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Streptogramin B Biosynthesis in *Streptomyces pristinaespiralis* and *Streptomyces virginiae*: Molecular Characterization of the Last Structural Peptide Synthetase Gene

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***Streptomyces pristinaespiralis* and *S. virginiae* both produce closely related hexadepsipeptide antibiotics of the streptogramin B family. Pristinamycins I and virginiamycins S differ only in the fifth incorporated precursor, di(mono)methylated amine and phenylalanine, respectively. By using degenerate oligonucleotide probes derived from internal sequences of the purified *S. pristinaespiralis* SnbD and SnbE proteins, the genes from two streptogramin B producers, *S. pristinaespiralis* and *S. virginiae*, encoding the peptide synthetase involved in the activation and incorporation of the last four precursors (proline, 4-dimethylparaaminophenylalanine [for pristinamycin I_A] or phenylalanine [for virginiamycin S], pipecolic acid, and phenylglycine) were cloned. Analysis of the sequence revealed that SnbD and SnbE are encoded by a unique *snbDE* gene. SnbDE (4,849 amino acids [aa]) contains four amino acid activation domains, four condensation domains, an N-methylation domain, and a C-terminal thioesterase domain. Comparison of the sequences of 55 amino acid-activating modules from different origins confirmed that these sequences contain enough information for the performance of legitimate predictions of their substrate specificity. Partial sequencing (1,993 aa) of the SnbDE protein of *S. virginiae* allowed comparison of the proline and aromatic acid activation domains of the two species and the identification of coupled frameshift mutations.**

Pristinamycins I (PI) and virginiamycins S (VS) are members of the so-called streptogramin B or mikamycin B group (7, 29). These two cyclohexadepsipeptides differ only by the presence of 4-dimethylparaaminophenylalanine (DMPAPA) in PI_A or 4-monomethylparaaminophenylalanine (MMPAPA) in PI_B and of phenylalanine in the VS macrocycle (Fig. 1). Like other antibiotics in this family, PI is synthesized nonribosomally by a multienzymatic peptide synthetase (PPS) complex (28). PPSs are composed of modules each involved in the incorporation of one amino acid. Both limited proteolysis data and analysis of the amino acid sequence show that these modules are multifunctional. Domains involved in activation, thioesterification, condensation, and, in some cases, modification (N-methylation or epimerization) have been identified (recently reviewed by Stein and Vater [27] and Kleinkauf and von Döhren [17]).

The *Streptomyces pristinaespiralis* peptide synthetases involved in PI synthesis had been purified previously (28). SnbA is involved in the activation of the 3-hydroxypicolinic acid starter unit, SnbC is involved in the incorporation of the first and second residues (threonine and L-aminobutyric acid), and SnbDE is involved in the activation of the last four residues of the PI macrocycle (proline, DMPAPA or MMPAPA, L-pipecolic acid, and phenylglycine). SnbDE was purified as two major proteolytic fragments, SnbD and SnbE. Cloning and sequencing of the *snbA* and *snbC* genes previously confirmed the involvement of PPSs in the biosynthesis of PI (9).

Understanding the molecular basis of the substrate specificity of PPS enzymes is an important issue. It has been shown that the information is contained entirely in the activation domain, as replacing a given activation domain with another selectively changes the amino acid incorporated (26). Such a situation occurs in nature, as VS and PI differ only by the fifth residue. One can argue that no PI is produced by *S. virginiae* because the DMPAPA pathway is absent in this strain (Southern hybridization using probes derived from the DMPAPA precursor pathway genes does not give any positive signal with *S. virginiae* chromosomal DNA [3]). However, *S. pristinaespiralis* does not produce any VS although phenylalanine is available. Biochemical characterization of the affinities of SnbDE from both producers shows that the substrate specificity profiles of the modules responsible for the incorporation of the fifth residue are different (28). The comparison of the primary structures of the peptide synthetase domains involved in DMPAPA and phenylalanine activation in both organisms could therefore be informative on the molecular basis of specificity. The genes encoding the SnbDE enzymes in the two strains were cloned and sequenced.

