Cloning and Expression in *Escherichia coli* of Isopenicillin N Synthetase Genes from *Streptomyces lipmanii* and *Aspergillus nidulans*

BARBARA J. WEIGEL,* STANLEY G. BURGETT, VICTOR J. CHEN, PAUL L. SKATRUD, CHARLES A. FROLIK, STEPHEN W. QUEENER, AND THOMAS D. INGOLIA

Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285

Received 19 November 1987/Accepted 2 June 1988

β-Lactam antibiotics such as penicillins and cephalosporins are synthesized by a wide variety of microbes, including procaryotes and eucaryotes. Isopenicillin N synthetase catalyzes a key reaction in the biosynthetic pathway of penicillins and cephalosporins. The genes encoding this protein have previously been cloned from the filamentous fungi *Cephalosporium acremonium* and *Penicillium chrysogenum* and characterized. We have extended our analysis to the isopenicillin N synthetase genes from the fungus *Aspergillus nidulans* and the gram-positive procaryote *Streptomyces lipmanii*. The isopenicillin N synthetase genes from these organisms have been cloned and sequenced, and the proteins encoded by the open reading frames were expressed in *Escherichia coli*. Active isopenicillin N synthetase enzyme was recovered from extracts of *E. coli* cells prepared from cells containing each of the genes in expression vectors. The four isopenicillin N synthetase genes studied are closely related. Pairwise comparison of the DNA sequences showed between 62.5 and 75.7% identity; comparison of the predicted amino acid sequences showed between 53.9 and 80.6% identity. The close homology of the procaryotic and eucaryotic isopenicillin N synthetase genes suggests horizontal transfer of the genes during evolution.

The penicillin, cephalosporin, and cephamycin classes of β-lactam antibiotics share two common steps in their respective biosynthetic pathways: the joining of three amino acids to form 5-(l-α-aminoacetyl)-l-cysteinyl-d-valine (LLD-ACV) and the conversion of LLD-ACV to isopenicillin N. These two reactions are carried out in filamentous fungi and streptomycetes, including *Penicillium chrysogenum* and *Aspergillus nidulans* (producers of penicillin), *Cephalosporium acremonium* (producer of cephalosporin C), and *Streptomycetes lipmanii* and *Streptomyces clavuligerus* (producers of cephamycins) (16). We have studied the biochemical and genetic basis of the conversion of LLD-ACV to isopenicillin N by cloning and characterizing the gene encoding the isopenicillin N synthetase (IPNS) enzyme from *C. acremonium* and *P. chrysogenum* (6, 23). The IPNS genes from these fungi show a high degree of nucleotide and amino acid sequence identity. The 77% identity of the predicted amino acid sequences of the IPNSs from these fungi is so extensive that it is difficult to identify regions of potential functional importance. We therefore decided to extend the comparison to IPNS genes from other organisms which might be more distant evolutionarily.

The IPNS gene from *A. nidulans* was of particular interest because of the well-developed genetic techniques available for this organism. Also, mutant *A. nidulans* strains with altered β-lactam-biosynthetic ability have been described (10) and characterized (20). The IPNS gene from a gram-positive *Streptomyces* species is also of interest. Such a gene might be expected to be further separated evolutionarily from the fungal IPNS genes, and regions of similarity might therefore be more likely to be important for catalysis. The best characterized β-lactam-producing *Streptomyces* species is *S. clavuligerus*, and progress toward the cloning and characterization of the IPNS gene from *S. clavuligerus* has been reported (17). To increase the breadth of the comparison, and because we had mutants altered in β-lactam-biosynthetic ability available (12), we decided to study the IPNS gene from *S. lipmanii*. This *Streptomycetes* species is also known to synthesize cephamycins (21) and hence would be expected to contain an IPNS gene. In this paper we report the cloning, characterization, and expression in *Escherichia coli* of the IPNS genes from *A. nidulans* and *S. lipmanii*.

