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Pristinamycin I Biosynthesis in *Streptomyces pristinaespiralis*: Molecular Characterization of the First Two Structural Peptide Synthetase Genes

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Two genes involved in the biosynthesis of the depsipeptide antibiotics pristinamycins I (PI) produced by *Streptomyces pristinaespiralis* were cloned and sequenced. The 1.7-kb *snbA* gene encodes a 3-hydroxypicolinic acid:AMP ligase, and the 7.7-kb *snbC* gene encodes PI synthetase 2, responsible for incorporating L-threonine and L-aminobutyric acid in the PI macrocycle. *snbA* and *snbC*, which encode the two first structural enzymes of PI synthesis, are not contiguous. Both genes are located in PI-specific transcriptional units, as disruption of one gene or the other led to PI-deficient strains producing normal levels of the polyunsaturated macrolactone antibiotic pristinamycin II, also produced by *S. pristinaespiralis*. Analysis of the deduced amino acid sequences showed that the SnbA protein is a member of the adenylate-forming enzyme superfamily and that the SnbC protein contains two amino acid-incorporating modules and a C-terminal epimerization domain. A model for the initiation of PI synthesis analogous to the established model of initiation of fatty acid synthesis is proposed.

Pristinamycins I (PI), cyclohexadepsipeptide antibiotics produced by *Streptomyces pristinaespiralis*, are members of the streptogramin B group. They are coproduced with the polyunsaturated macrolactone antibiotics pristinamycins II (PII), members of the streptogramin A group (6, 52). Like many other small metabolites with a peptide structure, PI are synthesized nonribosomally by large multifunctional enzymes (49). More than 25 years ago, Lipmann emphasized the analogy between nonribosomal peptide synthesis and fatty acid synthesis (25). In his model, large multifunctional enzymes called peptide synthetases (PPSs) catalyze elongation of a peptide covalently linked to a phosphopantetheinyl arm by a thioester bond (for a review, see reference 23). The original version of the thiotemplate multienzymatic mechanism was revised recently, and a multiple-carrier model was proposed (39, 43, 46, 53). According to this model, each amino acid is activated as an aminoacyl adenylate and linked to the enzyme as a thioester with a phosphopantetheinyl group. Elongation then occurs by transfer of the activated carboxyl to the amino group of the next amino acid, thus effecting N-to-C stepwise condensation. Primary structure analysis of several PPS genes resulted in the identification of approximately 1,000-amino-acid (aa)-long modules selectively catalyzing activation and condensation of one amino or hydroxy acid (30, 51). The modules are organized such that they are colinear with the sequence of the oligopeptide. Each module can be subdivided in domains. The activation domain (500 aa) belongs to the large family of adenylate-forming enzymes that includes firefly luciferase and acyl coenzyme A (acyl-CoA) synthetases and contains nine core sequences (named boxes A to I by Pfeifer et al. [31]) identified as AMP, ATP-Mg binding, adenine binding, or ATPase sites (10, 12, 14, 28, 31, 44, 45, 51). This domain is also involved in the specific recognition of a given hydroxy or amino acid (7). The activation domain is followed by the acyl carrier protein (ACP) domain (80 aa) containing the phosphopantetheinyl attachment site, LGGXSI (39, 44, 45, 46). Recently a potential elongation domain (350 aa) containing the motif HHXXXDG, which is thought to be involved in acyl transfer, was identified (9). N-methylation domains (350 aa) containing an *S*-adenosylmethionine binding site (14) can be inserted between activation and ACP domains (15). Epimerization domains (500 aa) have been identified just after some ACP domains (7, 44, 45). PPSs can also contain a C-terminal thioesterase domain presumed to be involved in releasing the peptide from the enzyme (26).

In the accompanying report, it was shown that at least three PPSs could be involved in the activation and condensation of the seven PI precursors (49). The 3-hydroxypicolinic acid (3-HPA):AMP ligase (SnbA) activates the starter hydroxy acid of the PI molecule. PI synthetase 2 (SnbC) activates two amino acids, L-threonine and L-aminobutyric acid (L-Abu). PI synthetase 3 (SnbDE), which was isolated as two proteolytic fragments, SnbD and SnbE, activates the last four amino acids of the peptide: proline, dimethylpara-aminophenylalanine, pipecolic acid, and phenylglycine.

Several enzymes activating a branched aromatic acid have been studied in the case of actinomycin, etamycin, or enterobactin synthesis. However, information at the molecular level is available only in the case of enterobactin synthesis. Enterobactin is a siderophore produced by *Escherichia coli*. Two enterobactin PPSs, EntE, the 2,3-dihydroxybenzoate (DHB): AMP ligase (36), and EntF, the serine-activating enzyme (33, 35), have been identified, and the corresponding genes have been cloned and sequenced. Etamycin is a member of the streptogramin B class produced by *Streptomyces griseoviridus*, and the enzyme involved in activation of the chromophore

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TABLE 1. Bacterial strains, phage, cosmids, and plasmids used

Strain, phage, cosmid, or plasmid	Relevant properties	Reference or source
Strains		
E. coli		
TG1	*E. coli* K-12, Δ(*lac-pro*) *supE thi hsd* ΔS5/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	11
BL21(DE3)	*E. coli* B, F$^-$ *dcm ompT hsdS*(r_B^- m_R^-) *gal* λ (DE3)	Novagen
S. pristinaespiralis SP92	Natural isolate of *S. pristinaespiralis* ATCC 25486	4
Phage		
M13mp18/19	Multicloning site vector	Boehringer
Cosmids		
pHC79	Cosmid, Amp^r	18
pIBV2	Cosmid containing the *snbA* gene, Amp^r	This work
pIBV3	Cosmid containing the *snaA* and *snbC* genes, Amp^r	4
pIBV5	Cosmid overlapping pIBV3	This work
pVRC1100	Internal *Bam*HI deletion of pIBV3	This work
Plasmids		
pUC18/19	Multicloning site vector, Amp^r	New England Biolabs
pDH5	*Streptomyces* suicide vector, Amp^r Tsr^r	17
pET11a	T7-RNA polymerase-based expression vector, Amp^r	Novagen
pVRC402	5.5-kb *Eco*RI/*Bgl*II insert from pIBV2 in pUC19 containing *snbA*, Amp^r	This work
pVRC404	1,170-bp *Xho*I/*Hin*dIII insert from pVRC402 in pDH5, Amp^r Tsr^r	This work
pVRC1105	6.2-kb *Sph*I fragment from pVRC1100 in pUC19 containing part of *snbC*, Amp^r	This work
pVRC1108	pUC19 derivative containing the Am^r gene	8a
pVRC1110	3.0-kb *Bgl*II-*Sal*I insert from pVRC1108 in pVRC1105, Amp^r Am^r	This work
pVRC1111	7-kb *Sph*I insert from pVRC1110 in pDH5, Amp^r Am^r Tsr^r	This work
pVRC1210	pET11a derivative expressing the *snbC* gene, Amp^r	This work

