes are present in nonproducer strains. Our results show that DNA sequences that give positive hybridization signals under stringent conditions with the P. chrysogenum pcbC gene exist in the DNAs of S. griseus NRRL 3585, S. cattleya, S. lipmani, S. clavuligerus, and N. lactamadurans, five species known to produce cephamycins, and also in the DNAs of S. lividans and S. coelicolor, two species known not to synthesize cephamycins. Although positive hybridization signals with the P. chrysogenum pcbC gene do not prove the existence of a functional pcbC gene, the strong hybridization observed suggests that homologous sequences have been conserved in many actinomycetes (35).

Silent genes which are not expressed have been proposed to exist in Streptomyces species (17). S. lividans grown in GSPG medium, which is known to support good cephamycin
production, failed to synthesize any detectable trace of cephamycin. Furthermore, pcbC cloned from S. griseus (in the 6.4-kb S. griseus DNA fragment that carries the promoter and the structural gene) was not expressed to a detectable extent in S. lividans, whereas it was expressed and restored cephamycin C biosynthesis in the S. clavuligerus nce2 mutant (Fig. 4), which is blocked in the IPNS step (30). The fact that the pcbC gene was expressed in S. clavuligerus nce2 transformants but not in S. lividans with the same construction (pULMG13) suggests that expression requires a positively acting regulatory protein which occurs in S. clavuligerus (a cephamycin producer) but not in S. lividans. Similar results were observed by Cox et al. (6) and Fishman and co-workers (8) when they introduced the cloned tylF gene (encoding macrolin-O-methyltransferase, the last enzyme of the tylosin biosynthesis pathway) into S. lividans or S. fradiae. Since the cloned fragment contained a 500-bp sequence upstream of the tylF open reading frame in which the tylF promoter is located, it was proposed that a positive regulatory protein is required for effective expression of the tylF gene. One negative and three other positive regulatory genes have been reported to be involved in the control of expression of clusters of antibiotic biosynthesis genes (25).

A main transcription initiation sequence was detected by S1 nuclease mapping starting 44 nucleotides upstream of the ATG triplet; i.e., a promoter region is associated with the pcbC gene. A comparison of the nucleotide sequences immediately upstream of the initiation sites with the known sequences of Streptomyces promoters (13) revealed a puta-
to obtain maximal alignment. Shaded amino acids are introduced of Streptomyces species precedents for rule S1 nuclease mapping in (25).

The amino acid residues are indicated by IPNSs.

Although Streptomyces pcbC, the gene determined of precise transcript in (329 kb) of 1.1-kb mRNA. Preliminary results (31) indicated that the pcbC gene is clustered with other cephapin biosynthesis genes that may be transcribed as separate units.

The pcbC gene of S. griseus contains a 12-bp inverted repeat 52 nucleotides downstream from the open reading frame termination triplet. This sequence probably results in the formation in the RNA of a stable stem and loop structure (two alternative pairings are possible) that may be involved in transcription termination.

The size (329 amino acids; Mw, 37,371) of the protein encoded by the pcbC gene of S. griseus (987 nucleotides) correlates well with the size of the IPNS found in extracts of nce2 transformants as determined by gel filtration (38 kDa)

FIG. 7. Comparative analysis of the deduced amino acid sequences of the S. griseus IPNS and six other IPNSs. Gaps (asterisks) have been described nucleotide downstream from (329 kb). This gene contains the S. griseus cephamycin biosynthesis genes as a part. pcbC gene of S. griseus (987 nucleotides in the same manner as did S. clavuligerus NRRL 3585 (no hybridization signal). The RNAs were probed with an S. griseus 0.39-kb AvaI DNA fragment that was internal to the pcbC gene and that was labeled by nick translation. E. coli rRNAs (1.5 and 2.9 kb) used as molecular weight standards are indicated at the right.
and of a protein (38 kDa) observed by SDS-PAGE (Fig. 5). The size of the enzyme of *S. griseus* is similar to that of the purified IPNSs of *S. clavuligerus* (33 kDa) (16) and *N. lactamdurans* (26.5 kDa) (5); the cloned *pcbC* from *S. clavuligerus* encoded a protein with a deduced *M* of 36,917 (20). The IPNSs of *S. clavuligerus* and *S. jumonjiniensis* contain 329 amino acids, and the enzyme of *S. lipmanii* contains 333 (20, 35, 38).

The alignment of the amino acid sequence of the IPNS of *S. griseus* with those of six other known IPNSs revealed extensive sequence similarities (shaded areas in Fig. 7) (23, 24). As a result, it is not possible to define precisely those regions involved in substrate binding and/or catalytic activity. Two cysteines (amino acid residues at positions 104 and 251) that have been proved to be important for the activity of the IPNS of *C. acremonium* (33) are also conserved in the IPNS of *S. griseus* and in all known IPNSs (arrowheads in Fig. 7). Both of them are located in a stretch of conserved amino acids and may be important in forming a thiol bridge via an iron atom (33), a cofactor of fungal and bacterial IPNSs (5, 16, 29). The *S. griseus* IPNS also contains two nonconserved cysteine residues at positions 37 and 142 that exist in some procaryotic IPNSs but not in the fungal enzymes.

*S. griseus* IPNS contains a tyrosine at position 197 which is conserved in all IPNSs, including that of *P. chrysogenum* AS-P-78 but with the exception of that of a strain (23-X-80-269-37-2) producing large amounts of penicillin (2, 4). This change from tyrosine to isoleucine may have been introduced during the strain improvement program, but the role of this mutation in IPNS activity is unclear.

ACKNOWLEDGMENTS

This research was supported in part by a grant from Gist-Brocades, Delft, Holland. M.-G.-D. received a fellowship from FISS, Madrid, Spain.

We acknowledge the help of L. Laiz in enzyme purification and the excellent technical assistance of B. Martín, M. P. Puertas, and M. I. Corrales and thank J. L. Barredo for the gift of the *P. chrysogenum* *pcbC* gene.

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