

Research Paper

Cloning and characterization of a phosphopantetheinyl transferase from *Streptomyces verticillus* ATCC15003, the producer of the hybrid peptide–polyketide antitumor drug bleomycin

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Abstract

Background: Phosphopantetheinyl transferases (PPTases) catalyze the posttranslational modification of carrier proteins by the covalent attachment of the 4'-phosphopantetheine (P-pant) moiety of coenzyme A to a conserved serine residue, a reaction absolutely required for the biosynthesis of natural products including fatty acids, polyketides, and nonribosomal peptides. PPTases have been classified according to their carrier protein specificity. In organisms containing multiple P-pant-requiring pathways, each pathway has been suggested to have its own PPTase activity. However, sequence analysis of the bleomycin biosynthetic gene cluster in *Streptomyces verticillus* ATCC15003 failed to reveal an associated PPTase gene.

Results: A general approach for cloning PPTase genes by PCR was developed and applied to the cloning of the *svp* gene from *S. verticillus*. The *svp* gene is mapped to an independent locus not clustered with any of the known NRPS or PKS clusters. The Svp protein was overproduced in *Escherichia coli*, purified to homogeneity, and shown to be a monomer in solution. Svp is a PPTase capable of modifying both type I and type II acyl carrier proteins

(ACPs) and peptidyl carrier proteins (PCPs) from either *S. verticillus* or other *Streptomyces* species. As compared to Sfp, the only 'promiscuous' PPTase known previously, Svp displays a similar catalytic efficiency (k_{cat}/K_m) for the BImI PCP but a 346-fold increase in catalytic efficiency for the TcmM ACP.

Conclusions: PPTases have recently been re-classified on a structural basis into two subfamilies: ACPS-type and Sfp-type. The development of a PCR method for cloning Sfp-type PPTases from actinomycetes, the recognition of the Sfp-type PPTases to be associated with secondary metabolism with a relaxed carrier protein specificity, and the availability of Svp, in addition to Sfp, should facilitate future endeavors in engineered biosynthesis of peptide, polyketide, and, in particular, hybrid peptide–polyketide natural products. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Phosphopantetheinyl transferase; Bleomycin; Acyl carrier protein; Peptidyl carrier protein; *Streptomyces verticillus*

Abbreviations: ACP, acyl carrier protein; ArCP, aryl carrier protein; BLM, bleomycin; CoA, coenzyme A; FAS, fatty acid synthase; MW, molecular weight; MBP, maltose binding protein; NRPS, nonribosomal peptide synthetase; PCP, peptidyl carrier protein; PCR, polymerase chain reaction; pI, isoelectric point; PKS, polyketide synthase; P-pant, 4'-phosphopantetheine; PPTase, phosphopantetheinyl transferase; RBS, ribosome binding site; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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1. Introduction

Fatty acid synthases (FASs), polyketide synthases (PKSs), and nonribosomal peptide synthetases (NRPSs) all contain one or more small proteins, either as integrated domains (type I) or as discrete subunits (type II), that function as carrier proteins for the growing intermediate in the biosynthesis of fatty acids, polyketides, and nonribosomal peptides. Depending on the nature of the growing intermediate, these carrier proteins are called acyl carrier proteins (ACPs), aryl carrier proteins (ArCPs), or peptidyl carrier proteins (PCPs). Carrier proteins are also involved in other processes, such as transacylation of bacterial oligosaccharides [1,2] and proteins [3,4], α -aminoadipate reduction in yeast [5], and formation of

β -alanyl-dopamine in *Drosophila* [6]. A characteristic feature of carrier proteins is the covalently bound 4'-phosphopantetheine (P-pant) prosthetic group. The P-pant group plays a dual role in these multienzyme systems – acting as a nucleophile to activate acyl groups by a thioester linkage to the terminal thiol and providing a flexible arm (20 Å) to channel the growing intermediates between the neighboring active sites [7,8]. In order to be functional, all carrier proteins must be converted from the inactive apo-forms to the functional holo-forms by the covalent attachment of the P-pant group to a conserved serine residue of the carrier proteins. This posttranslational modification is catalyzed by a superfamily of enzymes known as phosphopantetheinyl transferases (PPTases) that derive the P-pant group from coenzyme A (CoA) [9,10].