3-HPA was characterized (38). As emphasized by Schlumbohm and Keller (38), the actinomycin half molecule and PI are both branched depsipeptides, and their syntheses are most certainly catalyzed by similar enzymes. The first two structural enzymes of the actinomycin biosynthesis pathway, actinomycin synthetase I (ACMSI) and ACMSII, have been extensively studied (22, 47, 48). We report in this study the cloning of the two first structural genes of PI macrocycle synthesis, *snbA* and *snbC*. Careful analysis of the sequences of these genes led us to propose a model for the initiation of PI synthesis.

MATERIALS AND METHODS

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

Media and bacteriological conditions. *Streptomyces* strains were maintained on HT agar medium (32) and grown in YEME medium (19) at 30°C. Nosiheptide (for selection of thiostrepton resistance [Tsr^r]) was used at concentrations of 400 μg/ml for solid media and 2 μg/ml for liquid media. Geneticin, for selection of apramycin resistance (Am^r), was used at final concentrations of 250 μg/ml for soft agar overlays on R2YE medium (19) and 10 μg/ml in HT agar medium. Liquid cultures for pristinamycin production were made as described by Thibaut et al. (50) with an inoculum step of 44 h and a production step of 32 h. Extraction and quantification of the PI and PII components were performed as described elsewhere (50). *E. coli* strains were grown in LB medium at 37°C (27). Selection was made with ampicillin (100 μg/ml) in LB agar or liquid media and geneticin (10 μg/ml) in LB agar or liquid media. *E. coli* TG1 was used as the recipient strain for plasmid transformation and for obtaining single-stranded DNA from M13 vectors. For expression of the *snbC* gene, the following protocol was followed. Overnight cultures (37°C, with ampicillin) of *E. coli* BL21λ(DE3) (Novagen) strains containing the pET11a derivatives were diluted in LB-ampicillin and grown at 30°C until they reached an optical density (A_{600}) of 1. Isothiogalactopyranoside (IPTG) was then added at a final concentration of 1 mM to induce expression of the T7 RNA polymerase and transcription of *snbC* starting from the T7 RNA polymerase-driven promoter. An expression time of 2.5 h was generally used.

Transformation. Competent *E. coli* cells were prepared and transformed as described by Chung and Miller (5). For transformation, *S. pristinaespiralis* cells were grown in YEME medium supplemented with 0.25% glycine at 30°C for 40 h. Protoplasts were prepared and transformed as described by Hopwood et al. (19).

DNA procedures. Total DNA from *S. pristinaespiralis* strains was obtained by lysozyme treatment and phenol-chloroform extraction as described by Hopwood et al. (19). Plasmid DNA was purified by alkaline extraction procedures as described by Maniatis et al. (27). Single-stranded DNA was extracted by the phenol-chloroform procedure (27) and dialyzed for 45 min against water prior to sequencing. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures (27) under conditions described by the manufacturer. DNA fragments were isolated from agarose gels, using a Geneclean kit from Bio 101 (La Jolla, Calif.). Hybridization with the *S. pristinaespiralis* genomic bank was done as described by Blanc et al. (4). Oligonucleotide probes were labeled with [γ-^{32}P]dATP, using T4 polynucleotide kinase, as described by Maniatis et al. (27). DNA segments for sequencing were purified and subcloned into M13mp18 and M13mp19 vectors. The nucleotide sequence of the corresponding single-stranded DNA was determined by the dideoxy-chain termination method (37), using universal and synthetic oligonucleotides primers. Reactions were made by using dye-labeled dideoxy terminators from the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and analyzed on an Applied Biosystems model 370A DNA sequencer. Sequencing was performed with fluorescence-based dideoxy terminators and Ampli Taq polymerase (Applied Biosystems); sequencing reactions were run on an Applied Biosystems sequencer (model 373A). Both universal M13 primers and appropriate primers from within the sequenced region were used. Alternatively, sequencing was performed with a Sequenase 2.0 kit (U.S. Biochemical) and [α-^{35}S]dATP.

Oligonucleotide probes. As previously reported (49), the N-terminal sequence of SnbA is MLDGSVPWPEDVAAKYRAAGY. Internal sequences obtained by tryptic digestion (49) were identified as VSA-EVEGHLGAHPDVQQAA for SnbA and LAAFNDTARPVPR for SnbC. Degenerate primers or oligonucleotide probes derived from part of the N-terminal or internal sequence of SnbA (VPWPED-VAAKY or EVEGHLGAHPDVQQ) and SnbC (AAFNDTARP) were synthesized and are as follows (degenerate positions are shown in parenthesis: OL1, 5′-GT(CG)CC(CG)TGGCC(CG)GAGGACGT(CG)GC(CG)AA GTAC-3′; OL2, 5′-GAGGT(CG)GAGGG(CG)CACCT(CG)GG(CG)GC(CG) CACCC(CG)GACGT(CG)CAGCAGGC-3′; and OL3, 5′-GC(GC)GC(CG)TT CAACGACAC(GC)GC(CG)CG(CG)CC-3′.

