JOURNAL OF BACTERIOLOGY, Sept. 1995, p. 5206–5214 Vol. 177, No. 18
0021-9193/95/$04.00+0

Cloning and Analysis of Structural Genes from *Streptomyces pristinaespiralis* Encoding Enzymes Involved in the Conversion of Pristinamycin II_B to Pristinamycin II_A (PII_A): PII_A Synthase and NADH:Riboflavin 5′-Phosphate Oxidoreductase

VÉRONIQUE BLANC,* DELPHINE LAGNEAUX, PATRICK DIDIER, PATRICIA GIL, PATRICIA LACROIX, AND JOËL CROUZET

Unité de Biologie Moléculaire, Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer S.A., 94403 Vitry-sur-Seine Cedex, France

Received 17 April 1995/Accepted 27 June 1995

In *Streptomyces pristinaespiralis*, two enzymes are necessary for conversion of pristinamycin II_B (PII_B) to pristinamycin II_A (PII_A), the major component of pristinamycin (D. Thibaut, N. Ratet, D. Bisch, D. Faucher, L. Debussche, and F. Blanche, J. Bacteriol. 177:5199–5205, 1995); these enzymes are PII_A synthase, a heterodimer composed of the SnaA and SnaB proteins, which catalyzes the oxidation of PII_B to PII_A, and the NADH:riboflavin 5′-phosphate oxidoreductase (hereafter called FMN reductase), the SnaC protein, which provides the reduced form of flavin mononucleotide for the reaction. By using oligonucleotide probes designed from limited peptide sequence information of the purified proteins, the corresponding genes were cloned from a genomic library of *S. pristinaespiralis*. SnaA and SnaB showed no significant similarity with proteins from databases, but SnaA and SnaB had similar protein domains. Disruption of the *snaA* gene in *S. pristinaespiralis* led to accumulation of PII_B. Complementation of a *S. pristinaespiralis* PII_A^- PII_B^+ mutant with the *snaA* and *snaB* genes, cloned in a low-copy-number plasmid, partially restored production of PII_A. The deduced amino acid sequence of the *snaC* gene showed no similarity to the sequences of other FMN reductases but was 39% identical with the product of the *actVB* gene of the actinorhodin cluster of *Streptomyces coelicolor* A(3)2, likely to be involved in the dimerization step of actinorhodin biosynthesis. Furthermore, an *S. coelicolor* A(3)2 mutant blocked in this step was successfully complemented by the *snaC* gene, restoring the production of actinorhodin.

Pristinamycin belongs to the family of streptogramin antibiotics, also called virginiamycin-like or mikamycin-like antibiotics. Streptogramins are a small and homogeneous group composed of related compounds such as pristinamycin, virginiamycin, mikamycin, and vernamycin (9, 10, 58). They are protein synthesis inhibitors (9, 10). The special feature of the family is that each member is a complex of two structurally different components exhibiting a synergistic antibacterial activity (2, 10). The two types of compounds are both macrocyclic lactone peptolides, but their structures are notably different. They belong to one of the two following distinct groups: the streptogramin A type (Sa) corresponding to polyunsaturated cyclic peptolides and the streptogramin B type (Sb) corresponding to branched cyclic hexadepsipeptides. The proportion of Sa and Sb in the complex varies from one antibiotic to another. Moreover, the major form of each component is accompanied by several structurally different minor forms.

Pristinamycin, produced by *Streptomyces pristinaespiralis*, consists of approximately 30% pristinamycins I (PI), the Sb type molecules, and 70% pristinamycins II (PII), the Sa type molecules. In industrial strains, PII is produced mainly in two forms, PII_A and PII_B, in a 80:20 ratio. The difference between PII_A and PII_B is the presence of a dehydroproline instead of a proline in the macrocycle (Fig. 1). Thibaut et al. (57) reported high levels of conversion of radiolabelled PII_B to PII_A both in vivo and in vitro with several strains of *Streptomyces* spp. that produce pristinamycins. The same type of observation was made with *Streptomyces virginiae*, the producer of virginiamycin, closely related to pristinamycin (49). These results indicated that PII_B is the biosynthetic precursor of PII_A, and so the oxidation of the proline residue into a dehydroproline residue appears to be the last step of PII_A biosynthesis.

Thibaut et al. (57) also showed that two enzymes are involved in the conversion of PII_B to PII_A (Fig. 1). Both were purified to homogeneity. The first, called PII_A synthase, is a heterodimer composed of two polypeptides, SnaA and SnaB, with M_rs of 50,000 and 35,000, respectively. It catalyzes the oxidation of the proline residue of PII_B in the presence of molecular oxygen and reduced flavin mononucleotide ($FMNH_2$). The second is an NADH:riboflavin 5′-phosphate oxidoreductase (hereafter called FMN reductase), SnaC, with an apparent M_r of 30,000 which provides the reduced FMN necessary for the oxidation of PII_B.

In this study, we describe the cloning, sequencing, and characterization of the structural genes for PII_A synthase (*snaA* and *snaB*) and FMN reductase (*snaC*) from *S. pristinaespiralis* and provide evidence for their functions. We believe that this is the first report of the cloning of genes involved in the synthesis of a streptogramin.

MATERIALS AND METHODS

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

Media and bacteriological techniques. *Streptomyces* strains were maintained on HT agar medium (48) and grown in YEME medium (28) at 30°C. Liquid cultures for pristinamycin production were prepared by the method of Thibaut et al. (57), with an inoculum step of 44 h and a production step of 32 h. Extraction

* Corresponding author. Mailing address: Unité de Biologie Moléculaire, Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer S.A., 13 Quai Jules Guesde, B.P. 14, 94403 Vitry-sur-Seine Cedex, France. Phone: (33-1) 45 73 77 85. Fax: (33-1) 45 73 77 96.

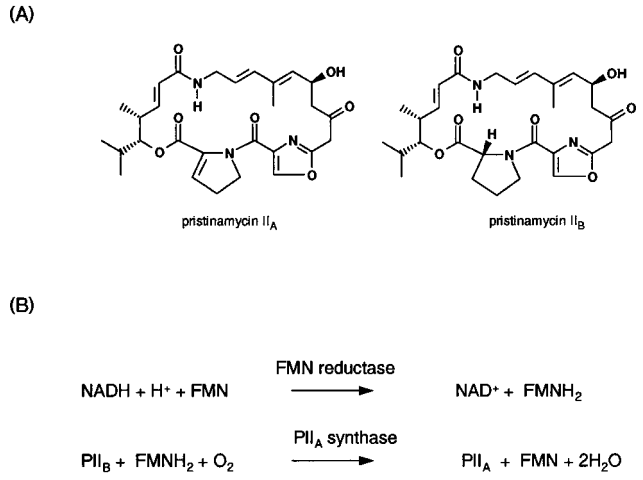


FIG. 1. (A) Structures of PII$_A$ and PII$_B$. (B) Reactions catalyzed by PII$_A$ synthase and FMN reductase.

and quantitation of the PII components were performed as described elsewhere (57).

Nosiheptide was used as an alternative to thiostrepton to select for the presence of the *tsr* gene, at a concentration of 400 μg/ml for solid media and of 2 μg/ml for liquid media. *Escherichia coli* strains were grown in LB medium at 37°C (44). Selection was made with 100 μg of ampicillin per ml in LB agar or liquid media.

DNA isolation and manipulation. Total DNA from *S. pristinaespiralis* SP92 was obtained by lysozyme treatment and phenol-chloroform extraction as described by Hopwood et al. (28). Plasmid DNA was purified by alkaline extraction procedures as described by Hopwood et al. (28) for *Streptomyces* species and by Maniatis et al. (44) for *E. coli*. Single-stranded DNA was extracted by the phenol-chloroform procedure (44) and dialyzed against water for 45 min prior to sequencing. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures (44) under conditions described by the manufacturer. DNA fragments were isolated from agarose gels with the Geneclean kit from Bio101 (La Jolla, Calif.).

Transformations. Competent *E. coli* cells were prepared and transformed by the method of Chung and Miller (8). For transformation, *S. pristinaespiralis* and *S. coelicolor* cells were grown in YEME medium supplemented with 0.25 and 0.5% glycine, respectively, at 30°C for 40 h. Protoplasts were prepared and transformed by the method of Hopwood et al. (28). Only unmethylated DNA, isolated from *E. coli* ET12567, was used for transformation of *S. coelicolor* (41).