In organisms containing multiple P-pant-requiring pathways, each pathway has been suggested to have its own posttranslational modifying PPTase activity [9,10]. This hypothesis has been proved to be true in several organisms. For example, three different PPTase activities are known in the yeast *Saccharomyces cerevisiae*, encoded by the *PPT2*, *FAS2* (subunit α of cytoplasmic FAS where the PPTase is integrated as a C-terminal domain), and *LYS5* genes. The three PPTases specifically modify different apo-proteins – PPT2 acts on the type II ACP of mitochondrial FAS, the PPTase domain of FAS2 acts intermolecularly on the type I ACP domain located at the N-terminus of the FAS subunit α itself, and Lys5 acts on the type I PCP domain of the α -amino adipate reductase Lys2 [5,11,12]. *PPT2*, *FAS2*, and *Lys5* mutants show distinct phenotypes, affecting either respiration [11], fatty acid synthesis [13,14], or lysine biosynthesis [15], respectively. Similarly, there are three PPTases in *Escherichia coli* – ACPS for the specific modification of the type II ACP of the FAS complex, EntD for the activation of the ArCP and type I PCP domains of the NRPS complex involved in enterobactin biosynthesis, and YhhU (also called o195) whose protein substrate remains unknown [9,16]. (Very recently, it has been reported that YhhU is able to modify apo-ACP in vivo and to complement an *acpS* mutation, suggesting that *E. coli* has evolved two enzymes capable of modifying apo-ACP due to its essential role in cell survival [17].) Furthermore, in vivo genetic studies have established that several PPTases, such as Sfp in *Bacillus subtilis* (for surfactin), Gsp in *Bacillus brevis* (for gramicidin), and EntD in *E. coli* (for enterobactin), are required for non-ribosomal peptide biosynthesis in these organisms, but are not essential for their survival [18,19]. The latter results suggest additional unidentified PPTases in the *Bacillus* organisms specific for the ACPs of their respective FAS, which, like *E. coli* ACPS, will be essential for cell viability. All these examples support the view of co-evolution of PPTases with their respective P-pant-requiring pathways.

Genes encoding the production of secondary metabolites, such as nonribosomal peptides and polyketides, are often clustered in one region of the bacterial chromosome.

Then, if every PKS or NRPS has its own dedicated PPTase, one should expect to find a PPTase gene within each cluster. This has indeed been the case for several gene clusters encoding nonribosomal peptide biosynthesis, such as those for enterobactin in *E. coli* [18], surfactin in *B. subtilis* [20], and gramicidin in *B. brevis* [21], and polyketide biosynthesis, such as those for nystatin in *Streptomyces noursei* [22] and possibly landomycin in *Streptomyces cyanogenus* [23]. One could then envisage the use of known PPTase genes as additional probes for the cloning of novel polyketide and nonribosomal peptide biosynthetic gene clusters. However, exceptions to the clustering of PPTase and NRPS genes are already known, such as the mycobactin biosynthetic gene cluster from *Mycobacterium tuberculosis*, which lacks the corresponding PPTase gene [24]. In fact, PPTase genes have not been found to be clustered with PKS genes in most of the polyketide biosynthetic gene clusters known to date.

Until very recently, research in polyketide and nonribosomal peptide biosynthesis was handicapped by the frequent inability to produce fully active, holo-forms of PKSs or NRPSs in heterologous hosts, probably because the host PPTases either fail to recognize or act inefficiently on these carrier protein substrates. For that reason the identification of the PPTase family and utilization of various PPTases, either in vivo or in vitro, to facilitate the production of active PKSs and NRPSs have remarkably accelerated the progress in this field [25–27]. We have been studying the biosynthesis of bleomycin (BLM) in *Streptomyces verticillus* ATCC15003 as a model system to investigate intermodular communication between NRPS and PKS modules [28–32]. BLM is an antitumor drug belonging to a group of natural products of hybrid peptide–polyketide origin, synthesized by a hybrid NRPS–PKS, the Blm NRPS/PKS/NRPS megasynthetase [30]. This prompted us to search for a PPTase from *S. verticillus* in order to use it either in vitro or in vivo for the production of properly modified, fully active Blm NRPS and PKS proteins for our studies. We were particularly interested in finding out (1) if the PPTase gene is clustered with their respective NRPS or PKS genes, as we know that *S. verticillus* contains at least three additional NRPS gene clusters and one additional PKS gene cluster [28], and (2) if a PPTase from a hybrid peptide–polyketide metabolite-producing organism exhibits relaxed substrate specificity towards both ACPs and PCPs. Here, we report a general approach for identifying and cloning PPTase genes from *Streptomyces* species, its application to the cloning of an *S. verticillus* PPTase gene (*svp*), and the biochemical characterization of Svp as a PPTase. The *svp* gene seems not to be clustered to any of the NRPS or PKS loci cloned previously from *S. verticillus*. The Svp PPTase can efficiently 4'-phosphopantetheinylate both type I and type II ACPs and PCPs from either *S. verticillus* or other *Streptomyces* species. Hence Svp should be of great utility in engineered biosynthesis of polyketide,