**MATERIALS AND METHODS**

Cloning of the *A. nidulans* IPNS gene. *A. nidulans* FGSC-4 was obtained from W. Timberlake (University of Georgia, Athens). Spores of *A. nidulans* FGSC-4 were used to inoculate YG (2% glucose, 0.5% yeast extract) broth medium. The inoculated medium was incubated at 37°C for 48 h with agitation (250 rpm). Mycelia were harvested by vacuum filtration (Whatman no. 1 filter) and washed with an equivalent volume of sterile water. The washed mycelia were transferred to a mortar containing liquid nitrogen and ground to a fine powder with a pestle. Pulverized mycelia were gently suspended in 2 ml of lysis buffer per g of cells (lysis buffer: 2% sodium dodecyl sulfate [SDS], 0.1 M EDTA, 10 mM Tris hydrochloride [pH 7.8], 100 μg of proteinase K per ml) and incubated for 1 h at 60°C. After being heated, solid NaCl was added to a final concentration of 1.0 M and refrigerated (4°C) for at least 1 h. Cell debris was removed by centrifugation (Sorvall RC-5B, GSA rotor, 6,000 rpm, 20 min). Nucleic acids were precipitated from the supernatant with isopropanol and harvested by centrifugation. The pellet, containing nucleic acids, was suspended in TE buffer (10 mM Tris hydrochloride [pH 7.5], 0.1 M EDTA). DNase-free RNase was added to remove RNA, and the remaining protein was removed by extraction with phenol and chloroform. DNA was precipitated with ethanol and harvested by spoiling. The spoold DNA was air-dried and resuspended in TE buffer.

* Corresponding author.
A. nidulans DNA was partially digested with Sau3AI to achieve an average fragment size of 10 to 20 kilobases (kb). These fragments were ligated into the BamHI site in EMBL-3 lambda phage arms (Vector Cloning Systems, Inc.) and packaged according to the manufacturer’s recommendations (Gigapack; Vector Cloning Systems, Inc.). The library was screened without amplification.

The C. acremonium IPNS gene contained on plasmid pLT336 (23) and the P. chrysogenum IPNS gene contained on pXL2 (6) were radiolabeled with $^{32}$P by nick translation (22). The radiolabeled IPNS genes were used separately to screen portions of the bacteriophage lambda library immobilized on nitrocellulose filters. Hybridizations were carried out in 30% formamide-5 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C and then washed in 5 x SSC at 65°C as described previously (6). A recombinant bacteriophage was isolated which hybridized to both IPNS genes, and an approximately 4.5-kb HindIII fragment containing the cross-hybridizing DNA was subcloned into pUC18 (7) to create pOG04 (Fig. 1A).

**DNA sequencing.** The DNA sequence of the A. nidulans IPNS gene on pOG04 was determined by using the dideoxy chain termination method of Sanger et al. (26) after subcloning fragments into M13 cloning vectors. The sequencing strategy is shown in Fig. 1A. Some of the sequencing data were obtained with an Applied Biosystems model 370A automatic DNA sequencer.

**Construction of the E. coli expression vector pOG0216.** Expression of the A. nidulans IPNS gene in E. coli was accomplished by using an expression vector containing the lambda $\lambda_{PL}$ promoter and cI857 gene to drive and control transcription, a two-cistron translation-enhancing element (27), a modified tetracycline resistance gene from pBR322, a unique NcoI restriction site at the translation initiation site, and a unique BamHI site downstream of the NcoI site (Ron Schoner, personal communication). The A. nidulans IPNS gene was reconstructed at the amino-terminal coding region through use of a synthetic linker which incorporated an NcoI restriction site at the translation initiation site. The linker extended to the Clai restriction site which encodes amino acids 12 and 13, and had the sequence

$$5'$$-CATGGTTTCAGTCAGCAAGCCAATGTTCCAAAGAT$$3'$$  
$$3'$$-CCAAGTCAGTCGTTCGGTTACAAGGTTTCTAGC-$$5'$$

The remainder of the A. nidulans IPNS gene was isolated on an approximately 1.6-kb Clai-BglII restriction fragment. The DNA was isolated from a dam mutant of E. coli (GM48) because the Clai site was preceded by a G residue, creating a methylation site which prevented Clai from cleaving DNA prepared from dam+ strains. This Clai-BglII fragment was ligated into the NcoI and BamHI sites of the vector together with the NcoI-Clai linker to create the expression vector pOG0216.