DNA-DNA hybridization. Transfer of denatured DNA from agarose gels or colonies to Biodyne nylon membranes (Pall Corporation, Portsmouth, England) were performed by standard procedures (44). DNA fragments were labelled by random priming with [α-^{32}P]dCTP by using the random primer labelling kit (Amersham International, Little Chalfont, Buckinghamshire, England), as described by the supplier. Oligonucleotide probes were labelled with [γ-^{32}P]dATP with T4 polynucleotide kinase by the method of Maniatis et al. (44). Hybridization experiments were performed by the method of Maniatis et al. (44).

Oligonucleotide probes. As previously reported (57), the N-terminal sequences of SnaA, SnaB, and SnaC are TAPR(R/W)RITLAGIIDGPGG, TAPILVATLDTRGPAATLGTIT, and TGADDPARPAVGPQSFRDAMAQLASPV, respectively. Internal sequences obtained by tryptic digestion (57) were identified as GADGFNIDFPYLPGSADDFV for SnaA, GL(-)DSFDDDAFVHDR for SnaB, and FAGGEFAAWDGTGVPYLPDAK and TGDPAKPPLLWYR for SnaC. Degenerate primers or oligonucleotide probes derived from part of the N-terminal or internal sequence of SnaA (IDFPYLPG), SnaB (FDDDAFVH), and SnaC (FRDAMAQLA, FAGGEFAAWDGTG, and DPAKPPLLWYR) were synthesized and are as follows: (degenerate positions shown in parentheses): A, 5′-ATCGA(C,T)TT(C,T)CC(C,G,A,T)TA(C,T)CT(C,G)CC(C,G)GG-3′; B, 5′-TTCGACGA(T,C)GA(T,C)GC(A,T,C,G)TTCGT(C,G)CA(T,C)GAC-3′; C1, 5′-TTCCG(C,G)GACGC(C,G)ATGGC(C,G)CAGCT(C,G)GC-3′; C2, 5′-TTCGC(C,G)GG(C,G)GG(C,G)GAGTTCGC(C,G)GC(C,G)TGGGACGGCAC(C,G)GG-3′; and C3, 5′-GACCC(C,G)GC(C,G)AAGCC(C,G)CC(C,G)CT(G,C)CT(G,C)TGGTACCG-3′, respectively.

Preparation of antiserum. Rabbits were immunized by repeated subcutaneous inoculations of the two subunits of the purified PII$_A$ synthase. The protocol was based on three injections of 100 μg of proteins (in complete Freund adjuvant at days 0, 15, and 30) and one injection of the same dose (in incomplete Freund adjuvant at day 37). Blood was harvested 10 days after the last injection.

Preparation of cell extracts. Portions (5 ml) of *S. pristinaespiralis* cell suspensions were harvested after 16, 18, 20, or 22 h of culture in production medium (57). The washing buffer was phosphate-buffered saline (44) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 5 mM ethylene glycol-bis(β-amino ethyl) tetraacetic acid (EGTA). The pellet was kept frozen at −20°C. Prior to sonication, cells were thawed and resuspended in 1.5 to 2 ml of the same buffer. Cells were disrupted with the Bioruptor type UEC-200 (Eurogentec, Seraing, Belgium) by the following procedure: four rounds of 5-min oscillating pulses (48 s on, 24 s off; power of 200 W). The obtained lysate was centrifuged for 15 min in an Eppendorf tube at 10,000 × *g*, and the resulting supernatant was referred to as cell extract. Protein concentration was determined by the method of Lowry et al. (39).

Assays of PII$_A$ synthase and FMN reductase activities. Enzymatic activities were assayed from cell extracts obtained with cells from 30 ml of fermentation broth, as described elsewhere (57).

Western blot (immunoblot) analysis. Proteins, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (36), were electroblotted onto nitrocellulose membranes [Cellulosenitrat(E); Schleicher and Schuell, Dassel, Germany) by using the Biometra Fastblot (Biometra Inc., Tampa, Fla.). Antigenic proteins were stained by using the Vectastain ABC Mouse IgG kit (Vector Laboratories, Biosys S.A., Compiègne, France) and anti-rabbit immunoglobulin G-horseradish peroxidase conjugate according to the procedures suggested by the manufacturer.

Construction of *S. pristinaespiralis* genomic library. A partial *Sau*3A digestion of *S. pristinaespiralis* genomic DNA was fractionated on a 20 to 40% sucrose gradient as described by Maniatis et al. (44). DNA fragments (35 to 45 kb) were ligated with pHC79 linearized with *Bam*HI. In vitro packaging with the Gigapack II Gold Packaging Extract (Stratagene, La Jolla, Calif.) was performed as described by the manufacturer, using HB101 or DH1 as the recipient strain. A total of 1,500 colonies for each transfection were selected on LB agar supplemented with ampicillin. Selected clones were individually grown in 200 μl of Hogness medium (19) in 96-well microplates and stored at −80°C.

DNA sequence analysis. A 4-kb *Sac*I-*Bam*HI fragment from pXL2045 containing the *snaA* and *snaB* genes was digested with different restriction enzymes (*Sac*I, *Not*I, *Nru*I, *Eco*RI, *Pst*I, and *Bam*HI). The resulting DNA fragments were subcloned in M13mp18 and M13mp19 vectors. The nucleotide sequence of the corresponding single-stranded DNA was determined by the dideoxy-chain termination method (51) with universal and synthetic oligonucleotides primers. Reactions were performed with dye-labelled dideoxy terminators from the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on the Applied Biosystems model 370A DNA Sequencer (Applied Biosystems). In the case of the *snaC* gene, a 1.5-kb *Xho*I-*Pst*I fragment included in the 4-kb *Bam*HI-*Bam*HI fragment from pVRC509 was cloned in M13mp18 and M13mp19 and was partially sequenced as described previously with universal and synthetic primers.

Analysis of sequence data. Nucleic acid and amino acid sequences were analyzed by using CITI2 facilities (13). The nucleotide sequences were analyzed by the program of Staden and McLachlan (55), using codon preference to identify the coding sequences. A codon preference table was established with 19,673 codons from *Streptomyces* species, obtained from GenBank. Amino acid sequences were compared with Genbank, NBRF, and Swissprot databases by using either the FASTA (13) or Kanehisa (31) program. Multiple alignments were performed with the CLUSTAL multiple-alignment program of Higgins and Sharp (23).

Integrative transformation of *S. pristinaespiralis*. The *snaA* gene was disrupted by homologous recombination by an integration construction containing a fragment internal to the N-terminal part of the gene. A 800-bp *Pst*I-*Eco*RI fragment was subcloned from pXL2045 in the suicide vector pDH5 to create pVRC505. The recombinant plasmid was used to transform *S. pristinaespiralis*, and recombinants were selected for the ability to grow on nosiheptide-containing plates. After 7 days, the resistant colonies were passed through one step of single-colony purification on HT medium containing nosiheptide.

Homologous expression of *snaA* and *snaB* in *S. pristinaespiralis*. Because the *snaA* gene started 31 bp after the *Bam*HI site, we isolated a 7.3-kb *Sac*I fragment from pIBV1, corresponding to an extra 3-kb fragment upstream of the *snaA* gene. This fragment was first subcloned in pUC1813 to give pVRC506. The 7.3-kb fragment was then isolated from pVRC506 by *Hin*dIII digestion and cloned in *Hin*dIII-linearized pIJ903. The recombinant plasmid, named pVRC507, contained *snaA* and *snaB* downstream of the *tet* promoter of pIJ903, albeit separated from each other by ORF401 oriented in the opposite direction from that of *snaA* and *snaB*.

Heterologous expression of *snaC* in *S. coelicolor*. The 1.5-kb *Xho*I-*Pst*I fragment containing the *snaC* gene and the 3′ end of the upstream open reading frame (ORF) was isolated from pVRC509 and cloned into pUC19 linearized by double digestion with *Sal*I and *Pst*I, giving pVRC518. A DNA fragment containing the *ermE** promoter from *Saccharopolyspora erythraea* (6) was purified from pVRC1116 after digestion with *Eco*RI and *Bam*HI and cloned into pVRC518 digested with *Eco*RI and *Bam*HI. The recombinant plasmid was named pVRC519. The *Eco*RI-*Hin*dIII fragment containing the *snaC* gene under control of the *ermE** promoter was purified and cloned in pIJ903 linearized by digestion with *Eco*RI and *Hin*dIII. The recombinant plasmid was named pVRC520. Transformation of *E. coli* ET12567 with pVRC520 allowed the preparation of unmethylated DNA necessary for transformation of *S. coelicolor*.