Integrative transformation of *S. pristinaespiralis*. The *snbA* gene was disrupted by homologous recombination by an integration construction containing a fragment internal to the gene. A 1,170-bp *Xho*I-*Hin*dIII DNA fragment isolated from pVRC402 was cloned in the suicide vector pDH5 linearized by *Sma*I digestion to give pVRC404 (Fig. 1). The *snbC* gene was disrupted by double homologous

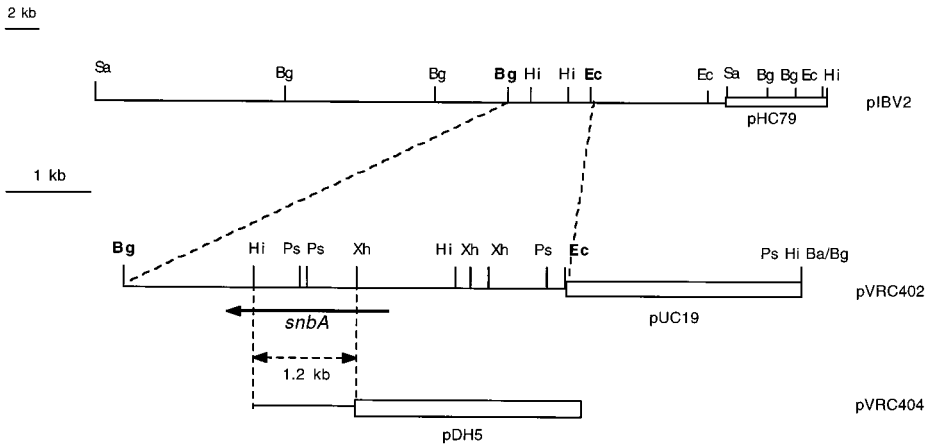

FIG. 1. Restriction maps of cosmid pIBV2 of the pVRC402 vector containing the 5.5-kb *Eco*RI-*Bgl*II fragment from pIBV2 and of the disruption plasmid pVRC404. The arrow corresponds to the identified ORF. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Hi, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sac*I; Xh, *Xho*I; Sl, *Sal*I; Sp, *Sph*I.

recombination events leading to the insertion of an (Am^r) gene in the *snbC* gene. A 6.2-kb *Sph*I DNA fragment containing a portion of the *snbC* gene was subcloned from pVRC1100 in pUC19 to give plasmid pVRC1105 (Fig. 2C). A 3.0-kb *Bgl*II-*Sal*I fragment containing the Am^r gene (derived from pVRC1108 [8a]) was cloned in the *Bgl*II-*Xho*I sites of pVRC1105, yielding plasmid pVRC1110 (Fig. 2C). A 2-kb fragment internal to the *snbC* gene was therefore deleted as the Am^r marker was inserted. The 7-kb *Sph*I insert of pVRC1110 was cloned in the *Sph*I site of the suicide vector pDH5, yielding plasmid pVRC1111. The recombinant plasmids pVRC404 and pVRC1111 were used to transform *S. pristinaespiralis*, and recombinants were selected for the ability to grow on nosiheptide- and geneticin-containing plates, respectively. After 7 days, the resistant colonies were passed through one step of single-colony purification on HT medium containing nosiheptide or geneticin.

Heterologous expression of *snbC* in *E. coli*. An *Nde*I site was introduced at the *snbC* start codon by a PCR amplification step performed with a Perkin-Elmer thermocycler. The template for the PCR reaction was an M13mp18 derivative with a 0.8-kb *Sal*I insert containing the 5′ end of the *snbC* gene. Reverse sequencing primer −40 and the mutagenic oligonucleotide 4258 (5′-GTCGAC GCGGTGCAGGGTGAAGGGGACGTCGTCGGCGAC) were used. The reaction mixture contained 10 ng of denatured template, 1 μg of each primer, 4% dimethyl sulfoxide, 5 mM each deoxynucleoside triphosphate in LiCl, and 1 U of Perkin-Elmer *Taq* DNA polymerase in 2 mM $MgCl_2$–10 mM Tris (pH 8.5)–50 mM KCl–0.01% gelatin. The sample was subjected to 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (1 min at 72°C). Reaction products were analyzed on a 3% NuSieve agarose gel. DNA of the correct size (350 bp) was purified and subcloned in M13mp18, and insertions of