**Purification of the S. lipmanii IPNS protein.** Washed S. lipmanii mycelia were broken by sonication in a buffer consisting of 25 mM MOPS (morpholinopropanesulfonic acid), pH 8.0, 0.3 mM nitroacetic acid, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and the cell debris was removed by centrifugation. The supernatant was treated with ammonium sulfate, and a 40 to 80% ammonium sulfate cut was collected and desalted by passage through a Sephadex G-25 column equilibrated in 25 mM MOPS, pH 8.0. After the ammonium sulfate step, the protein mixture was sequentially subjected to Celluline A-800 ion exchange, Pharmacia Q Sepharose fast flow, and high-pressure liquid Spherogel Beckman TSK-DEAE-5PW chromatographies. The first two columns were equilibrated in 25 mM MOPS, pH 8.0, and eluted with a linear NaCl gradient, while the third column was equilibrated in 0.02 M triethanolamine, pH 8.4, and developed with a linear gradient of NaCl. IPNS activity was followed throughout the chromatographic steps by using the biological plate assay described previously (26). At the end of this purification scheme, a major band of the expected molecular mass (approximately 35,000 daltons, as estimated by SDS-polyacrylamide gel electrophoresis [PAGE]) accounted for about 70% of the visible material on the gel, based on densitometric scanning of Coomasie blue-stained SDS-polyacrylamide gels (18).

Protein prepared in this manner was further purified by SDS-PAGE. KCl-stained protein (15) was cut out of the gel and electroeluted with a C.B.S. Scientific Company (Del Mar, Calif.) electroeluter following the supplier’s recommended conditions. Protein purified in this manner was subjected to amino-terminal sequence analysis, and 23 amino-terminal amino acid residues were determined.

**Cloning of the S. lipmanii IPNS gene.** A 60-base-pair (bp) “guess-mer” probe was designed and synthesized by assuming typical Streptomyces codon bias that would encode amino acids 3 to 22 of the experimentally derived amino-terminal amino acid sequence. This probe was used to screen a lambda library of S. lipmanii DNA. S. lipmanii 16884.3 (provided by O. Godfrey) was grown and DNA was isolated as described (8). Partially digested S. lipmanii DNA was cloned into lambda EMBL-3 as described above for the
A. nidulans lambda bank. The S. lipmanii lambda bank was screened with the \(^{32}\)P-labeled 60-bp guess-mer probe essentially as described for the cloning of the A. nidulans IPNS gene except that the hybridizations were carried out at 37°C in 40% formamide. A recombinant lambda phage which repeatedly hybridized to the probe was purified, and a 2.8-kb SalI fragment from this phage was subcloned into pUC18 to create pOGO239 (Fig. 1B). The region of pOGO239 which hybridized to the probe was sequenced as described above; the strategy is summarized in Fig. 1B.

Expression of the S. lipmanii IPNS in E. coli. An NdeI site containing the translation-initiating methionine codon was incorporated into the S. lipmanii IPNS gene via site-directed mutagenesis (1). The gene was then isolated on an approximately 1.4-kb NdeI-BamHI fragment. The digestion with BamHI was a partial digest because the S. lipmanii IPNS gene contains a BamHI site within the protein-coding region as well as a BamHI site about 300 bp downstream of the translation termination site. The NdeI-BamHI fragment containing the entire protein-coding region of the S. lipmanii IPNS gene as well as about 300 bp of 3' noncoding DNA was cloned into an E. coli expression vector to create pOGO249. The expression vector used for the S. lipmanii IPNS gene was derived from pCZR111 (27) and contains genetic elements similar to those on pOGO216 described above. A notable difference is the NdeI site at the translation initiation site of pOGO249, whereas pOGO216 contains an Ncol site at the translation initiation site. E. coli K-12 JM109 cells containing pOGO216 or pOGO249 were grown at 30°C in broth containing 50 μg of kanamycin per ml and then diluted 1:100 into fresh broth containing antibiotic, and the temperature was shifted to 42°C. The cells were incubated with shaking at 42°C for about 6 h, during which time gene expression occurred and the foreign proteins accumulated inside the cells. After incubation at 42°C, the cells were found to contain inclusion bodies or granules when observed under phase-contrast microscopy. To be sure that the material in the granule was dissolved for SDS-PAGE analysis, the cells were dissolved in sample buffer containing 2% SDS, 6 M urea, and 1% β-mercaptoethanol.