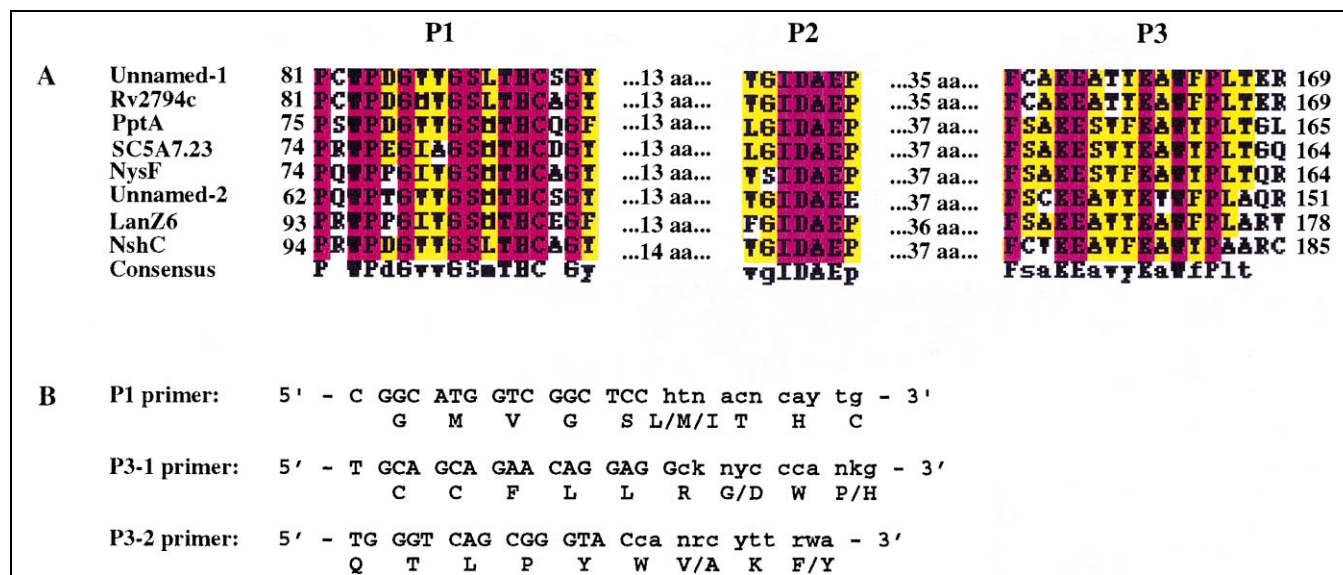


Fig. 1. (A) The three conserved motifs of PPTases from actinomycetes. The accession numbers are given in parentheses: Unnamed-1 (CAB36579), Rv2794c (A70885), Svp (AF210331), SC5A7.23 (T35172), NysF, (AF263912), Unnamed-2 (BAA22407), LanZ6 (AAD13565), and NshC (AAB17877). The numbers at two ends indicate the position of amino acids in the respective proteins, and the numbers between the motifs indicate the distance in amino acids. (B) Primers designed for the P1 and P3 motifs according to the CODEHOP strategy [35]. H, C/A; K, G/T; N, A/C/T/G; R, A/G; W, T/A; Y, C/T.

nonribosomal peptide, and, in particular, hybrid peptide–polyketide natural products, many of which are clinically important drugs such as BLM, rapamycin, and epothilone.