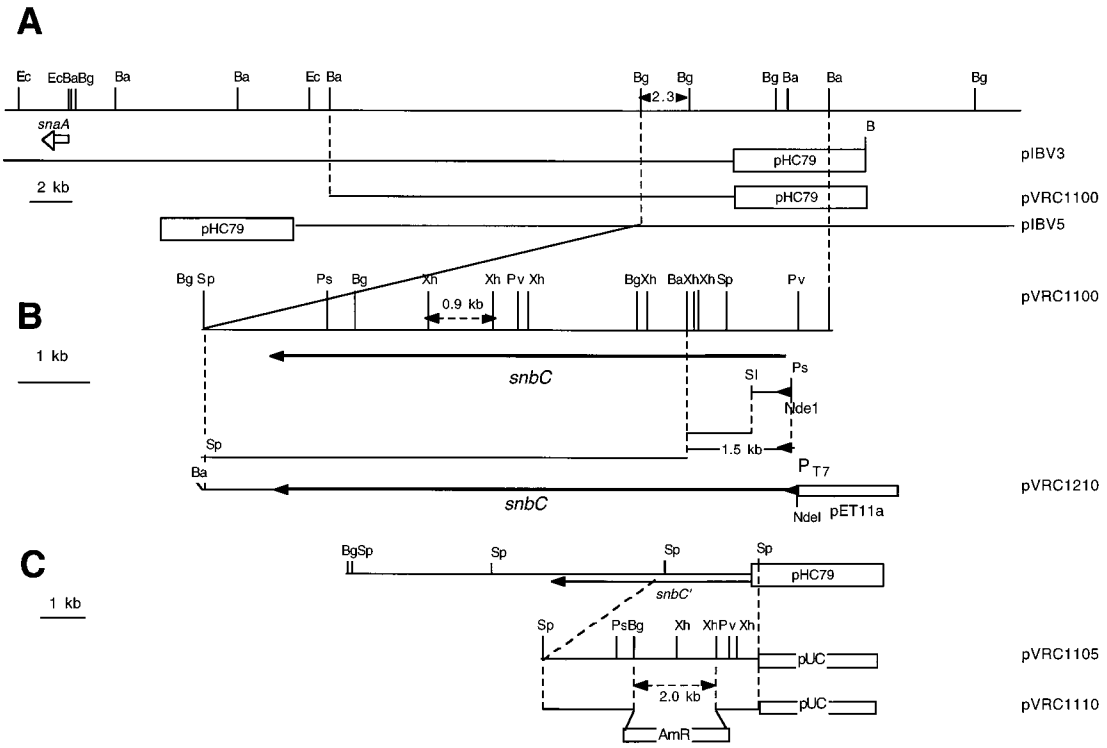

FIG. 2. (A) Restriction map of the *S. pristinaespiralis* chromosomal region covered by cosmids pIBV3, pIBV5, and pVRC1100. (B) Restriction map of the sequenced region and cloning strategy leading to the construction of pVRC1210. The arrow corresponds to the identified ORF. (C) Cloning strategy leading to the construction of plasmid pVRC1110 used for preparing the disruption plasmid pVRC1111. Abbreviations are defined in the legend to Fig. 1.

two clones were checked by sequencing. The 350-bp *Pst*I-*Sal*I fragment containing the 5′ end of the *snbC* gene was excised from one of these clones and cloned into pUC19. The 950-bp contiguous *Sal*I-*Bam*HI fragment was then inserted in the resulting plasmid (Fig. 2B). The 1.5-kb *Nde*I-*Bam*HI fragment was cloned in pET11a (Novagen), and finally the 6.8-kb *Bam*HI fragment containing the end of the *snbC* gene was inserted to give the T7-based expression plasmid pVRC1210, containing the whole *snbC* gene (Fig. 2B).

Computer analysis. Sequence analysis and comparison were performed with the programs of the Wisconsin package (10a); sequences were also aligned with ClustalW (16).

Assay and purification of SnbC in *E. coli*. *E. coli* cell extracts were obtained as described by Blanc et al. (4); purification, ATP-PP_i exchange, and aminoacylation were performed as described by Thibaut et al. (49). Pellets and supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24), and the solubility of SnbC was assessed by comparing total and soluble protein fractions. Proteins and protein standards were stained with Coomassie brilliant blue R.

Nucleotide sequence accession numbers. The nucleotide sequences from *S. pristinaespiralis* described in this report have been submitted to GenBank under accession numbers X98515 for the region containing *snbA* and X98690 for the region containing *snbC*.

RESULTS

Cloning and sequencing of the *snbA* gene encoding the 3-HPA:AMP ligase. Oligonucleotide probes OL1 and OL2 were synthesized on the basis of N-terminal and internal amino acid sequences of the SnbA protein. They were used to screen 1,500 colonies of the genomic library of *S. pristinaespiralis* SP92, previously constructed in *E. coli* DH1 (4). Six clones, hybridizing with both probes, were identified. One of them, named pIBV2, containing a 47-kb insert, was further studied (Fig. 1). Restriction mapping analysis and Southern experiments using OL1 and OL2 as probes showed that the hybridizing sequence was contained in a 5.5-kb *Eco*RI-*Bgl*II DNA fragment. This fragment was cloned in pUC19 linearized with *Eco*RI and *Bam*HI to give pVRC402 (Fig. 1). The nucleotide sequence of 1,879 bp from the 5.5-kb *Eco*RI-*Bgl*II fragment contained in pVRC402 was determined as described in Materials and Methods, using *Hin*dIII and *Pst*I as restriction sites for subcloning in M13mp18 and M13mp19. Analysis of the obtained nucleotide sequence revealed one open reading frame (ORF) with a typical *Streptomyces* codon usage, starting at position 110 (ATG) and ending at position 1858 (TGA). A typical Shine-Dalgarno sequence (AAGGA) was found six nucleotides upstream the start codon. The identified ORF encoded a polypeptide of 582 aa with a predicted M_r of 61,400. The N-terminal region of its deduced amino acid sequence was identical to the N-terminal amino acid sequence of the purified SnbA protein. Moreover, the internal sequence of the protein matched exactly an internal segment (aa 448 to 467) of the identified ORF product. A good correlation was also observed between the calculated M_r (61,400) of the gene product and the estimated M_r of SnbA (67,000) (49). This ORF was named *snbA*.

Cloning and sequencing of the *snbC* gene. The oligonucleotide probe OL3 was synthesized on the basis of the internal amino acid sequence of the SnbC protein. This oligonucleotide was used as a hybridization probe in Southern blots analysis of *S. pristinaespiralis* genomic DNA and of cosmids of the genomic bank that had been isolated in previous studies of pristinamycin biosynthesis genes (4). OL3 hybridized with a 20-kb *Bam*HI fragment present on the cosmid pIBV3 isolated by Blanc et al. (4) as containing the *snaA* gene encoding one of the two subunits of PII_A synthase (the last enzyme involved in PII_A biosynthesis) (Fig. 2A). An internal *Bam*HI deletion of pIBV3 was performed, and the corresponding cosmid was named pVRC1100. Southern hybridization experiments with the cloned and chromosomal DNAs revealed that OL3 hybridized with a 0.9-kb *Xho*I fragment, located at one end of the cloned insert (Fig. 2A). The size of the purified SnbC protein had been estimated to be 240 kDa (49), which would correspond to a gene of 6 to 7 kb. To ensure cloning of the whole gene, a 2.3-kb *Bgl*II DNA fragment located at the end of the pVRC1100 insert DNA (Fig. 2) was used to probe the *S. pristinaespiralis* genomic bank. A cosmid containing an insert of approximately 40 kb overlapping the pIBV3 cosmid over 21 kb was isolated and named pIBV5.