MATERIALS AND METHODS

Bacterial strains, phages, cosmids, and plasmids. The bacterial strains, phages, cosmids, and plasmids used in this study are listed in Table 1.

Media and bacteriological conditions. *Streptomyces* strains were maintained on HT agar medium (24) and grown in YEME medium (16) at 30°C. *Escherichia coli* strains were grown in LB medium at 37°C (22). Selection was done with ampicillin at 100 μg/ml or tetracycline at 12 μg/ml in LB agar or liquid medium. *E. coli* TG1 was used as the recipient strain for plasmid transformation and to obtain single-stranded DNA from M13 vectors.

DNA procedures. All of the DNA procedures used in this study have been described previously (9). After restriction mapping of the DNA fragment to be sequenced, restriction fragments of sizes varying from 200 to 2,000 bp were subcloned in M13 vectors and the sequence on both strands was obtained as described previously (9).

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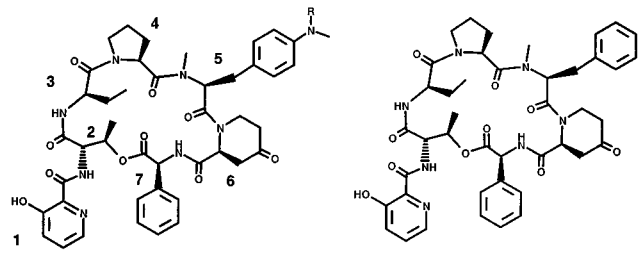

FIG. 1. Structures of PI_A, PI_B, and VS_1.

Transformation. Competent *E. coli* cells were prepared and transformed as described by Chung and Miller (6).

Oligonucleotide probes. As previously reported (28), internal sequences obtained by tryptic digestion were identified as VTPYRAYALAHLAG for SnbD and VTVFLNNTRLIQNFRPR for SnbE. The following degenerate oligonucleotide probes, derived from parts of the internal sequences of SnbD (VTPYRAY AL) and SnbE (TRLIQNFRPR), were synthesized (degenerate positions are in parentheses): OL1, 5′-GT(CG)AC(CG)CC(GC)TACCG(CGT)GC(CG)TAC-3′; OL2, 5′-AC(GC)CG(CGT)CT(CG)ATCCAGAACTTCCG(CGT)CC-3′.

Construction of an *S. virginiae* genomic library. A partial *Bgl*II digest of *S. virginiae* genomic DNA was fractionated on a 20 to 40% sucrose gradient as described by Maniatis et al. (22). DNA fragments (15 to 25 kb) were ligated with pXL667 linearized with *Bam*HI. In vitro packaging using the Gigapack II Gold Packaging Extract (Stratagene, La Jolla, Calif.) was performed as described by the manufacturer. Transfections of the packaged DNA were performed with strain HB101. Two thousands colonies were selected on LB agar supplemented with tetracycline. Selected clones were individually grown in 200 μl of Hogness medium (4) in 96-well microplates and stored at −80°C.

Analysis of sequence data. Nucleic and amino acid sequences were analyzed by using the GCG package (Program Manual for the Wisconsin Package, version 8, September 1994; Genetics Computer Group, Madison, Wis.). A codon preference table was previously established (4). Amino acid sequences were compared with the GenBank, NBRF, and SwissProt databases by using either FASTA or BLASTP (1). Multiple alignments were performed by using the CLUSTAL program (14).

Nucleotide sequence accession numbers. The nucleotide sequences described in this report have been submitted to EMBL under accession numbers Y11548, for the region containing the *S. pristinaespiralis snbDE* gene, and Y11547, for the region containing the *S. virginiae snbDE* gene.