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

Strain, phage, cosmid, or plasmid	Relevant properties	Source or reference
Strains		
E. coli		
HB101	F$^-$ *supE44 hsdS3*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	7
DH1	F$^-$ *gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44*	38
TG1	K-12 Δ(*lac-pro*) *supE thi hsd* ΔS5/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	20
DH5α	F$^-$ *E44 ΔlacU169 φ80 lacZ*ΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	22
ET12567	F$^-$ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44*	42
S. pristinaespiralis		
SP92	Natural isolate of *S. pristinaespiralis* ATCC 25486	Rhône-Poulenc Rorer
SP119	PI$^-$ PII_A^+ PII_B^+; mutant of *S. pristinaespiralis* SP92 obtained by chemical mutagenesis	Rhône-Poulenc Rorer
SP120	PI$^-$ PII_A^- PII_B^+; mutant of *S. pristinaespiralis* SP119 obtained by chemical mutagenesis	
S. coelicolor A3(2)		
B135	*hisA1 uraA1 strA1* SCP1$^-$ SCP2$^+$ *actVB-235*	50
Phages		
M13mp18, M13mp19	Multicloning site vector	Boehringer
Cosmids		
pHC79	Cosmid; Amp^r	26
pIBV1	Cosmid containing the PII_A synthase genes; Amp^r	This work
pIBV3	Cosmid overlapping with pIBV1; Amp^r	This work
pIBV4	Cosmid containing the FMN reductase gene; Amp^r	This work
Plasmids		
pUC18, pUC19	Multicloning site vector; Amp^r	Biolabs
pUC1813	Multicloning site vector; Amp^r	33
pBKS$^-$	Multicloning site vector; Amp^r	Stratagene
pIJ702	*Streptomyces* high-copy-number plasmid; *mel* Tsr^r	32
pIJ903	*E. coli* and *Streptomyces* shuttle vector (low-copy-number); Amp^r Tsr^r	40
pDH5	*Streptomyces* suicide vector; Amp^r Tsr^r	25
pXL2045	6-kb *Bam*HI-*Bam*HI insert from pIBV1 in pBKS$^-$ containing *snaA* and *snaB*; Amp^r	This work
pVRC509	4-kb *Bam*HI-*Bam*HI insert from pIBV4 in pUC19 containing *snaC*; Amp^r	This work
pVRC505	800-bp *Pst*I-*Eco*RI insert from pXL2045 in pDH5; Amp^r Tsr^r	This work
pVRC506	7.3-kb *Sac*I-*Sac*I insert from pXL2045 in pUC1813; Amp^r	This work
pVRC507	*Streptomyces* expression vector containing the entire *snaA* and *snaB* genes in pIJ903; Amp^r Tsr^r	This work
pVRC1116	*ermE** promoter region cloned in pIC20H from pUC1070; Amp^r	12
pVRC518	1.5-kb *Xho*I-*Pst*I insert from pVRC509 in pUC19; Amp^r	This work
pVRC519	*ermE** promoter cloned upstream *snaC* in pVRC518; Amp^r	This work
pVRC520	*Streptomyces* expression vector of *snaC*, cloned in pIJ903; Amp^r Tsr^r	This work

Nucleotide sequence accession number. The nucleotide sequences from *S. pristinaespiralis* described in this paper have been submitted to GenBank under accession numbers U21215 for the region containing *snaA*, *snaB*, and ORF401 and U21216 for the region containing *snaC*.

RESULTS

Identification and cloning of the *snaA* and *snaB* genes. Oligonucleotide probes A and B were synthesized on the basis of internal amino acid sequences of the SnaA and SnaB proteins of the PII_A synthase, respectively. They were used to screen 3,000 colonies of the genomic library of *S. pristinaespiralis* SP92 by colony hybridization. Five clones hybridizing with either one or both probes were identified. Four of the recombinant cosmids contained a 6-kb *Bam*HI fragment hybridizing with both probes. One clone, named pIBV1, with a 33-kb insert, was studied further. The fifth clone, named pIBV3, with a 34-kb insert, did not contain the 6-kb *Bam*HI fragment described previously, but as pIBV1, a 2.5-kb *Eco*RI fragment hybridizing with probe A only. Restriction maps of these two cosmids were constructed (Fig. 2). They shared a 8-kb region containing the 2.5-kb *Eco*RI fragment. The 6-kb *Bam*HI fragment from pIBV1 was cloned in pBKS$^-$ to give pXL2045 (Fig. 2). The nucleotide sequence of 3,573 bp from the 4-kb *Sac*I-*Bam*HI fragment from pXL2045 was determined as described in Materials and Methods. Analysis of the obtained nucleotide sequence revealed three ORFs (ORF1, ORF2, and ORF3) with a typical *Streptomyces* codon usage, ORF2 being on the strand opposite to that carrying ORF1 and ORF3 (Fig. 2). ORF1, ORF2, and ORF3 encoded polypeptides of 422, 401 or 402, and 277 amino acids, with M_rs of 46,500, 45,200, and 28,700, respectively (Fig. 3). Typical Shine-Dalgarno sequences (56) (GGAG, GGAG, and AGGA) were found upstream of ORF1, ORF2, and ORF3, respectively (Fig. 3), indicating that in the case of ORF2, the GTG is most probably the start codon. No significant inverted repeat was found between the intergenic regions or at the end of ORF3.

The N-terminal region of ORF1 was identical to the N-terminal amino acid sequence of the purified large subunit of