The nucleotide sequence of a 7,920-bp DNA fragment surrounding the 0.9-kb *Xho*I fragment was determined on both strands, using the restriction sites noted in Fig. 2A for subcloning in M13mp18 and M13mp19. A 7,776-bp ORF following typical *Streptomyces* codon usage starting with the putative initiation codon GTG at position 115 and ending with a TGA at position 7889 was identified. The sequence of the tryptic fragment derived from the purified SnbC protein was found at position 1490 of the deduced amino acid sequence. A good correlation was also observed between the calculated M_r (270,000) of the gene product and the estimated M_r of SnbC (240,000) (49). This gene was therefore named *snbC*.

SnbA contains one amino acid activation domain. Comparison of SnbA with proteins present in the databases showed that SnbA was highly similar to the EntE proteins of *E. coli* (43.6% identity) (42) and *Bacillus subtilis* (51.8% identity) (GenBank accession number U26444). SnbA is also highly similar to the *Yersinia pestis* protein YbtE (46.4% identity), (GenBank accession number U50364), thought to be involved in yersiniabactin biosynthesis. The NH_2-terminal sequence of SnbA presents 62 and 58% identity with the NH_2-terminal sequences of the *S. griseoviridis* 3-HPA-activating enzyme (SwissProt accession number P80437) and the *Streptomyces triostinicus* chinoxalin-2-carboxylic acid-activating enzyme (SwissProt accession number P80436), respectively. Further sequence comparison analysis showed that SnbA, like EntE, is a member of the superfamily of adenylate-forming enzymes and is part of the subfamily composed of coumarate-CoA ligases, acetyl-CoA synthetases, and firefly luciferase (13, 53). The main characteristic of members of this subfamily is that they contain the signature sequences thought to be involved in ATP binding and amino acid adenylation but lack the ACP domain involved in thioester formation. An alignment of the sequences of SnbA and EntE with the sequences of 44 other peptide synthetase activation modules enabled us to determine the positions of activation domain core sequences A to I (31). As shown in Fig. 3, members of the EntE subfamily, composed of the EntE, YbtE, and SnbA proteins, contain core sequences A, C, E, F, and G. Core sequences B, D, and I are present but less conserved. The lysine residue of the PPS I motif (NGK), which has been shown to be involved in ATP binding (28), is not conserved in the SnbA family, but several other lysines (512, 530, 533, and 534 as numbered in the alignment) are conserved in the neighboring region. Core sequence H, thought to be involved in adenine binding (29), is not found in the EntE subfamily.

SnbC contains two amino acid activation domains and an epimerization domain. Previous compilation of PPS modules enabled us to define consensus sequences for the different PPS domains (9). These consensus sequences were used to recognize the different domains of SnbC. As shown on Fig. 4, SnbC contains two amino acid activation domains (453 to 995 and 1499 to 2049), two elongation domains (1 to 452 and 996 to 1498), and one C-terminal epimerization domain (2074 to 2590). The activation domains contain the nine core sequences A to I defined by Pfeifer et al. (31) (data not shown). The sequences of the phosphopantetheiyl attachment sites as well as the sequences of the putative elongation sites are shown in Fig. 4. The epimerization domain contains all the conserved

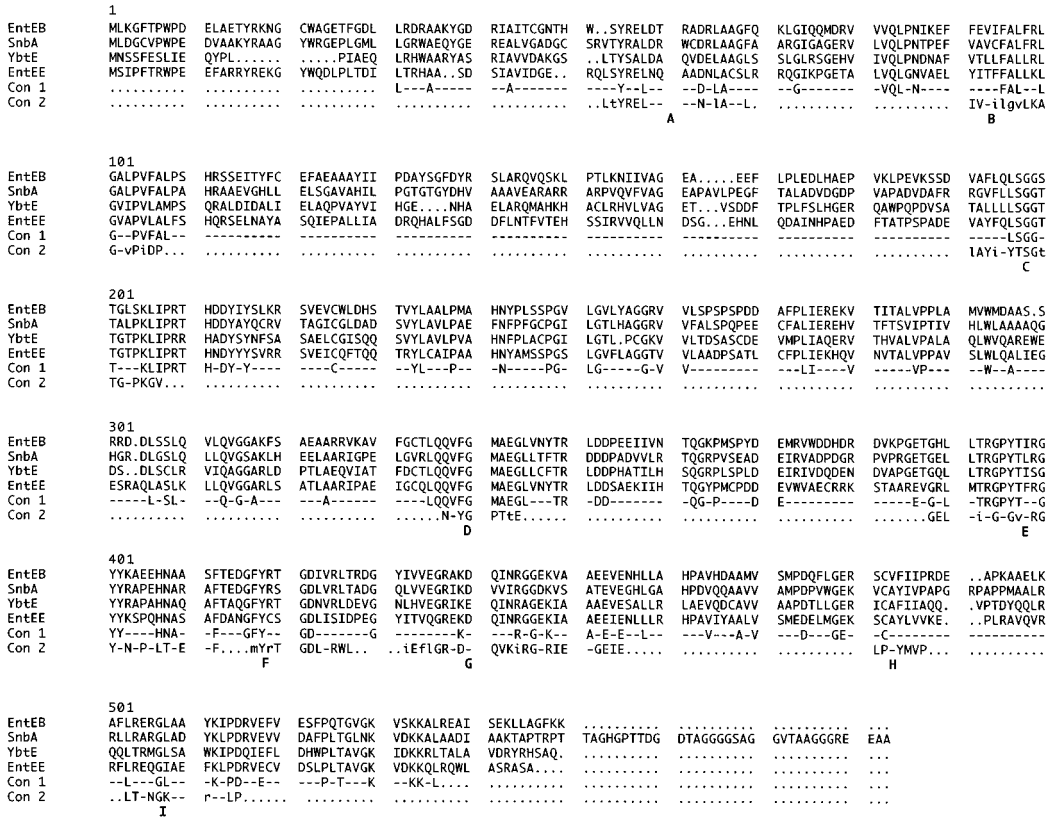

FIG. 3. Alignments of amino acid sequences of SnbA of *S. pristinaespiralis* with EntE of *E. coli* (EntEE), EntE of *B. subtilis* (EntEB), and YbtE of *Y. pestis*. Consensus sequence 1 (Con 1) corresponds to the amino acids conserved in the SnbA-YbtE-EntE subfamily. Consensus sequence 2 (Con 2) corresponds to the positions of core sequences A to I defined by Pfeifer et al. (31) after alignment of SnbA with 44 PPS activation domains (data not shown).

motifs (boxes K to Q) described by Pfeifer et al. (31) (data not shown). The HHXXXDGXSW motif (or M box), for which we have previously proposed a possible catalytic role in epimerization (9), is strictly conserved in SnbC (Fig. 4).