RESULTS AND DISCUSSION

Cloning of the *snbDE* gene of *S. pristinaespiralis*. To clone the structural genes encoding SnbDE, oligonucleotide probes OL1 and OL2 were synthesized on the basis of the internal amino acid sequences of the SnbD and SnbE protein fragments. These oligonucleotides were used as hybridization probes for Southern blot analysis of *S. pristinaespiralis* genomic DNA and of cosmids of the genomic library previously isolated and containing genes involved in PI biosynthesis (4, 9). Both oligonucleotide probes hybridized with the pIBV3 cosmid isolated previously as containing part of the *snbC* gene (Fig. 2A) (9). Southern blot hybridization revealed that OL1 hybridized with a 250-bp *Bgl*II-*Sph*I fragment (fragment A in Fig. 2B) and OL2 hybridized with a 1.5-kb *Pst*I-*Pst*I fragment (fragment B in Fig. 2B), both on the cosmid and on the chromosomal DNA (data not shown). The sequence of a 15-kb DNA fragment located downstream of the *snbC* gene was determined on both strands. A 14,547-bp open reading frame (ORF) with a typical *Streptomyces* codon bias throughout was identified with a possible ATG start codon overlapping the *snbC* TGA stop codon and ending with a TGA stop codon. The codon usage and the presence of a possible Shine-Dalgarno sequence (GGAAGG) located 8 bp upstream of the putative ATG codon made us favor this initiation position but another candidate could be a GTG codon located 8 bp upstream. This ORF encodes a putative protein of 4,849 amino acids (aa) with a predicted molecular mass of 521,767 Da. The sequences of the tryptic fragments derived from the purified SnbD and SnbE protein fragments were found at positions 178 to 190 and 4764 to 4780, respectively, of the deduced amino acid sequence. The SnbD and SnbE proteins are encoded by a unique gene that was named *snbDE*. Cases of partial proteolysis during purification are frequent with peptide synthetases and have been discussed previously (28).

Previous compilation of the sequences of known peptide synthetase modules had enabled us to define consensus sequences for the different peptide synthetase domains (10). These consensus sequences were used to delimit the different domains of SnbDE. As shown in Fig. 3A, SnbDE contains four amino acid activation and thioesterification domains (positions 464 to 1008, 1514 to 1963 and 2349 to 2451, 2948 to 3483, and 3998 to 4549), four elongation domains (positions 1 to 463, 1009 to 1513, 2452 to 2947, and 3484 to 3997), one N-methylation domain (positions 1963 to 2348), and one C-terminal thioesterase domain (positions 4603 to 4821). The assignment of which amino acid was activated by which domain was proposed with the assumption that a strict correlation exists between the order of the amino acid domains and the sequence of the peptide. This hypothesis has been confirmed in several systems (27). Characterization of the respective amino acid ATP-pyrophosphate exchanges of the SnbD and SnbE fragments performed by Thibaut et al. (28), as well as the position of the N-methylation domain on the sequence, confirmed the colinearity hypothesis. It has also been shown previously that SnbDE activates not 4-oxopipecolic acid but L-pipecolic acid and that the oxidation is done after synthesis of the cyclic molecule (28).

Cloning and partial sequencing of the *snbC* and *snbDE* genes from *S. virginiae*. The *S. pristinaespiralis* 3.6-kb *Xho*I

TABLE 1. Bacterial strains, phages, cosmids, and plasmids used in this study

Bacterium or DNA	Relevant properties	Source or reference
E. coli strains		
HB101	F⁻ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	22
TG1	*E. coli* K-12 Δ(*lac-pro*) *supE thi hsd* ΔS5/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ* ΔM15	12
S. pristinaespiralis	SP92 natural isolate of *S. pristinaespiralis* ATCC 25486	4
S. virginiae	ATCC 13161	
Phage M13mp18/19	Multicloning site vector	Boehringer
Cosmids		
pHC79	Amp^r	15
pXL667	pLFRA1 derivative; Tet^r	11
pIBV3	pHC79 derivative containing *snaA* and *snbC snbDE* genes of *S. pristinaespiralis*; Tet^r	4
pIBV30	pXL667 derivative containing *snbC* and *snbDE* genes of *S. virginiae*; Amp^r	This work