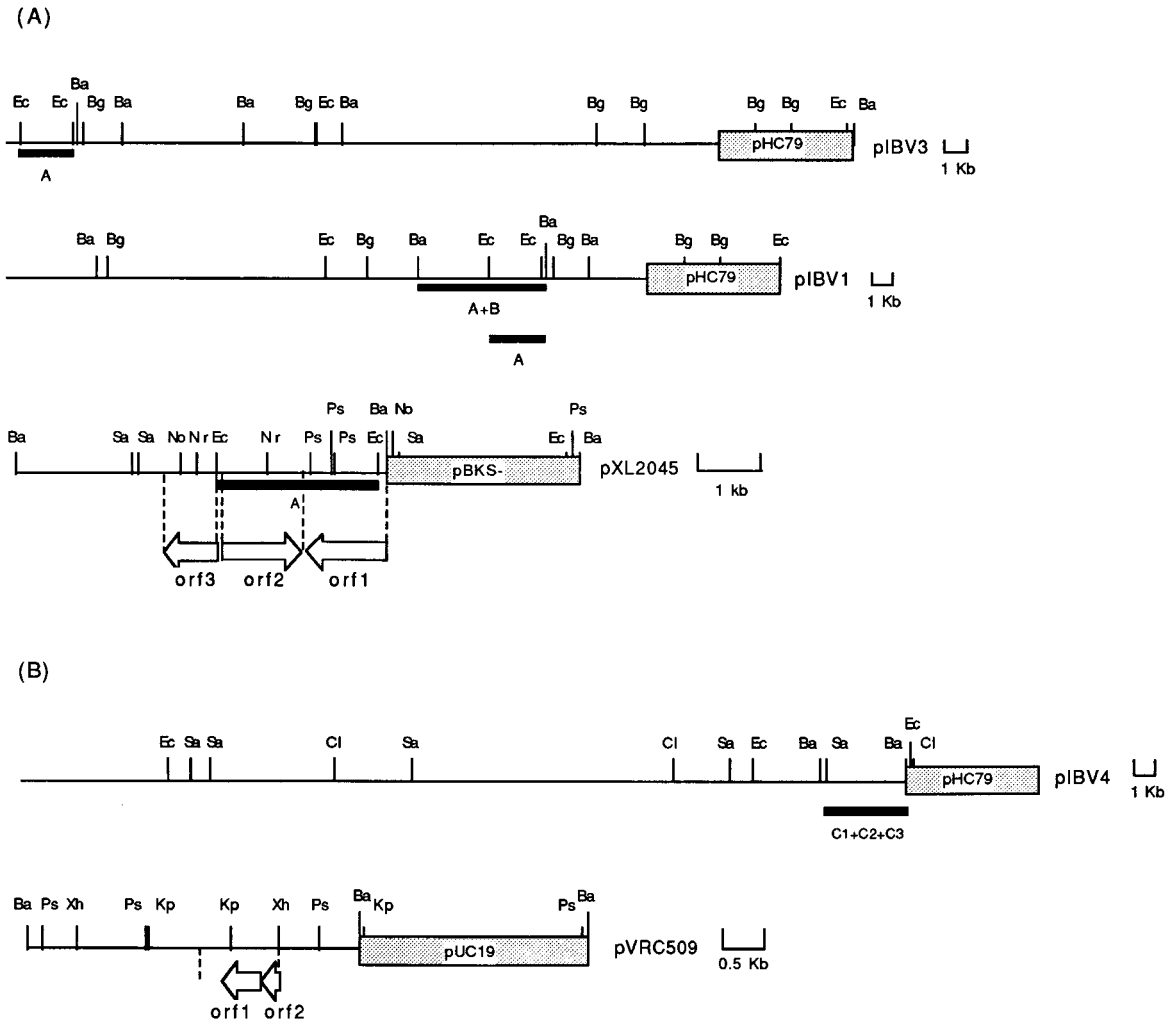


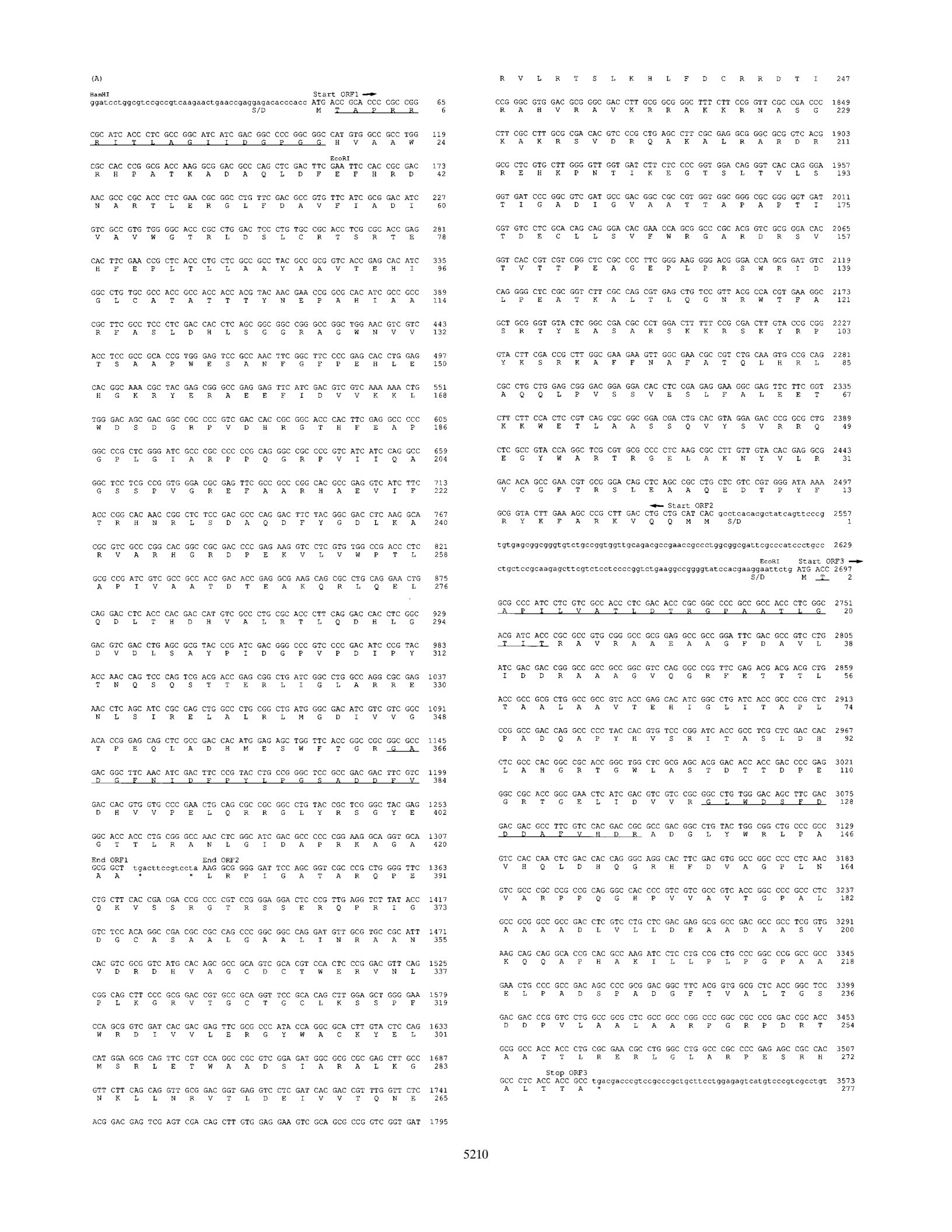
FIG. 2. (A) Restriction maps of cosmids pIBV1 and pIBV3 and the pXL2045 vector containing the 6-kb *Bam*HI fragment from pIBV3. (B) Restriction map of cosmid pIBV4 and the pVRC509 vector containing the 4-kb *Bam*HI fragment from pIBV4. Arrows correspond to the identified ORFs. The black boxes show the fragments hybridizing with probes described in the text. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Cl, *Cla*I; Ec, *Eco*RI; Kp, *Kpn*I; No, *Not*I; Nr, *Nru*I; Ps, *Pst*I; Sa, *Sac*I; Xh, *Xho*I.

the PII$_A$ synthase, except that the amino-terminal methionine was missing (removal of the methionine residue has been proposed to occur when the penultimate amino acid is threonine [24]). The N-terminal region of the ORF3 product was identical with the N-terminal amino acid sequence of the purified small subunit of PII$_A$ synthase. Moreover, the internal amino acid sequences obtained from tryptic digestion of SnaA and SnaB were found in the polypeptides encoded by ORF1 (amino acids 365 to 384) and ORF3 (amino acids 122 to 136). A good correlation was observed between the calculated M_rs of the ORF1 and ORF3 products, respectively, 46,500 and 28,700, and the ones estimated from the purified subunits of PII$_A$ synthase, 50,000 and 35,000, respectively (57).

These results demonstrated that ORF1 and ORF3 corresponded to the large and small subunits of PII$_A$ synthase, and we named the corresponding genes *snaA* and *snaB*. They were separated by 1.4 kb containing ORF2. ORF2 was named ORF401 for the size of the corresponding polypeptide. The average G+C content of the sequenced region was around 71.5%.

Identification and cloning of the *snaC* gene. Degenerate oligonucleotide probes C1, C2, and C3 were designed from the N-terminal and two internal peptide sequences of the purified FMN reductase. None of them hybridized with the five previously described cosmids isolated with probes specific for PII$_A$ synthase genes. Hybridization of the library with C1, C2, and C3 probes allowed the identification of two cosmids which contained a common 4-kb *Bam*HI fragment hybridizing with the three probes. One cosmid, pIBV4, containing a 41-kb insert (Fig. 2), was further studied. The 4-kb *Bam*HI fragment from this cosmid was subcloned in pUC19 to give pVRC509 (Fig. 2). The nucleotide sequence of the 770-bp fragment internal to the 4-kb *Bam*HI fragment was determined (Fig. 3). Two adjacent ORFs showing a typical *Streptomyces* codon usage were found (Fig. 3). The average G+C content of the region was 76%. ORF2 started with a GTG at nucleotide 212, finished with TGA at nucleotide 731, and had a putative ribosome-binding site (AGGAG) 5 bp upstream of the start codon. ORF2 encoded a polypeptide of 176 amino acids with an M_r of 18,300. Only the 3′ end of ORF1 was present on the sequenced fragment.

The N-terminal sequence of the ORF2 product was identical to the N-terminal sequence of the purified FMN reductase (57), except that the N-terminal methionine was missing.