Disruption of the *snbA* and *snbC* genes. To confirm the role of the SnbA and SnbC proteins in PI biosynthesis, we disrupted the *snbA* and *snbC* genes in *S. pristinaespiralis* SP92 by gene replacement. *S. pristinaespiralis* protoplasts were transformed with pVRC404, a pUC19 derivative containing an internal fragment of the *snbA* gene, as described in Materials and Methods. A few clones resistant to nosiheptide were studied. Southern blot analysis with pVRC404 as a probe showed that several clones had stably integrated pVRC404, through a single homologous recombination event (data not shown). Transformation of *S. pristinaespiralis* by pVRC1111 with geneticin selection and subsequent screening for nosiheptide sensitivity enabled us to isolate three Am^r Tsr^s clones that had integrated the Am^r gene in the chromosomal *snbC* gene by a double-crossover events. The structure of each of three *snbC*::Am^r mutants was confirmed by Southern hybridization experiments using the pVRC1105 plasmid as probe (data not shown).

Fermentation experiments with two independent *snbA*::pVRC404 clones and two independent *snbC*::Am^r clones showed that the four mutant clones produced no detectable levels of PI (<1% of the wild-type level) but produced normal levels of PII (90% of the level in the wild-type *S. pristinaespiralis* strain). These results indicated that the *snbA* and *snbC* genes are located in PI-specific transcriptional units. In the case of the *snbA* mutant, it was also shown that PI production could not be restored by adding any of the PI precursors in the culture medium.

Expression of the *snbC* gene in *E. coli*. To produce subsequent amounts of SnbC and pursue its biochemical characterization, an attempt was made to overexpress the *snbC* gene in

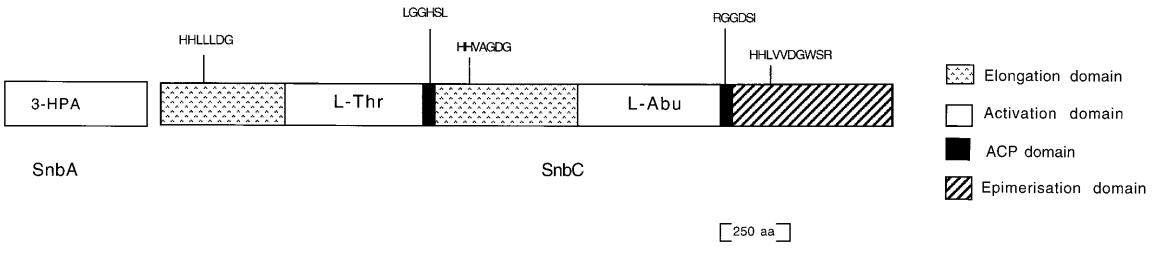

FIG. 4. Distribution of the PPS domain regions within SnbA and SnbC.

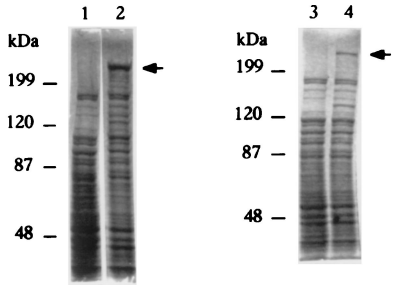

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the SnbC protein expressed in *E. coli*. Proteins of total crude extracts (lanes 1 and 2) or of soluble protein fractions (lanes 3 and 4) of *E. coli* BL21(DE3) cells grown in inducing conditions (1 mM IPTG) and containing the control plasmid pET11a (lanes 1 and 3) or plasmid pVRC1210 (lanes 2 and 4) were separated on an 8% polyacrylamide gel and stained with Coomassie blue. The molecular masses of the molecular size markers (prestained high-range markers from Bio-Rad) are indicated on the left.

E. coli by using the T7 RNA polymerase expression system from Novagen. As shown in Fig. 2B, the entire *snbC* gene was inserted into the *Nde*I-*Bam*HI sites of pET11a, following a mutagenic PCR step to insert an *Nde*I site at the *snbC* start codon and a series of cloning steps to reconstitute the whole gene (see Materials and Methods). The final construct, containing the *snbC* gene under the control of the T7 promoter, was named pVRC1210 and was introduced into *E. coli* BL21 (DE3) harboring on its chromosome the T7 RNA polymerase. As shown in Fig. 5, under inducing conditions, the strain carrying plasmid pVRC1210 expressed a protein of high molecular mass (around 250 kDa) which is absent from the control strain carrying plasmid pET11a. This protein is most likely SnbC, as it comigrated with the native SnbC protein purified from the wild-type *S. pristinaespiralis* strain (data not shown). In the expression conditions used (see Materials and Methods), SnbC was estimated to constitute around 12% of total protein, and its solubility was estimated to be around 40%.

In attempts to purify the SnbC protein produced in *E. coli*, we observed that when produced in a heterologous host, this protein was found in the exclusion fraction and was not retained by the Mono Q column, whereas the control SnbC protein produced from its natural *S. pristinaespiralis* SP92 host was normally retained on the ion-exchange column. No ATP-PP_i exchange with L-Abu could be detected in the exclusion fraction containing the SnbC expressed in *E. coli*. These results suggested that the SnbC protein overexpressed in *E. coli* was not in its native conformation, and its purification was not pursued.

DISCUSSION

Several if not all peptide synthetase enzymes involved in PI biosynthesis have been purified (49). Using oligonucleotide probes derived from partial sequences obtained from PI synthetase 1 (SnbA) and PI synthetase 2 (SnbC), two genes, *snbA* and *snbC*, were cloned. All indications suggest that these genes encode the first two structural enzymes of the PI biosynthesis pathway.