**IPNS assays.** IPNS assays were carried out on cell extracts of E. coli or S. lipmanii by monitoring production of isopenicillin N from LLD-ACV by the antibiotic activity of isopenicillin N against Micrococcus luteus in an agar plate assay, as described previously (28).

**Synthesis of DNA oligonucleotides.** DNA oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer according to the manufacturer's recommended protocols.

**RESULTS**

Cloning of A. nidulans and S. lipmanii IPNS genes. The A. nidulans and S. lipmanii IPNS genes were isolated from lambda banks by using DNA hybridization probes. The probes used to isolate the A. nidulans gene were the C. acremonium and P. chrysogenum IPNS genes described previously (6, 23). The P. chrysogenum probe pXL2 was found to hybridize more strongly to the A. nidulans DNA, but both probes hybridized to a group of the same recombinant lambda phages. A portion of the insert from one such phage was cloned into pUC18 to create pOGO4 (Fig. 1A).

The probe used to isolate the S. lipmanii IPNS gene was a 60-bp synthetic DNA fragment based on amino acid sequences of purified IPNS. The S. lipmanii IPNS protein was purified and subjected to amino-terminal sequencing, and the amino-terminal amino acid sequence obtained was NH\(^2\)-Met-Pro-Val-Leu-Met-Pro-Ser-Ala-Asp-Val-Pro-Thr-Ile-Asp-Ile-Ser-Pro-Leu-Phe-Gly-Thr-Asp-Pro. The DNA probe based on this sequence encoded all but the first two and the last amino acids and had the sequence 5'-GTCTCGTAG CCGTCCGCCAACGTTCCGCCACCATCGAATCTCCCG CTTGTCGGCAGCAGC. The DNA probe was designed to utilize preferred codons from Streptomyces genes, which typically have a high G+C content (4). The experimentally derived amino acid sequence included aspartate residues at positions 9 and 22, whereas the fungal IPNS genes contained asparagine residues. Since asparagine might be artifically produced from asparagine during purification or sequencing, we incorporated asparagine codons at these positions. It turned out that our probe was incorrect at these positions and that aspartate was genetically encoded at these positions. However, the probe was still successfully used to isolate the IPNS gene. Even with the two incorrect amino acid codons, the probe matched the actual sequence at 31 of 60 bases.

**Sequence analysis of the IPNS genes.** The sequence of the S. lipmanii IPNS gene is shown in Fig. 2 and that of the A. nidulans IPNS gene is shown in Fig. 3. The primary DNA sequences of the S. lipmanii and A. nidulans IPNS genes are shown in Fig. 3 and differ from the E. coli K-12 IPNS and P. chrysogenum IPNS genes (6, 23). The identities at the DNA level of the protein-coding regions of the four IPNS genes are summarized in the lower left of Fig. 4.

The predicted amino acid sequences of the IPNS genes are also very similar, as shown by the degree of identity in the upper right of Fig. 4. The pairwise comparisons show amino acid identities ranging from 53.9 to 80.6%. A less stringent comparison is illustrated in Fig. 5, in which a manual alignment of the four amino acid sequences is presented. In this comparison, if two pairs of matches occurred at a single amino acid residue position, then all four were boxed. The comparison showed that the A. nidulans IPNS amino acid sequence matched at least one of the other three IPNS sequences at 88% of the residues; the P. chrysogenum IPNS matched one of the others at 87% of the residues; the C. acremonium IPNS matched one of the others at 82% of the residues; and the S. lipmanii IPNS matched one of the others at 68% of the amino acid residues. These comparisons account only for identical amino acid matches. No consideration is given for amino acids with similar side chains.

**Expression of the IPNS genes in E. coli.** While the close homology between the A. nidulans and S. lipmanii genes described in this paper and the previously described fungal IPNS genes (6, 23) strongly suggests that the former are indeed IPNS genes, proof was provided by expression of the IPNS activity in E. coli. The open reading frames from the A. nidulans and S. lipmanii genes were inserted into an E. coli expression vector as described in Materials and Methods. The proteins encoded by the open reading frames were expressed at high levels in E. coli, as shown in Fig. 6. Extracts from E. coli cells containing the expression vectors were assayed for IPNS activity by testing their ability to convert LLD-ACV to a molecule with penicillinase-sensitive antibiotic activity against M. luteus (presumably isopenicillin N). Portions of the in vitro reaction mixtures were incorporated into the wells of agar plates seeded with M. luteus, and the zone of inhibition of growth of the organism around the wells was measured as an indication of IPNS activity. Zone sizes of 12 mm and above are indicative of IPNS activity, and zone sizes at least 20 to 25 mm were observed with extracts from both the A. nidulans and S.
FIG. 2. DNA sequence of the A. nidulans IPNS gene.
S. LIPMANNII AND A. NIDULANS ISOPENICILLIN N SYNTHETASE