(A)
BamHI
Start ORF1
S/D
M T A P R R
R I T L A G I I D G P G G H V A A W
EcoRI
R H P A T K A D A Q L D F E F H R D
N A R T L E R G L F D A V F I A D I
V A V W G T R L D S L C R T S R T E
H F E P L T L L A A Y A A V T E H I
G L C A T A T T T Y N E P A H I A A
R F A S L D H L S G G R A G W N V V
T S A A P W E S A N F G F P E H L E
H G K R Y E R A E E F I D V V K K L
W D S D G R P V D H R G T H F E A P
G P L G I A R P P Q G R P V I I Q A
G S S P V G R E F A A R H A E V I F
T R H N R L S D A Q D F Y G D L K A
R V A R H G R D P E K V L V W P T L
A P I V A A T D T E A K Q R L Q E L
Q D L T H D H V A L R T L Q D H L G
D V D L S A Y P I D G P V P D I P Y
T N Q S Q S T T E R L I G L A R R E
N L S I R E L A L R L M G D I V V G
T P E Q L A D H M E S W F T G R G A
D G F N I D F P Y L P G S A D D F V
D H V V P E L Q R R G L Y R S G Y E
G T T L R A N L G I D A P R K A G A
End ORF1
End ORF2
A A * * L R P I G A T A R Q P E
Q K V S S R G T R S S E R Q P R I G
D G C A S A A L G A A L I N R A A N
V D R D H V A G C D C T W E R V N L
P L K G R V T G C T G C L K S S P F
W R D I V V L E R G Y W A C K Y E L
M S R L E T W A A D S I A R A L K G
N K L L N R V T L D E I V V T Q N E
R V L R T S L K H L F D C R R D T I
CCG GGC GTG GAC GCG GGC GAC CTT GCG GCG GGC TTT CTT CCG GTT CGC CGA CCC 1849
R A H V R A V K R R A K K R N A S G 229
CTT CGC CTT GCG CGA CAC GTC CCG CTG AGC CTT CGC GAG GCG GGC GCG GTC ACG 1903
K A K R S V D R Q A K A L R A R D R 211
GCG CTC GTG CTT GGG GTT GGT GAT CTT CTC CCC GGT GGA CAG GGT CAC CAG GGA 1957
R E H K P N T I K E G T S L T V L S 193
GGT GAT CCC GGC GTC GAT GCC GAC GGC CGC CGT GGT GGC GGG CGC GGG GGT GAT 2011
T I G A D I G V A A T T A P A P T I 175
GGT GTC CTC GCA CAG CAG GGA CAC GAA CCA GCG GCC CGC ACG GTC GCG GGA CAC 2065
T D E C L L S V F W R G A R D R S V 157
GGT CAC CGT CGT CGG CTC CGC CCC TTC GGG AAG GGG ACG GGA CCA GCG GAT GTC 2119
T V T T P E A G E P L P R S W R I D 139
CAG GGG CTC CGC GGT CTT CGC CAG CGT GAG CTG TCC GTT ACG CCA CGT GAA GGC 2173
L P E A T K A L T L Q G N R W T F A 121
GCT GCG GGT GTA CTC GGC CGA CGC CCT GGA CTT TTT CCG CGA CTT GTA CCG CGG 2227
S R T Y E A S A R S K K R S K Y R P 103
GTA CTT CGA CCG CTT GGC GAA GAA GTT GGC GAA CGC CGT CTG CAA GTG CCG CAG 2281
Y K S R K A F F N A F A T Q L H R L 85
CGC CTG CTG GAG CGG GAC GGA GGA CAC CTC CGA GAG GAA GGC GAG TTC TTC GGT 2335
A Q Q L P V S S V E S L F A L E E T 67
CTT CTT CCA CTC CGT CAG CGC GGC GGA CGA CTG CAC GTA GGA GAC CCG GCG CTG 2389
K K W E T L A A S S Q V Y S V R R Q 49
CTC GCC GTA CCA GGC TCG CGT GCG CCC CTC AAG CGC CTT GTT GTA CAC GAG GCG 2443
E G Y W A R T R G E L A K N Y V L R 31
GAC ACA GCC GAA CGT GCG GGA CAG CTC AGC CGC CTG CTC GTC CGT GGG ATA AAA 2497
V C G F T R S L E A A Q E D T P Y F 13
Start ORF2
GCG GTA CTT GAA AGC CCG CTT GAC CTG CTG CAT CAC gcctcacacgctatcagttcccg 2557
R Y K F A R K V Q Q M M S/D 1
tgtgagcggcgggtgtctgccggtggttgcagacgccgaaccgccctggcggcgattcgcccatccctgcc 2629
EcoRI
Start ORF3
ctgctccgcaagagcttcgtctcctccccggtctgaaggccggggtatccacgaaggaatttctg ATG ACC 2697
S/D M T 2
GCG CCC ATC CTC GTC GCC ACC CTC GAC ACC CGC GGC CCC GCC GCC ACC CTC GGC 2751
A P I L V A T L D T R G P A A T L G 20
ACG ATC ACC CGC GCC GTG CGG GCC GCG GAG GCC GCC GGA TTC GAC GCC GTC CTG 2805
T I T R A V R A A E A A G F D A V L 38
ATC GAC GAC CGG GCC GCC GCC GGC GTC CAG GGC CGG TTC GAG ACG ACG ACG CTG 2859
I D D R A A A G V Q G R F E T T T L 56
ACC GCC GCG CTG GCC GCC GTC ACC GAG CAC ATC GGC CTG ATC ACC GCC CCG CTC 2913
T A A L A A V T E H I G L I T A P L 74
CCG GCC GAC CAG GCC CCC TAC CAC GTG TCC CGG ATC ACC GCC TCG CTC GAC CAC 2967
P A D Q A P Y H V S R I T A S L D H 92
CTC GCC CAC GGC CGC ACC GGC TGG CTC GCG AGC ACG GAC ACC ACC GAC CCC GAG 3021
L A H G R T G W L A S T D T T D P E 110
GGC CGC ACC GGC GAA CTC ATC GAC GTC GTC CGC GGC CTG TGG GAC AGC TTC GAC 3075
G R T G E L I D V V R G L W D S F D 128
GAC GAC GCC TTC GTC CAC GAC CGC GCC GAC GGC CTG TAC TGG CGG CTG CCC GCC 3129
D D A F V H D R A D G L Y W R L P A 146
GTC CAC CAA CTC GAC CAC CAG GGC AGG CAC TTC GAC GTG GCC GGC CCC CTC AAC 3183
V H Q L D H Q G R H F D V A G P L N 164
GTC GCC CGC CCG CCG CAG GGC CAC CCC GTC GTC GCC GTC ACC GGC CCC GCC CTC 3237
V A R P P Q G H P V V A V T G P A L 182
GCC GCG GCC GCC GAC CTC GTC CTG CTC GAC GAG GCG GCC GAC GCC GCC TCG GTG 3291
A A A A D L V L L D E A A D A A S V 200
AAG CAG CAG GCA CCG CAC GCC AAG ATC CTC CTG CCG CTG CCC GGC CCG GCC GCC 3345
K Q Q A P H A K I L L P L P G P A A 218
GAA CTG CCC GCC GAC AGC CCC GCG GAC GGC TTC ACG GTG GCG CTC ACC GGC TCC 3399
E L P A D S P A D G F T V A L T G S 236
GAC GAC CCG GTC CTG GCC GCG CTC GCC GCC CGG CCC GGC CGC CCG GAC CGC ACC 3453
D D P V L A A L A A R P G R P D R T 254
GCG GCC ACC ACC CTG CGC GAA CGC CTG GGC CTG GCC CGC CCC GAG AGC CGC CAC 3507
A A T T L R E R L G L A R P E S R H 272
Stop ORF3
GCC CTC ACC ACC GCC tgacgacccgtccgcccgctgcttcctggagagtcatgtcccgtcgcctgt 3573
A L T T A * 277

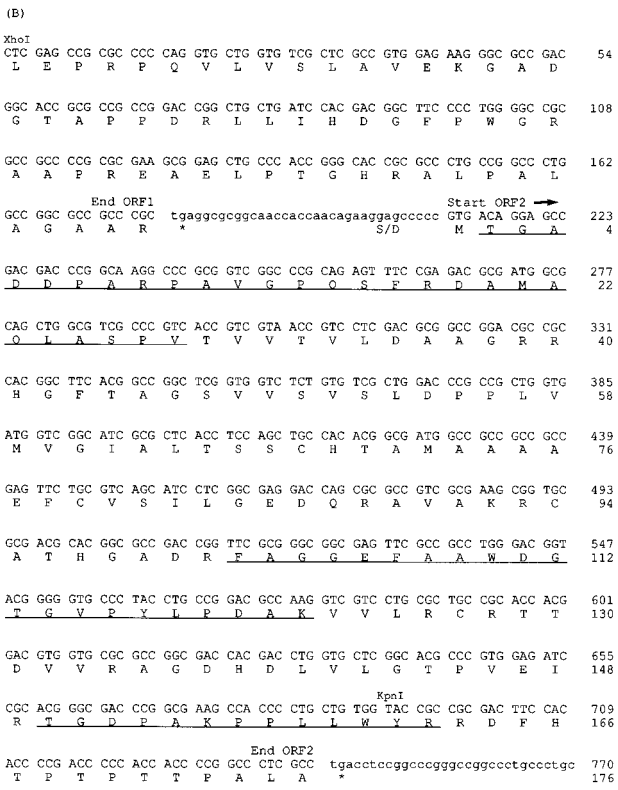


FIG. 3. (A) Nucleotide and derived amino acid sequences of a 3,573-bp region from the *Bam*HI-*Sst*I fragment carrying *snaA* and *snaB*. (B) Nucleotide sequence of a 770-bp fragment containing *snaC*. All the ORFs except ORF2 in panel A are on the strand shown. For ORF2, the amino acid sequence of the putative encoded protein was deduced from the other strand. The amino acid sequences determined from the N-terminal sequences and internal sequences of the purified SnaA, SnaB, and SnaC proteins are underlined. Noncoding DNA is represented in lowercase letters. The putative ribosome-binding sites (Shine-Dalgarno sequences [S/D] are shown. Relevant restriction sites are indicated over the nucleotide sequence.