Analysis of the sequence of the 3-HPA:AMP ligase (SnbA) confirmed that this enzyme is part of the superfamily of adenylate-forming enzymes (13, 53). It contains one activation domain, which is consistent with the fact that it activates only one precursor. SnbA is highly homologous to EntE, the 2,3-DHB:AMP ligase involved in the biosynthesis of the *E. coli* enterobactin. They both lack the ACP domain containing the LGG (D/S)I phosphopantetheinyl attachment site characteristic of the PPS subfamily, and a ClustalW multialignment (data not shown) clusters both enzymes in the firefly luciferase–acyl-CoA synthetase–coumarate-CoA ligase subfamily. Both the biochemical and the sequence data suggest that SnbA activates 3-HPA as an adenylate. The fate of the 3-HPA–AMP will be discussed further, but as SnbA is a member of the acyl-CoA ligase superfamily, the hydroxyacyl is likely to be transferred to the thiol acceptor of CoA (or of a phosphopantetheinyl arm).

The second enzyme of PI biosynthesis pathway is SnbC, PI synthetase II. SnbC has an estimated molecular weight of 240,000 and was shown to activate and bind L-threonine and L-Abu (49). The corresponding gene *snbC* was cloned and sequenced. Analysis of the SnbC amino acid sequence showed that SnbC is composed of two PPS modules. We propose that the first module is involved in activation and binding of L-threonine and the second is involved in the activation and binding of L-Abu, for the following reasons: (i) the correlation between the order of amino acid domains and the sequence of the peptide is one of the main hypothesis of the thiotemplate mechanism and has been confirmed in several systems (7, 51), and (ii) an epimerization domain was identified at the C terminus of SnbC, following the second activation domain, and only L-Abu has to be epimerized in the final PI molecule (49).

Expression of the entire *snbC* gene in *E. coli* confirmed that it encoded a protein of approximately 250 kDa comigrating with the native SnbC protein. However, under the expression conditions used, heterologously expressed protein was inactive for adenylation of L-Abu and L-threonine. Several authors have shown that PPS proteins obtained after overexpression in *E. coli* of the corresponding genes have very low aminoacylation activities, as the overexpressed proteins are not posttranslationally modified by 4′-phosphopantetheine (12, 14, 31). However, in all cases, adenylation reactions could be catalyzed, albeit at a low rates (31). This is the first time, to our knowledge, that a PPS protein containing two modules has been overexpressed in *E. coli* by using the T7 RNA polymerase system, and the failure of the overexpressed SnbC protein to catalyze the adenylation reactions could be due to a misfolding of the enzyme in the absence of the phosphopantetheinyl arm. It seems now certain that *E. coli* is not the ideal host for overexpression of heterologous PPS or polyketide synthetase genes (8, 34), as the products are not correctly posttranslationally modified. Direct purification from *S. pristinaespiralis* gives yields too low for performance of mechanistic studies. The solution could therefore be to overexpress the PPS genes in *Streptomyces lividans* or *Streptomyces coelicolor* as has been done successfully for the genes encoding the polyketide synthase (PKS) involved in erythromycin synthesis (20).

It is interesting that *snbC* is not contiguous to *snbA*. Both genes are located in regions containing PI synthesis operons, as disruption of either *snbA* or *snbC* led to mutants which did not produce any PI but still produced standard levels of PII. However, the first structural gene of the PI biosynthesis pathway, *snbA*, and the second structural gene, *snbC*, are 130 kb apart and separated by genes involved in PII synthesis and in PI precursor biosynthesis (3). A similar situation is found with the peptide synthetase gene *phsA* of *Streptomyces viridochromogenes*, which is thought to encode the first structural gene involved in phosphinotricin tripeptide synthesis and is not juxtaposed to other PPS genes (40).

Determination of the sequences of the first two structural enzymes involved in PI synthesis should shed some light on the first steps involved in the formation of this depsipeptide. Like the 2,3-DHB:AMP ligase catalyzing the first step of enterobactin synthesis or the 4-methyl-3-hydroxyanthranilic acid (4-