FIG. 3. DNA sequence of the S. lipmanii IPNS gene.
lipmanii IPNS expression vectors. These zones were eliminated when penicillinase was added to the wells along with the reaction mixture, and appearance of zones was dependent on the addition of the substrate LLD-ACV and appropriate E. coli extract to the in vitro assay.

**Features of the IPNS genes.** The DNA sequences reported in Fig. 2 and 3 include several features characteristic of transcribed and translated genes. Open reading frames can often be identified by examining the G+C content bias as a function of codon position, especially for organisms with unusually high or low G+C content in their genome. Since Streptomyces species often have unusually high G+C content, we examined the G+C content as a function of codon position for the S. lipmanii IPNS gene by using an algorithm described by Bibb et al. (5). The Streptomyces IPNS gene had a pronounced G+C content bias as a function of codon position over the entire open reading frame, as shown in Fig. 7A. The overall G+C content of this gene was about 66%. Interestingly, even though the A. nidulans IPNS gene was only 52.4% G+C, the codon usage bias allowed discrimination of the open reading frame by using the Bibb algorithm, as shown in Fig. 7B.

The Streptomyces IPNS gene contained a Shine-Dalgarno binding sequence (13) in the 5' noncoding region and an inverted repeat in the 3' noncoding region, as is typical for procaryotic genes. The sequence 5'-GGAGG was separated from the translation initiation site by 5 bases, which is also characteristic of procaryotic Shine-Dalgarno sites (13). The sequence 5'-GCGGGGCACAGGCTGGTCCCGGC was found 35 bases downstream of the translation termination codon. This sequence contained a possible stem-and-loop structure, with 9 bp (8 of them G+C pairs) in the stem and 6 bp in the loop. This type of structure is commonly found downstream of Streptomyces genes (4).

**DISCUSSION**

The IPNS genes from A. nidulans and S. lipmanii have been cloned and characterized. Proof of the identity of the genes was obtained by expressing the genes in E. coli and obtaining IPNS activity from the E. coli cell extracts. The assignments of the translation initiation sites for expression of the open reading frames in E. coli were based primarily on a comparison with the predicted amino acid sequences of previously characterized IPNS genes, although other evidence supports the assignments. In the case of the S. lipmanii IPNS, the experimentally derived amino acid sequence of the protein purified from S. lipmanii cells was consistent with translation initiation at the methionine codon shown in Fig. 2, and a typical Shine-Dalgarno site with appropriate spacing was found adjacent to this methionine codon. Also, the codon bias of the predicted amino acid sequences indicates that the open reading frame should start near the methionine codons indicated in Fig. 2 and 3. As shown in Fig. 7, analysis of the G+C content as a function of codon position strongly indicated a protein-coding region exactly coinciding with the predicted boundaries of the IPNS open reading frames. It is particularly interesting that this type of analysis is meaningful for the Aspergillus gene, since the overall G+C content of this S. lipmanii IPNS is only 52.4%, compared with 65.3% for the S. lipmanii IPNS.

The high levels of expression obtained in E. coli with the A. nidulans and S. lipmanii open reading frames (Fig. 6) should provide a useful source of protein for biochemical analysis of these IPNS proteins. It is likely that the proteins purified from E. coli would be representative of the native proteins, since previous studies have shown that the C. acremonium IPNS protein purified from E. coli is indistinguishable, by stringent criteria, from the C. acremonium IPNS protein purified from C. acremonium cells (2).

The A. nidulans and S. lipmanii IPNS genes are closely homologous to each other and to previously characterized IPNS genes from two other fungi, C. acremonium and P. chrysogenum. The percent identities for pairwise comparisons of the aligned DNA sequences of the four IPNS genes ranged from 62.5 to 75.7%, and the identities for the predicted amino acid sequences ranged from 53.9 to 80.6%, as summarized in Fig. 4.