Moreover, the two internal sequences of the protein matched exactly with internal segments of the ORF2 product (Fig. 3). The calculated molecular mass of ORF2 was smaller than the estimated 30 kDa of the purified FMN reductase. The identity of the deduced amino acid sequence with the three identified peptide sequences from the purified FMN reductase proves that ORF2 is the structural gene *snaC* encoding FMN reductase.

Sequence homology studies. No significant identity was found between SnaA, SnaB, and proteins in databases. However, the two proteins showed 37% identity over the whole sequences (Fig. 4). Gaps were introduced in the SnaB protein because of the smaller size of this subunit. High conserved regions between these gaps justified their presence.

The deduced protein corresponding to ORF401 was 50% identical with the product of ORF425 from IS*1136* from *S. erythraea*, the erythromycin producer (14). In addition, the entire gene products of *vsdF* from *Salmonella dublin* (35) and ORFE from *Salmonella typhimurium* (21), were 33% identical with the 100 N-terminal amino acids of the ORF401-encoded protein. The central 200 amino acids of ORF401 were 36% identical with the C-terminal portion of the gene product of 402 amino acids of an ORF found in the insertion sequence IS*891* from the cyanobacterium *Anabaena* sp. strain M131 (4).

Comparison of the *snaC* gene product with databases showed 39% identity with the *actVB* gene product (Fig. 4), involved in actinorhodin synthesis in *S. coelicolor* A(3)2 (17).

Disruption of the *snaA* gene in *S. pristinaespiralis*. To confirm the function of the SnaA protein, we disrupted the *snaA* gene in *S. pristinaespiralis* SP92 by single homologous recombination. *S. pristinaespiralis* protoplasts were transformed with 1 μg of pVRC505, containing an internal fragment of the *snaA* gene, as described in Materials and Methods. A few clones resistant to nosiheptide were studied. Southern blot analysis with pVRC505 as the probe showed that one clone named SP92::pVRC505 had stably integrated pVRC505 through homologous recombination (data not shown). This strain and SP92 (as control) were grown in fermentation broth and PI and PII components were extracted as described elsewhere (57). The mutant strain SP92::pVRC505 produced only PII$_B$, whereas the parental strain produced 80% PII$_A$ and 20% PII$_B$. PI production was identical in both strains. Western blotting showed that SnaA protein was absent from the mutant and, surprisingly, that SnaB was also undetectable (Fig. 5).

Homologous expression of *snaA* and *snaB* genes in *S. pristinaespiralis* SP120. SP120, isolated by chemical mutagenesis, had the same phenotype as that of SP92::pVRC505 for PII production, namely, accumulation of PII$_B$ and no immunologically cross-reacting bands with polyclonal antibodies raised against SnaA and SnaB proteins. Morever, SP120 did not produce PI. This mutant was used to perform complementation experiments with the *snaA* and *snaB* genes. Mutant SP120 was transformed with pVRC507, and nosiheptide-resistant clones were selected. Two transformants, SP120(pVRC507)-1 and SP120(pVRC507)-2, were studied further, with SP120 containing pIJ903 as a control. These clones regained the ability to oxidize PII$_B$ to PII$_A$, but complementation was partial, since PII$_A$ represented only 14% of the total PII in comparison to 80% in SP92. Expression of the *snaA* and *snaB* genes was confirmed by assay of PII$_A$ synthase activity (Table 2). PII$_A$ synthase activity of SP119 was assayed as the reference activity. FMN reductase activity was assayed as a control of the enzymatic assay. The results showed an increase in PII$_A$ synthase activity in SP120(pVRC507) clones; however and as predicted by the partial complementation, the increase was below the wild-type level (Table 2).

Heterologous complementation of the *S. coelicolor* B135 mutant by *snaC*. In order to demonstrate identity of the enzymatic activities of the SnaC protein and the product of the *actVB* gene, we expressed the *snaC* gene under the control of the *ermE** promoter in *S. coelicolor* B135, an *actVB* mutant. After transformation of the mutant B135 with unmethylated pVRC520, many transformants resistant to nosiheptide were isolated. These transformants were grown on R2YE medium (28), with nosiheptide as the selecting marker, and after 5 days, they became blue (data not shown). This color, specific for actinorhodin production (43, 53), did not appear when B135 was transformed with pIJ903.

DISCUSSION

The structural genes *snaA*, *snaB*, and *snaC* coding for the two enzymes involved in the last step of PII$_A$ biosynthesis were cloned, sequenced, and characterized. Three lines of evidence confirmed that *snaA* and *snaB* were the structural genes for PII$_A$ synthase: (i) disruption of *snaA* in *S. pristinaespiralis* resulted in strains producing only PII$_B$ and defective in SnaA and SnaB proteins; (ii) the SnaA and SnaB proteins were absent also in a PII$_A$ synthesis-deficient mutant SP120; and (iii) mutant SP120 was partially complemented for PII$_A$ production and PII$_A$ synthase activity by extra copies of *snaA* and *snaB*

FIG. 4. Alignments of amino acid sequences by the program of Kanehisa (31). (A) *S. pristinaespiralis* SnaA and SnaB proteins. (B) *S. pristinaespiralis* (SP) SnaC protein and *S. coelicolor* (SC) A(3)2 *actVB* gene product. Identical amino acids among the different sequences are shaded. Gaps in the alignments are indicated (-).

cloned in pIJ903. Although complementation of SP120 by the *snaA* and *snaB* genes was incomplete, this is unlikely to reflect the presence of a second mutation in SP120, because Sezonov (52) achieved complete complementation of this mutant with the *snaA* and *snaB* genes under the control of *ermE** promoter, using an integrative vector. The low level of complementation could be explained by the absence of a promoter in the cloned fragment and the transcription of *snaA* and *snaB* from the *tet* promoter of pIJ903.

Disruption of *snaA* led to the absence of both SnaA and SnaB proteins in Western blots. One hypothesis could be that the presence of SnaA stabilizes SnaB. However, because of the dramatic effect, it is more likely that *snaA* and *snaB* are cotranscribed. The transcript would then also include the antisense sequence of ORF401. This organization is similar to that for *eryAI* and *eryAII*, which encode multifunctional polypeptides involved in erythromycin biosynthesis in *S. erythraea* (14). These two genes are separated by an ORF, ORF425, similar to that of IS*891* from *Anabaena* sp. strain M131 in the opposite orientation. The ORF401 product is 50% identical to that of ORF425. In both cases, the low G+C content observed in the intergenic regions suggested an insertion of an external DNA fragment (14). Meanwhile, transcription of *snaA* and *snaB* in the *snaA* disruption mutant and the wild-type strain of *S. pristinaespiralis* should be examined to confirm this organization.

The conversion of PII_B to PII_A is similar to the reaction involved in the production of light by the luciferase of bioluminescent bacteria (for reviews, see references 46 and 47): luciferase catalyzing also the oxidation of a substrate (a long-chain aldehyde), coupled to the oxidation of a reduced flavin. The reaction also needs an NAD(P)H:FMN oxidoreductase. The luciferase is a heterodimer composed of two subunits, α with an M_r of 40,000 to 45,000 and β with an M_r of 35,000 to 40,000. We compared the small and large subunits of the PII_A synthase with the α and β subunits of luciferases from different bioluminescent bacteria, such as *Vibrio harveyi* (16) and *Vibrio fischeri* (18), and found only a weak identity between them. The highest scores obtained (17 to 19% identity) were always between SnaB and the α or β luciferase subunits, in the N-terminal regions. However, in all cases, a common motif was conserved (L-D-Q/H-M/L-S/A-X-G-R) in the N-terminal regions of these different proteins. Up to now, no role has been assigned to it. These proteins have similar functions, different substrates, and low identity. Nevertheless, an interesting point was the homology observed between SnaA and SnaB proteins. The same type of identity was observed between the α and β

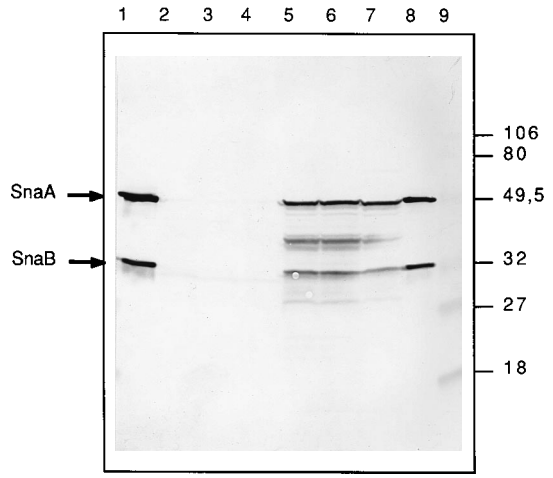


FIG. 5. Analysis of protein extracts after disruption of *snaA* gene in *S. pristinaespiralis* SP92 producing strain. Fermentation experiments were performed with SP92 and SP92::pVRC505 for 18, 20, and 22 h. Extracts were obtained by sonication of samples at each stage, and proteins were separated by electrophoresis with a SDS–12% polyacrylamide gel. The Western blot was obtained by using antibodies raised against the two subunits of the PII$_A$ synthase and stained with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate. Lanes: 1 and 8, purified PII$_A$ synthase; 2, 3, and 4, extracts from 22, 20, and 18 h of fermentation of the mutant strain SP92::pVRC505, respectively; 5, 6, and 7, extracts from 22, 20, and 18 h of fermentation of the parental strain SP92, respectively; 9, molecular weight markers (in thousands). The positions of SnaA and SnaB are indicated to the left of the gel.

subunits of the different luciferases, which commonly shared 30% identity (29, 46, 47). However, the subunits of the luciferases are closer in size than are SnaA and SnaB. These observations suggested that both protein complexes shared a similar evolutionary pathway, probably a gene duplication event (46).