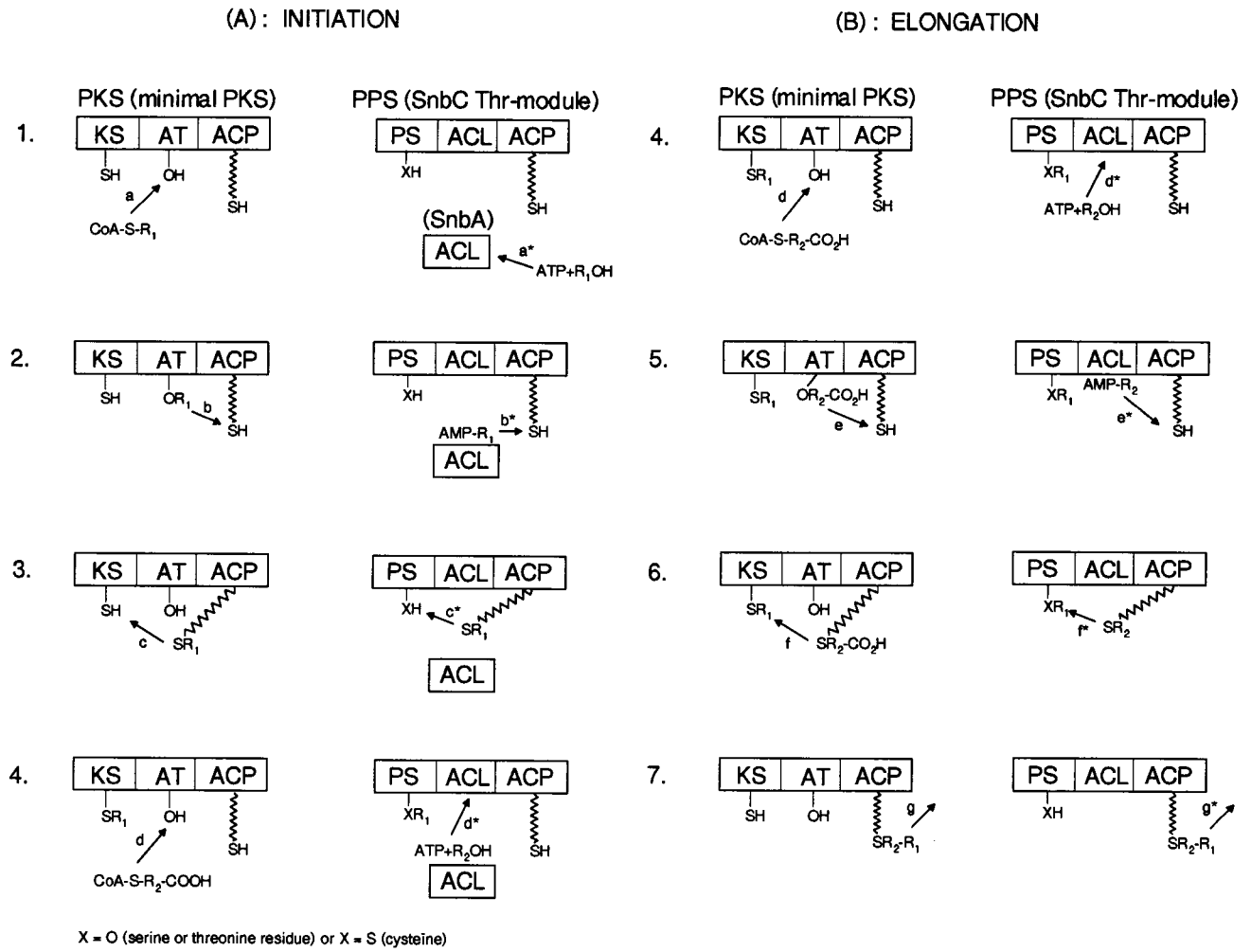

FIG. 6. Parallelism between polyketide and nonribosomal peptide synthesis. KS, β-ketoacylsynthase; AT, acyltransferase; PS, peptide synthase (elongation domain containing the HHxxxDG motif); ACL, acyl-CoA ligase (adenylate-forming domain). Steps 1 to 4, initiation. a and a*, transfer of the starter chain to the loading site; b and b*, transfer to the 4′-phosphopantetheinyl arm; c and c*, transfer to the waiting/elongation site. Steps 4 to 7, elongation. d and d*, transfer of the elongation chain to the loading site; e and e*, transfer to the 4-phosphopantetheinyl arm; f and f*, condensation of the elongation chain on the 4-phosphopantetheinyl arm with the starter chain at the waiting/elongation site; g and g*, transfer of the elongated chain to the following waiting/elongation site.

MHA):AMP ligase catalyzing the first step of actinomycin synthesis, SnbA activates 3-HPA as an adenylate. The question of the mechanism of formation of the amide bond between the first aromatic hydroxy acid of PI and the following threonine precursor remains open. Stindl and Keller (47, 48) have proposed that in actinomycin synthesis, the formation of the amide bond of the final molecule is catalyzed by the second enzyme of the ACMSII pathway. SnbC begins with a potential acyl transfer domain (Fig. 4) (9); we therefore propose that SnbC catalyzes the formation of the 3-HPA–threonine amide bond. Stindl and Keller (47) showed that the 4-MHA analog *p*-toluic acid, after activation as an adenylate, was transferred as a thioester on a phosphopantetheinyl cofactor present on the ACMSII enzyme. Analysis of the SnbC sequence reveals only two, not three, phosphopantetheinyl binding sites, suggesting that the three precursors (3-HPA, L-Thr, and L-Abu) cannot be charged simultaneously on the SnbC enzyme. We propose the model for initiation of PI synthesis shown in Fig. 6, which parallels the mechanism of initiation of fatty acid synthesis by fatty acid synthase (FAS) (54). The starter hydroxyacyl adenylate would first be charged on the phosphopantetheinyl arm of the threonine-activating module before being transferred to a waiting position located in the N-terminal elongation domain of SnbC. The threonine adenylate would then be charged on the same phosphopantetheinyl arm, positioned to the elongation site, and elongation would occur (Fig. 6). The arm of the first module would at this stage be charged with the dipeptide 3-HPA–threonine. We propose that like in polyketide synthesis by PKS (21), the dipeptide would then be transferred on a waiting position in the second elongation domain. The third amide bond could then be formed with L-Abu which would have been previously charged on the second phosphopantetheinyl arm. 3-HPA–threonine–L-Abu would then be epimerized in the peptide stage (48).

The presence of a waiting position in the elongation domain is essential in this new model, which mixes both the initial and revised forms of Lipmann's thiotemplate models (25). The thiol of a cysteine, like in the β-ketosynthase domains of FAS and PKS, seems unlikely, as no cysteine is conserved in the PPS elongation domains. An ester intermediate on a serine, like the acyltransferase loading site of the FAS and PKS enzymes, or on a threonine could be a good candidate for a waiting posi-

tion. Comparison of the sequences of 55 condensing domains that was performed previously and led to the definition of seven conserved motifs (9) suggests several possible candidates. The best candidate is a serine present two amino acids downstream the His motif (HHXXXDGXXS). At this position, a serine is found in 85% of the sequences, a cysteine is found in 9%, and a threonine is found in 2%. Very recently, the entire sequence of biosynthetic gene cluster for the peptidylmacrolactone rapamycin was entirely determined (41). It is the first available sequence of a mixed (PPS, PKS) biosynthetic system. Analysis of the enzymatic domains in the modular rapamycin PKS suggests that the shikimate-derived starter unit is activated by an acyl-CoA ligase domain and transferred to the waiting position (directly or indirectly) of the following β-ketosynthase domain (2). The existence of enzymes containing both PPS and PKS domains as the rapamycin synthase or as a sequence of unknown function of *B. subtilis* (1) favors the strict parallel between nonribosomal peptide synthesis and polyketide synthesis proposed in our new model.

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