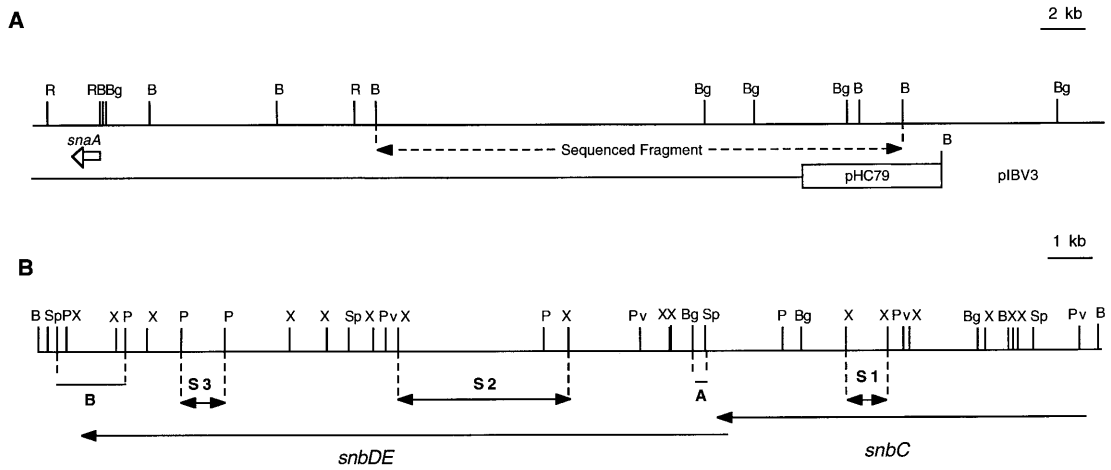

FIG. 2. (A) Restriction map of the *S. pristinaespiralis* chromosomal region containing the *snbC* and *snbDE* genes. (B) Restriction map of the sequenced fragment. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; X, *Xho*I; Sp, *Sph*I. A and B represent the fragments hybridizing with the OL1 and OL2 oligonucleotide probes, respectively. S1, S2, and S3 represent the pIBV3 restriction fragments used as probes for hybridization of *S. virginiae* chromosomal DNA.

fragment (S2 probe) (Fig. 2B) containing the region encoding the DMPAPA activation domain was used to screen the *S. virginiae* DNA library. Two clones hybridizing with the S2 probe were isolated. They both contained a 3-kb *Bam*HI doublet hybridizing with the probe. One of them, named pIBV30, containing a 22-kb insert, was studied further (Fig. 4). Restriction analysis of pIBV30 (Fig. 4) indicated that it corresponds to the cocloning of a small (1.9-kb) *Bgl*II fragment and a large (20-kb) *Bgl*II fragment. The larger one contained the region complementary to the S2 probe. This 3-kb *Bam*HI doublet could be separated by *Sac*I digestion, giving a 3-kb *Bam*HI fragment and a 2.2-kb *Bam*HI-*Sac*I fragment, both hybridizing with the S2 probe. This double hybridization was most certainly due to the strong sequence similarities found between modules of a PPS enzyme.

To determine if the *snbC* and *snbDE* genes are organized similarly in the two species, other probes from the *S. pristinaespiralis* genes were used. Both the S1 probe (0.9-kb *Xho*I fragment) and the S3 probe (1-kb *Pst*I fragment), shown in Fig. 2B, hybridized with pIBV30, suggesting that the genes encoding the structural streptogramin B enzymes are organized similarly in the two strains. The sequence of a 6-kb *Bam*HI-*Bam*HI fragment (Fig. 4) was determined on both strands. An ORF with typical *Streptomyces* codon usage throughout the whole fragment was identified. This ORF encoded a putative protein of 1,993 aa highly similar (64.2% identity) to the SnbDE protein of *S. pristinaespiralis*. As shown on Fig. 3B, the similarity started at the beginning of the proline-activating domain and finished at the end of the N-methylation domain.