Restriction analysis of pIBV1, pIBV3, and pIBV4 indicated that *snaC*, the structural gene for FMN reductase, was at least 24 kb distant from the PII$_A$ synthase genes. This was surprising because of the involvement of the three genes in the same biosynthetic step and the fact that PII$_A$ synthase and FMN reductase were expressed at the same time during fermentation (57). However, pulsed-field electrophoresis analysis of the *S. pristinaespiralis* genome showed that the three genes were present on a common 500-kb *Ase*I fragment (3). Further studies will clarify if they are part of the same cluster.

The *snaC* gene encodes a protein of 173 amino acids. SnaC is strikingly similar to the product of the *actVB* gene of the actinorhodin cluster from *S. coelicolor*. Actinorhodin biosynthesis has been well studied (5, 11, 62). From the observation of Cole et al. (11), it was proposed that the *actVB* product was involved in a late step of the pathway, corresponding to the dimerization of an intermediate, likely to be dehydrokalafungin. Recently Kendrew et al. (34) have purified the corresponding enzyme and shown that it is a flavin: NADH oxidoreductase. Dimerization of kalafungin is proposed to be a phenolic oxidation (45) and probably involves an hydroxylation step identical to the reaction involved in PII$_B$-to-PII$_A$ conversion, requiring reduced FMN. Heterologous complementation of the *actVB* mutant, B135, by *snaC* confirms the recent results of Kendrew et al. (34), showing that the *actVB* product is also an FMN reductase. The calculated and estimated (57) M_rs of SnaC, 18,000 and 30,000, respectively, are the same as those observed for the *actVB* product, which has been shown to be a dimer (34). Thibaut et al. (57) were able to oxidize PII$_B$ to PII$_A$ with purified PII$_A$ synthase and the FMN reductase from *Photobacterium fischeri*, a bioluminescent bacteria (commercial preparation from Boehringer Mannheim). Luminous bacteria usually contain several flavin reductases (15, 30, 59), and recently, genes encoding major and minor NAD(P)H-flavin oxidoreductases involved in bioluminescence reactions from different bacteria were cloned and sequenced (37, 60, 61). Although these reductases were all associated with the emission of light, they could be divided in three groups displaying no significant homology (37, 60, 61). These results underlined the diversity of flavin reductases that could be involved in the same type of reaction. Comparison of SnaC with these different FMN reductases and with the major flavin reductase of *E. coli*, Fre (1, 54), showed no significant homology. Amino acid similarity observed between SnaC and the *actVB* product and analysis of their biochemical properties (34, 57) suggested that these two enzymes belong to the same FMN reductase family and are different from the different types of FMN reductases purified from bioluminescent bacteria and from the major flavin reductase of *E. coli.*

TABLE 2. PII$_A$ synthase and FMN reductase activities of *S. pristinaespiralis* strains

Strain	Activity (μmol/h/mg) of FMN reductase	Activity (nmol/h/mg) of PII$_A$ synthase
SP119	0.23	90
SP120(pIJ903)	0.17	<0.2
SP120(pVRC507)-1	0.16	3.3
SP120(pVRC507)-2	0.09	3.9

Thus, genes corresponding to the two-enzyme system catalyzing the last step of PII$_A$ biosynthesis have been cloned and characterized. Disruption or overexpression of these genes will allow us to construct strains that selectively produce each of the two main forms of PII, PII$_B$ and PII$_A$, respectively. Morever, because of the general clustering of genes involved in the same biosynthetic pathway in *Streptomyces* sp. (27), these results give us the possibility to identify other genes involved in pristinamycin biosynthesis by chromosome walking.

ACKNOWLEDGMENTS

We are indebted to D. A. Hopwood (John Innes Center) for carefully reading of the manuscript and gifts of strains and plasmids. We thank J.-F. Mayaux for support during this work; N. Bamas-Jacques, V. de Crecy-Lagard, and D. Thibaut for discussions; W. Wohlleben (Universität des Saalardes) for gift of pDH5 vector; A. Crespo and N. Couteault for PII$_A$ synthase antiserum preparation; and T. Ciora for oligonucleotide synthesis.

This work has been done as part of the Bio Avenir programme supported by Rhône-Poulenc Rorer with the participation of the French Ministry of Research and the French Ministry of Industry.

REFERENCES

1. **Andrews, S. C., D. Shipley, J. N. Keen, J. B. C. Findlay, P. M. Harrisson, and J. R. Guest.** 1992. The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin NADP$^+$ reductases. FEBS Lett. **302:**247–252.
2. **Aumercier, M., S. Bouhallab, M. L. Capmau, and F. Le Goffic.** 1992. RP 59500: a proposal mechanism for its bactericidal activity. J. Antimicrob. Chemother. **30**(Suppl. A)**:**9–14.
3. **Bamas-Jacques, N., S. Lorenzon, P. Lacroix, and J. Crouzet.** 1994. Resolution of the *S. pristinaespiralis* chromosome by pulse-field electrophoresis and mapping of the genes involved in the pristinamycins I and II biosynthetic pathways, abstr. P1-12, p. 98. *In* Abstracts of the 9th International Symposium on Biology of Actinomycetes.
4. **Bancroft, I., and C. P. Wolk.** 1989. Characterization of an insertion sequence (IS*891*) of novel structure from the cyanobacterium *Anabaena* sp. strain M-131. J. Bacteriol. **171:**5949–5954.