The close relationship of the IPNS genes is even more apparent when overall comparisons are made. For example, the predicted amino acid sequences of the individual IPNS genes matched those of at least one of the other three IPNS genes at from 68 to 88% of the amino acids. The three fungal IPNS genes are particularly close, matching in this type of comparison at from 82 to 88% of the amino acids (Fig. 5). The close homology among the IPNS genes continues to frustrate attempts to identify regions important for catalysis based on conserved residues. We have previously shown that a cysteine residue in the C. acremonium IPNS is important for activity and substrate binding (24). This cysteine, residue 106 in the C. acremonium numbering, is found in all four of the IPNS proteins. Furthermore, four residues on each side of this cysteine residue are identical in the four IPNS proteins, except for a tryptophan on the amino-terminal side of the cysteine in the S. lipmanii protein, compared with a phenylalanine residue at this position in the fungal proteins. A similar amino acid sequence is found in another β-lactam-biosynthetic gene, the deacetoxycephalosporin C synthetase/deacetylcephalosporin C synthetase from C. acremonium (25), further suggesting that this region might be important for substrate binding. All four IPNS proteins contain another conserved cysteine, residue 253 in the C. acremonium numbering (S. lipmanii IPNS also contains two other cysteines not found in the fungal IPNS proteins). Mutation of this second conserved cysteine in the C. acremonium IPNS had little effect on activity or substrate binding (24). Further analysis of the active site of the IPNS will require genetic manipulation and physical analysis of the IPNS gene products.

**Evolutionary relationship of the IPNS genes.** The strong homology of the IPNS genes suggests a close evolutionary relationship among the genes. Particularly interesting is the close relationship between the procaryotic IPNS gene from E. coli and the fungal IPNS genes. Procaryotes and eucaryotes diverged about 2 billion years ago, based on differences in SS RNA sequences (15). The fungal genera *Cephalosporium*, *Penicillium*, and *Aspergillus* diverged
S. LIPMAVI AND A. NIDULANS ISOPENICILLIN N SYNTHETASE

<table>
<thead>
<tr>
<th>Alanine</th>
<th>Valine</th>
<th>Methionine</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Tyrosine</th>
<th>Phenylalanine</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>V</td>
<td>M</td>
<td>I</td>
<td>L</td>
<td>T</td>
<td>P</td>
<td>H</td>
</tr>
</tbody>
</table>

**Fig. 5.** Overall comparison of amino acid sequences of PNS genes. Abbreviations are the same as those used in Fig. 4. Where two pairs of matches occurred at a single site in the sequence, all four residues were boxed.

**Table:**

<table>
<thead>
<tr>
<th>Alanine</th>
<th>Valine</th>
<th>Methionine</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Tyrosine</th>
<th>Phenylalanine</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>V</td>
<td>M</td>
<td>I</td>
<td>L</td>
<td>T</td>
<td>P</td>
<td>H</td>
</tr>
</tbody>
</table>

**Legend:**

- **Alanine (A)**
- **Valine (V)**
- **Methionine (M)**
- **Isoleucine (I)**
- **Leucine (L)**
- **Tyrosine (T)**
- **Phenylalanine (P)**
- **Hydroxyproline (H)**

This table shows the comparison of amino acid sequences for PNS genes, indicating matches and differences. The abbreviations used are consistent with those in Fig. 4.
much more recently, probably several hundred million years ago (8, 29). The degree of conservation of amino acid sequences of a gene from different organisms can be used as a molecular clock to indicate approximately when the organisms diverged. The 75.2 to 80.6% identity among the fungal IPNS genes is fairly typical for organisms that diverged several hundred million years ago. However, the 53.9 to 56.9% identity among the S. lipmanii and the fungal IPNS genes is much higher than would be expected for organisms that diverged almost 2 billion years ago. As summarized by Bardwell and Craig (3), some of the strongest homologies known between procaryotes and eucaryotes are the approximately 52% identity between glyceraldehyde-3-phosphate dehydrogenase amino acid sequences, 45% for triose phosphate isomerase, 50% for heat shock protein Hsp70, and 42% for heat shock protein Hsp83. The only known eucaryotic genes that are more closely homologous to procaryotic genes are mitochondrial genes, which are believed to have been transferred horizontally long after the divergence of eucaryotes and procaryotes (30).