Comparison of the substrate specificity domains of the proline and DMPAPA (or phenylalanine)-activating domains of *S. pristinaespiralis* and *S. virginiae*. Cosmina et al. (8) had previously proposed that a 200-aa region of the activation domains located between nucleotide-binding motifs C (SGTTGxPKG) and E (GEL) (following the nomenclature of Stein and Vater [27]) is the amino acid recognition domain. Before comparing the corresponding regions of the DMPAPA-activating domain of *S. pristinaespiralis* and the phenylalanine-activating domain of *S. virginiae*, we wanted to confirm the study performed with 12 domains of *Bacillus subtilis* origin (8) by using a more extended database. The sequences of 62 peptide synthetase domains for which an amino acid activation activity had been assigned were collected. Seven of these domains activated an amino acid that was not activated by any other domain, and their sequence could therefore not be used in a prediction test. A database of the 55 remaining predictable

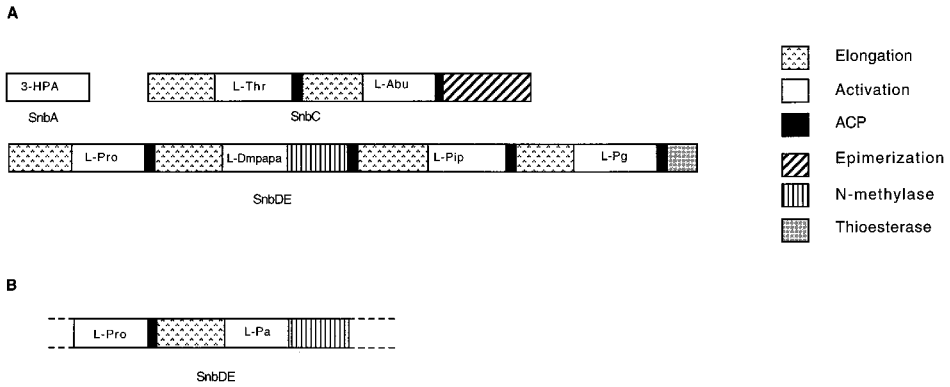

FIG. 3. Distribution of the peptide synthetase domain regions within SnbA, SnbC, and SnbDE of *S. pristinaespiralis* (A) and the sequenced fragment of the SnbDE protein of *S. virginiae* (B). ACP, acyl carrier protein; L-Abu, L-aminobutyric acid; 3-HPA, 3-hydroxypicolinic acid; L-Thr, L-threonine; L-Pro, L-proline; L-Pip, L-pipecolic acid; L-Pg, L-phenylglycine; L-Pa, L-phenylalanine.

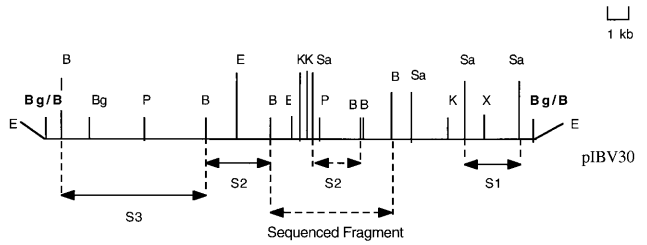

FIG. 4. Restriction map of the pIBV30 plasmid containing the *S. virginiae* peptide synthetase genes. Abbreviations are defined in the legend to Fig. 2. S1, S2, and S3 represent the pIBV30 restriction fragments hybridizing with the S1, S2, and S3 probes described in Fig. 2. K, *Kpn*I; Sa, *Sac*I; E, *Eco*RI.

domains was used. For each domain, the prediction procedure consisted of giving it the activity of the most similar domain among the other 54 domains. The similarity was estimated by using the BLASTP program. Forty-three percent of the predictions made were correct. To determine if this frequency was significantly higher than that obtained by random prediction, the amino acids were reassigned at random to the domains and the prediction procedure was repeated 100 times. The frequency of identical amino acid prediction after random assignment dropped to an average of 6.6% and was always less than 22%. We have now established with statistical significance that the sequences of the amino acid-activating domains contain enough information for performance of legitimate predictions of their amino acid-activating activity. From a practical point of view, however, one has to point out that the frequency of good prediction is relatively low (43%) even when all of the available sequences are used. It is hoped that better efficiency will be obtained when the size of the data bank increases.