5. **Bartel, P. L., C. B. Zhu, J. S. Lampel, D. C. Dosch, N. C. Connors, W. R. Strohl, J. J. Beale, and H. G. Floss.** 1990. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in *Streptomycetes*: clarification of actinorhodin gene functions. J. Bacteriol. **172:**4816–4826.
6. **Bibb, M. J., J. White, J. M. Ward, and G. R. Janssen.** 1994. The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. Mol. Microbiol. **14:**533–545.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. **41:**459–472.
8. **Chung, C. T., and H. Miller.** 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. **16:**3580.
9. **Cocito, C. G.** 1979. Antibiotics of the virginiamycin family, inhibitors which contain synergistic components. Microbiol. Rev. **43:**145–198.
10. **Cocito, C. G., and G. Hinali.** 1985. Molecular mechanism of action of virginiamycin-like antibiotics (synergimycins) on protein synthesis in bacterial cell-free systems. J. Antimicrob. Chemother. **16**(Suppl. A)**:**35–52.
11. **Cole, S. P., B. A. Rudd, D. A. Hopwood, C. J. Chang, and H. G. Floss.** 1987. Biosynthesis of the antibiotic actinorhodin analysis of blocked mutants of *Streptomyces coelicolor*. J. Antibiot. (Tokyo) **40:**340–347.
12. **de Crecy-Lagard, V. (Rhône-Poulenc Rorer).** 1994. Personal communication.
13. **Dessen, P. C., C. Fondrat, C. Valencien, and C. Mugnier.** 1990. BISANCE: a French service for access to biomolecular sequence databases. Comput. Appl. Biosci. **6:**355–356.
14. **Donadio, S., and M. J. Staver.** 1993. IS*1136*, an insertion element in the erythromycin gene cluster of *Saccharopolyspora erythraea*. Gene **126:**147–151.
15. **Duane, W., and J. W. Hasting.** 1975. Flavin mononucleotide reductase of luminous bacteria. Mol. Cell. Biochem. **6:**53–64.
16. **Escher, A., D. J. O'Kane, and A. A. Szalay.** 1991. The beta subunit polypeptide of *Vibrio harveyi* luciferase determines light emission at 42°C. Mol. Gen. Genet. **230:**385–393.
17. **Fernandez-Moreno, M. A., E. Martinez, L. Boto, D. A. Hopwood, and F. Malpartida.** 1992. Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. J. Biol. Chem. **267:**19278–19290.
18. **Foran, D. R., and W. M. Brown.** 1988. Nucleotide sequence of the *luxA* and *luxB* genes of the bioluminescent marine bacterium *Vibrio fischeri*. Nucleic Acids Res. **16:**777.
19. **Frey, J., M. Bagdarasian, D. Feiss, F. C. H. Franklin, and J. Dehusses.** 1983. Stable cosmid vectors that enable the introduction of cloned fragments into a wide range of Gram-negative bacteria. Gene **24:**299–308.
20. **Gibson, T. J.** 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. Cambridge University, Cambridge.
21. **Gulig, P. A., A. L. Caldwell, and V. A. Chiodo.** 1992. Identification, genetic analysis and DNA sequence of a 7.8-kb virulence region of the *Salmonella typhimurium* virulence plasmid. Mol. Microbiol. **6:**1395–1411.
22. **Hanahan, D.** 1983. Studies on transformation of *E. coli* with plasmids. J. Mol. Biol. **166:**557–580.
23. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73:**237–244.
24. **Hirel, P. H., J. M. Schmitter, P. Dessen, G. Fayat, and S. Blanquet.** 1989. Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. Proc. Natl. Acad. Sci. USA **86:**8247–8251.
25. **Hillemann, D., A. Pülher, and W. Wohlleben.** 1991. Gene disruption and gene replacement in *Streptomyces* via single stranded DNA transformation of integration vectors. Nucleic Acids Res. **19:**727–731.
26. **Hohn, B., and J. F. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11:**291–298.
27. **Hopwood, D. A., M. J. Bibb, K. F. Chater, G. R. Janssen, F. Malpartida, and C. Smith.** 1986. Regulation of gene expression in antibiotic-producing *Streptomyces*, p. 251–276. *In* I. R. Booth and C. F. Higgins (ed.), Regulation of gene expression—25 years on, Cambridge University Press, Cambridge.
28. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. A laboratory manual. The John Innes Foundation, Norwich, England.
29. **Illarionov, B. A., V. M. Blinov, A. P. Donchenko, M. V. Protopopova, V. A. Karginov, N. P. Mertvetsov, and J. I. Gitelson.** 1990. Isolation of bioluminescent functions from *Photobacterium leiognathi*: analysis of *lux*A, *lux*B, *lux*G and neighboring genes. Gene **86:**89–94.
30. **Jablonski, E., and M. DeLuca.** 1977. Purification and properties of the NADH and NADPH specific FMN oxidoreductases from *Beneckea harveyi*. Biochemistry **16:**2932–2936.
31. **Kanehisa, M.** 1984. Use of statistical criteria for screening potential homologies in nucleic acids sequences. Nucleic Acids Res. **12:**203–215.
32. **Katz, E., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129:**2703–2714.
33. **Kay, R., and J. McPherson.** 1987. Hybrid pUC vectors for addition of new restriction enzyme sites to the ends of DNA fragments. Nucleic Acids Res. **15:**2778.
34. **Kendrews, S. G., S. E. Harding, D. A. Hopwood, and N. G. Marsh.** Identification of a flavin:NADH oxidoreductase involved in the biosynthesis of actinorhodin: purification and characterization of the recombinant enzyme. Submitted for publication.
35. **Krause, M., C. Roudier, J. Fierer, J. Harwood, and D. Guiney.** 1991. Molecular analysis of the virulence locus of the *Salmonella dublin* plasmid pSDL2. Mol. Microbiol. **5:**307–316.
36. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
37. **Lei, B., M. Liu, S. Huang, and S.-C. Tu.** 1994. *Vibrio harveyi* NAD(P)H-flavin oxidoreductase: cloning, sequencing and overexpression of the gene purification and characterization of the cloned enzyme. J. Bacteriol. **176:**3552–3558.
38. **Low, B.** 1968. Formation of merodiploids in matings with a class of Rec-recipient strains of *E. coli* K12. Proc. Natl. Acad. Sci. USA **60:**160.
39. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
40. **Lydiate, D. J., F. Malpartida, and D. A. Hopwood.** 1985. The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene **35:**223–235.
41. **MacNeil, D. J.** 1988. Characterization of a unique methyl-specific restriction system in *Streptomyces avermitilis*. J. Bacteriol. **170:**5607–5612.
42. **MacNeil, T. (Merck Sharp & Dohme Research Laboratories).** 1990. Personal communication.
43. **Malpartida, F., and D. A. Hopwood.** 1986. Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. **205:**66–73.
44. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Mann, J.** 1987. Secondary metabolism. Clarendon Press, Oxford.
46. **Meighen, E. A.** 1988. Enzymes and genes from the *lux* operons of bioluminescent bacteria. Annu. Rev. Microbiol. **42:**151–176.
47. **Meighen, E. A.** 1993. Bacterial bioluminescence: organization, regulation and application of the *lux* genes. FASEB J. **7:**1016–1022.
48. **Pridham, T. G., P. Anderson, C. Foley, L. A. Lindenfelser, C. W. Hesseltine, and R. C. Benedict.** 1957. A selection of media for maintenance and taxonomic study of *Streptomyces*. Antibiotic Annu. **1956–1957:**947–953.
49. **Purvis, M. B., J. W. Le Fevre, V. L. Jones, D. G. I. Kingston, A. M. Biot, and F. Gosselé.** 1989. Biosynthesis of antibiotics of the virginiamycin family. 8. Formation of the dehydroproline residue. J. Am. Chem. Soc. **111:**5931–5935.
50. **Rudd, B. A., and D. A. Hopwood.** 1979. Genetics of actinorhodin biosynthesis by *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **114:**35–43.
51. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
52. **Sezonov, G. (Université Paris-Sud).** 1994. Personal communication.
53. **Sherman, D. H., E.-S. Kim, M. J. Bibb, and D. A. Hopwood.** 1992. Functional replacement of genes for individual polyketide synthase components in *Streptomyces coelicolor* A3(2) by heterologous genes from a different polyketide pathway. J. Bacteriol. **174:**6184–6190.
54. **Spyrou, G., E. Haggard-Ljungquist, M. Krook, H. Jörnval, E. Nilsson, and P. Reichard.** 1991. Characterization of the flavin reductase gene (*fre*) of *Escherichia coli* and construction of a plasmid for overproduction of the enzyme. J. Bacteriol. **173:**3673–3679.
55. **Staden, R., and A. D. McLachlan.** 1982. Codon preference and its use in identifying protein coding regions in long DNA sequences. Nucleic Acids Res. **10:**141–156.
56. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent *Streptomyces* promotors. Nucleic Acids Res. **20:**961–974.
57. **Thibaut, D., N. Ratet, D. Bisch, D. Faucher, L. Debussche, and F. Blanche.** 1995. Purification of the two-enzyme system catalyzing the oxidation of the D-proline residue of pristinamycin II_B during the last step of pristinamycin II_A biosynthesis. J. Bacteriol. **177:**5199–5205.
58. **Vazquez, D.** 1975. The streptogramin family of antibiotics. Antibiotics **3:**521–534.
59. **Watanabe, H., and J. W. Hasting.** 1982. Specificities and properties of three reduced pyridine nucleotide-flavin mononucleotide reductases coupling to bacterial luciferase. Mol. Cell. Biochem. **44:**181–187.
60. **Zenno, S., and K. Saigo.** 1994. Identification of the genes encoding NAD(P)H-flavin oxidoreductases that are similar in sequence to *Escherichia coli* Fre in four species of luminous bacteria: *Photorhabdus luminescens*, *Vibrio fischeri*, *Vibrio harveyi*, and *Vibrio orientalis*. J. Bacteriol. **176:**3544–3551.
61. **Zenno, S., K. Saigo, H. Kanh, and S. Inouye.** 1994. Identification of the gene encoding the major NAD(P)H-flavin oxidoreductase of the bioluminescent bacterium *Vibrio fischeri* ATCC 7744. J. Bacteriol. **176:**3536–3543.
62. **Zhang, H., X. G. He, A. Adefarati, J. Galluci, S. P. Cole, J. M. Beale, P. J. Keller, C. Chang, and H. G. Floss.** 1990. Mutactin, a novel polyketide from *Streptomyces coelicolor*. Structure and biosynthetic relationship to actinorhodin. J. Org. Chem. **55:**1682–1684.