There are at least two possible explanations for the unusually high homology of the IPNS genes. One explanation is that the IPNS gene is relatively slow to change during evolution, perhaps because of strict functional requirements

for many amino acids or because of the evolutionary importance of IPNS. However, the rate of evolutionary drift of the fungal IPNS genes is fairly typical, and the IPNS is a secondary metabolism gene which is not necessary for survival. A second and preferred explanation is that an IPNS gene was transferred horizontally well after the divergence between eucaryotes and procaryotes. We have suggested earlier, based on the complexity of the β-lactam-biosynthetic pathways found in the various producing organisms, that the pathway arose first in a procaryote and was then horizontally transferred to eucaryotes (6).
this transfer occurred, based on our DNA sequences and a rate of nucleotide substitution of $10^{-9}$ nucleotide changes per site per year (19), is 370 million years ago. A phylogenetic tree based on these data is shown in Fig. 8, which is consistent with a phylogenetic tree for these organisms based on SS rRNA sequence similarities (9). The horizontal transfer hypothesis shown in Fig. 8 predicts that other β-lactam-biosynthetic genes from procaryotes and eucaryotes will also be about 36% identical at the amino acid level and about 63% identical at the DNA level, since it is most likely that all the necessary β-lactam-biosynthetic genes were transferred concomitantly. Cloning and characterization of a deacetoxycephalosporin C synthetase gene from Streptomyces spp. will allow such a comparison, since the fungal equivalent has already been cloned and characterized (25). It will also be interesting to compare another gene common to both fungi and procaryotes, such as an amino acid-biosynthetic enzyme gene, to obtain an independent prediction of evolutionary divergence times. Our hypothesis predicts that the evolutionary divergence times for the fungi will be the same whether the IPNS or an amino acid-biosynthetic gene is used as a clock but that the amino acid-biosynthetic gene homologies will predict that Streptomyces spp. and fungi diverged much longer than 370 million years ago.

The horizontal-transfer hypothesis also provides an explanation for the observation that a wide variety of fungi, including species of Paeclomycetes, Polypaecilium, Emericellopsis, Trichophyton, and others (reviewed by Jensen [16]), are capable of synthesizing β-lactams, whereas other classes of lower eucaryotes, such as yeasts, have no β-lactam-biosynthetic ability. Perhaps the β-lactam-producing fungi diverged after their ancestor had received β-lactam-biosynthetic genes in a horizontal transfer from a procaryotic species, but yeasts split off from the fungi before the transfer occurred. Phylogenetic groupings based on SS rRNA sequence similarities do in fact indicate that yeasts split from the fungal line well before the divergence of the three fungal species studied in this report (9). An evolutionary window of time therefore exists for uptake of β-lactam-biosynthetic genes between the divergence of yeast from fungi about 500 million years ago and the divergence of the various fungal species about 300 million years ago. As this paper was being prepared for submission, the DNA sequence of the S. clavuligerus IPNS gene was provided to us before publication (18a). The S. clavuligerus IPNS gene is also about 63% identical to the fungal IPNS genes at the DNA level, indicating that it has the same ancestral relationship to the fungal genes as the S. lipmanii IPNS. The two Streptomyces IPNS genes are only 79% identical, suggesting that the two species are about as distantly related as are A. nidulans and P. chrysogenum.

ACKNOWLEDGMENTS

We thank Lilly Research Laboratories for support of this work. We also thank S. Samson, M. Slisz, D. Van Frank, S. Haley, R. Belagaje, B. Glover, S. Ly, I. Jenkins, J. Shepherd, and L. Carr for assistance and advice, R. H. Balz and B. Schoner for critical reading of the manuscript and helpful discussions, D. W. S. Westlake and S. E. Jensen for communication of their results before publication, and Barbara Fogelman for assistance with manuscript preparation.

ADDITIONAL IN PROOF

After this manuscript was submitted for review, a paper was published reporting the cloning of the IPNS gene from A. nidulans (D. Ramon, L. Carramolino, C. Patiño, F. Sanchez, and M. A. Petalva, Gene 57:171–181, 1987). This paper reported a DNA coding sequence for the A. nidulans IPNS gene which agrees with the DNA coding sequence reported in the present study.

LITERATURE CITED