The sequences of the amino acid recognition domains from the *S. pristinaespiralis* DMPAPA (MMPAPA)-activating domain and from the *S. virginiae* phenylalanine-activating domain present 62% identity and 75% similarity, whereas the two proline-activating domains present 73% identity and 84% similarity (Fig. 5A and B). It can be noticed that the nonconservative differences between the two proline domains never exceed one amino acid, whereas a stretch of four nonconservative amino acids can be identified between the aromatic activating domains. Translation in the three phases of the DNA encoding this stretch reveals that the sequence of one can be changed into the sequence of the other by a double frameshift. Comparison of the nucleotide sequences shows a two-base insertion and a two-base deletion (Fig. 5C). The sequence was double checked in this region in both species, and no error could be detected. By definition, these changes constitute a coupled frameshift mutation analogous to those described in a number of bacteria and phages (25). Site-directed mutagenesis has to be performed to test the role of these amino acids in substrate specificity.

Analysis of the sequences of the N-methylase and thioesterase domains. The determination of the sequences of the SnbDE N-methylation domains in *S. pristinaespiralis* and *S. virginiae* brings to 10 the number of sequenced peptide synthetase domains transferring the methyl group from *S*-adenosyl-L-methionine to the NH_2 of the amino acid activated as a thioester on the enzyme. Others are found in the sequence of enniatin synthetase (2, 13), and seven are found in cyclosporin A synthetase (18, 30). Detailed structural data available for a great number of *S*-adenosine-dependent methyltransferases have suggested a common structure for these enzymes (21, 23). The PPS methylase domains clearly contain signature motif I, which is involved in making direct contacts with the methionine moiety of *S*-adenosyl-L-methionine (23). As shown in Fig. 6A, motif I consists of the signature motif GXGXG located at a β-α turn followed by a conserved Asp or Glu residue located 17 to 19 residues downstream.

The sequences of 11 thioesterase domains of peptide synthetases are available in the data banks (Fig. 6, legend). Thioesterases are members of the superfamily of serine-dependent hydrolases including proteinases, lipases, and acetylcholinesterases. Members of this superfamily catalyze the hydrolysis of amide, ester, or thioester bonds with an acyl intermediate on the enzyme. The catalytic residues constitute what is called the chymotrypsin-like triad, where the nucleophile is usually a serine (but can also be a cysteine or an aspartate), a histidine abstracts a proton from the nucleophile during the reaction, and an aspartic acid (sometimes replaced by a glutamic acid or a tryptophan) is the third member of the triad. As shown in Fig. 6B, the only serine residue conserved in the 11 sequences is part of a G(Y/W)SXG motif consistent with the so-called lipase-esterase motif (5) which had been previously identified in PPSs by MacCabe et al. (20). Only one aspartic acid (the boxed Asp in the alignment) is conserved in all of the domains and can be proposed as the third member of the catalytic triad. At first sight, no histidine residue conserved in all 11 sequences could be identified in the expected C-terminal region (data not shown). However, histidine is an essential member of the triad and its role in catalysis by thioesterases has been confirmed by determination of the structure of the myristoyl-acyl carrier protein-specific thioesterase from *Vibrio harveyi* (19) and by site-directed mutagenesis (31). The alignments were therefore

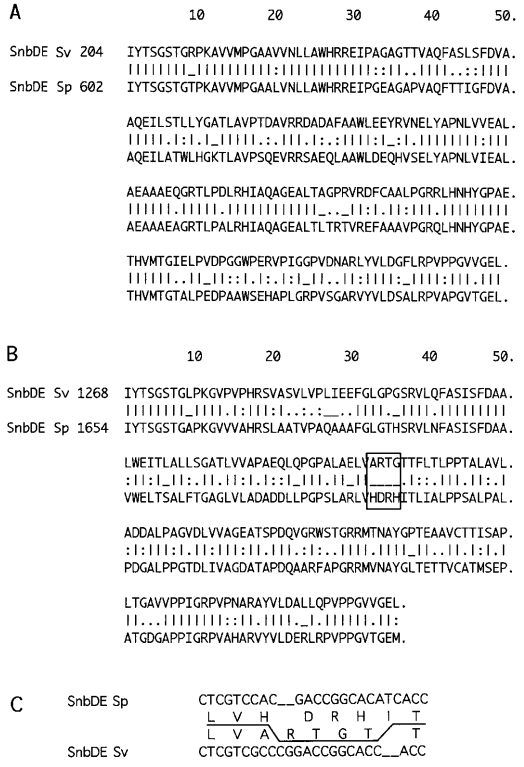

FIG. 5. Comparison (GAP program of the GCG package, default parameters) of the recognition domains for proline (A) and aromatic acids (B) of the *S. virginiae* (Sv) and *S. pristinaespiralis* (Sp) SnbDE enzymes. The nonconserved stretch is boxed. (C) Comparison of the nucleotide and amino acid sequences of the region boxed in B between the two strains.

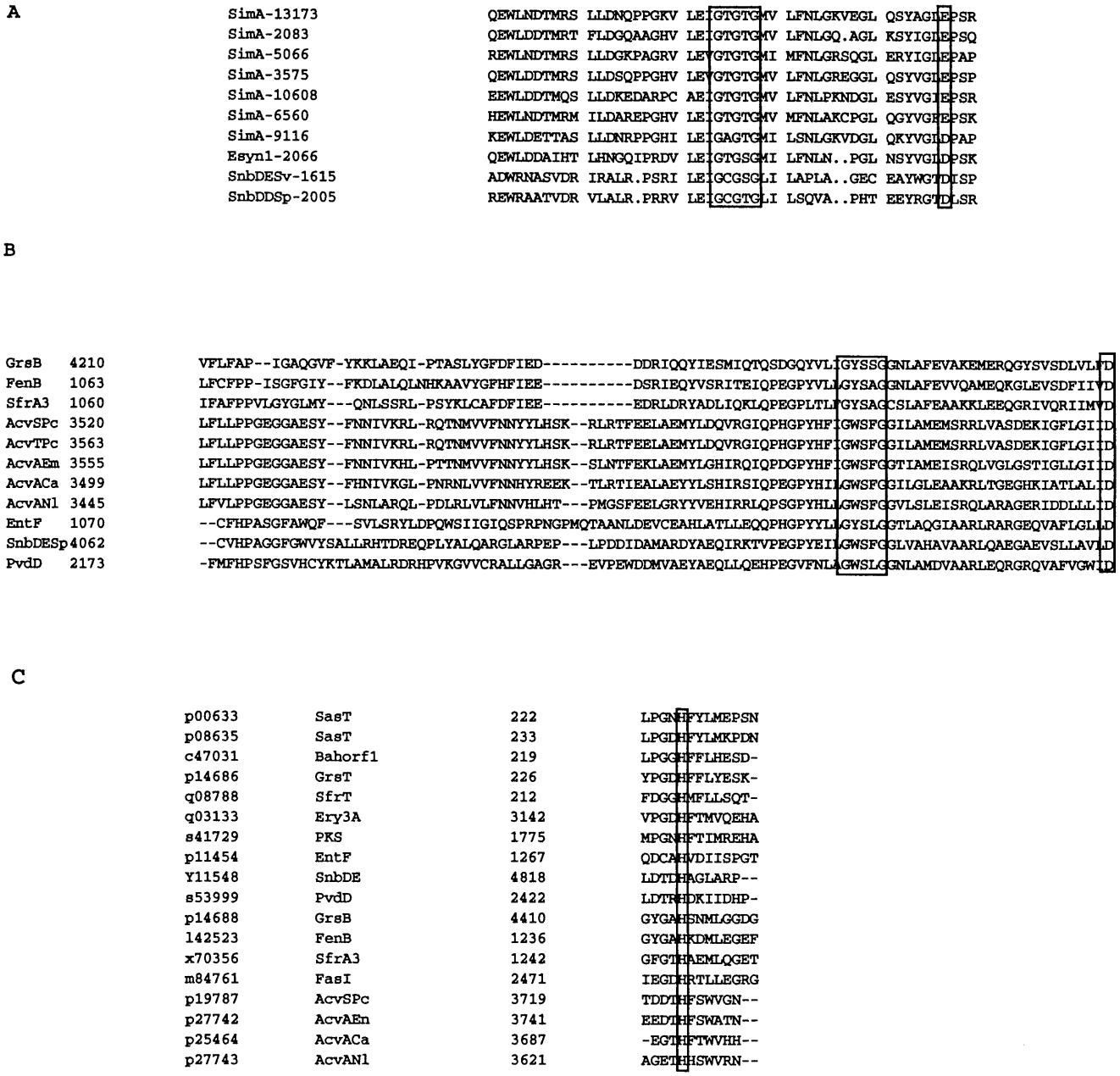

FIG. 6. Comparison of the amino acid sequences of several PPS enzymes in the regions containing the signature sequences of the N-methylase domains (A) and of the thioesterase domains (B and C). The conserved sequences are boxed. Accession numbers and the positions of the sequences are given. The peptide synthetases used in this study were the following: SimA, cyclosporin synthase (accession no. z28383); Esyn1, enniatin B synthetase (accession no. z18755); GrsB, gramicidin synthetase B; FenB, fengycin synthetase B; SfrA3, surfactin synthetase; EntF, enterobactin synthetase; PvdD, pyoverdin synthetase; AcvA, AcvS, and AcvT, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetases. The other thioesterase proteins or domains used in the alignment were the following: SasT, thioesterase II; Bahorf1, GrsT, and SfrT, proteins encoded by genes located near PPS genes; Ery3A and PKS, polyketide synthetase enzymes; FasI, fatty acid synthase. The origins and references of the enzymes can be retrieved with the accession numbers given. Abbreviations: Sv, *S. virginiae*; Sp, *S. pristinaespiralis*; Pc, *P. chrysogenum*; Em, *Emericella nidulans*; Ca, *Cephalosporium acremonium*; Nl, *Nocardia lactamdurans.*

analyzed further. It can be noted that the first halves of the thioesterase domains are more conserved than the second halves. The first 120 aa present 45 to 60% similarity in two-by-two comparisons, whereas similarities between the last 120 aa are much lower (30 to 45% similarity). The N-terminal halves contain the serine residue, which would make the acyl intermediate with the carbonyl of the thioester and therefore could be involved in recognition of the thioester bond of the pantetheinyl peptide. The C-terminal halves could be involved in recognition of the proton donor groups, which differ in the different systems. To improve the alignment in this C-terminal region, the sequences of thioesterase proteins or domains from other origins were included. As shown in Fig. 6C, the alignment of 18 thioesterase domains and proteins reveals the presence of a unique conserved histidine at the C-terminal end, corresponding to the catalytic histidine of the GXH motif of thioesterase II (31) (Fig. 6C). One exception is the AcvT synthetase from *Penicillium chrysogenum*. However, the C termi-

nus of this enzyme is very poorly conserved with respect to the sequences of the other δ-(L-α-aminoadipyl-L-cysteinyl-D-valine synthetases; this may have occurred either because a sequencing error was made or another histidine was recruited as a nucleophile in this enzyme. Structural analysis and site-directed mutagenesis are necessary to test these predictions.

As proposed previously (8, 20), the thioesterase domains of peptide synthetases are most certainly members of the lipase-serine esterase superfamily; they should therefore catalyze the transfer of the acyl intermediate from the SH group of the phosphopantetheinyl peptide to the OH group of the conserved serine residue and then the attack of the acyl intermediate by the proton donor, which can be a water molecule, a terminal amine, or a hydroxyl side chain of the elongated molecule. Like the acyl carrier protein-myristoyl acyltransferase (19), these enzymes can function as hydrolases or as acyltransferases. In PI synthesis, the C-terminal thioesterase domain could therefore be involved in the catalysis of the lactone bond formation.

Conclusions. All of the structural genes for the biosynthesis of the depsipeptide antibiotic PI have been cloned and sequenced. With gramicidin S, cyclosporin A, enniatin, HC toxin, and enterobactin (for reviews, see references 17 and 27), six complete biosynthetic systems are now available for analysis. The PI PPS enzymes are complex, as they catalyze two modification reactions (epimerization of L-aminobutyric acid and N-methylation of DMPAPA) and lactonize the peptide. Comparison of the sequences of the SnbDE proteins of *S. virginiae* and *S. pristinaespiralis* which activate the two substrates allowed the identification of candidate residues possibly involved in substrate specificity.

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