2. Results

2.1. Cloning of the *svp* gene from *S. verticillus* ATCC15003 by polymerase chain reaction (PCR)

The similarities among PPTases from different organisms are reduced to two short motifs: (V/I)G(V/I)D (motif P2) and (F/W)(S/C/T)XKE(A/S)hhK (h refers to a hydrophobic amino acid) (motif P3), separated by 40–45 residues [9,10]. PPTases have been previously classified according to their carrier protein specificity into two groups: PPTases for ACPs involved in fatty acid and polyketide biosynthesis and PPTases for ArCPs and PCPs involved in nonribosomal peptide and siderophore biosynthesis [10]. Since the Blm NRPS/PKS/NRPS megasynthetase contains at least 10 PCPs and only one ACP [30], we decided to bias our search towards a PCP-specific PPTase from *S. verticillus*. Several PCP-specific PPTase sequences were used to screen the databases for homologues, limiting only to those from actinomycetes. Seven proteins of unknown function were found: LanZ6 from *S. cyanogenus* (accession number AAD13565), NshC from *Streptomyces actuosus* (accession number AAB17877) [33], NysF from *S. noursei* (accession number AF263912) [22], Rv2794c from *Mycobacterium tuberculosis* (accession number A70885) [34], SC5A7.23 from *Streptomyces coelicolor* (ac-

cession number T35172), and two unnamed proteins from *Streptomyces* sp. strain TH1 (accession number BAA22407) [35] and *Mycobacterium leprae* (accession number CAB36579). The alignment of these actinomycete sequences not only confirmed the two conserved motifs, P2 and P3, but also revealed an additional motif, PXWPXGX₂GS(M/L)THCXGY (motif P1), located about 15 amino acids upstream of the P2 motif (Fig. 1A). We designed one forward primer for the P1 motif (P1 primer) and two reverse primers around the P3 motif (P3-1 and P3-2 primers) (Fig. 1B), using the recently developed CODEHOP strategy (Consensus-DEgenerate Hybrid Oligonucleotide Primer) of PCR primer design [36]. While no product was detected when the P1/P3-1 pair of primers was used, a distinct band with the expected size of approximately 250 bp was readily amplified from *S. verticillus* chromosomal DNA with the P1/P3-2 pair of primers. The PCR product was cloned, and eight randomly selected clones were sequenced. All eight clones yielded

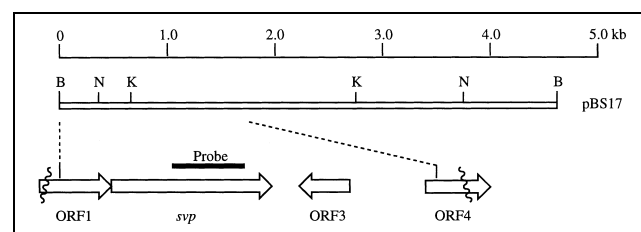


Fig. 2. Restriction map of the 4.7-kb *Bam*HI fragment in pBS17 and genetic organization of the sequenced *svp* locus. B, *Bam*HI; N, *Nco*I; K, *Kpn*I.

an identical sequence (except differences resulting from primer utilization), which shows high homology to the putative actinomycete PPTases (Fig. 1A). The PCR product was then used as a probe to screen an *S. verticillus* genomic library by colony hybridization. Of the 10 000 colonies screened, 25 positive clones were identified, and subsequently confirmed by Southern analysis to contain the same 4.7-kb *Bam*HI fragment hybridizing to the probe (Fig. 2).

2.2. Sequence analysis of the *svp* locus

The 4.7-kb *Bam*HI fragment containing the PCR-amplified probe was subcloned (pBS17), and the nucleotide (nt) sequence of a 1761-bp region was determined. Two incomplete open reading frames (ORFs), *orf1* and *orf4*, and two complete ORFs, *svp* and *orf3*, were identified within the sequenced region (Fig. 2). The incomplete *orf1* starts out of the analyzed area and ends with a TGA codon at nt 248. The deduced product of *orf1*, the 81-amino acid C-terminus, showed similarities to Rv2795c from *M. tuberculosis* (accession number CAA15590) and SC5A7.22 from *S. coelicolor* (accession number CAA19951), both of unknown function. The second ORF, *svp*, contains the PCR-amplified probe used for the cloning of this locus. It starts with a GTG codon at nt 245, which is translationally coupled to the TGA stop codon of *orf1*, and ends with a TAA codon at nt 983. The starting codon of *svp* is preceded by a potential ribosomal binding site (RBS), GGGAG. The overall (76.6%) and third codon position (93.9%) G+C contents and the codon usage of *svp* are similar to those found in other *Streptomyces* genes, with the exception of the stop codon (TAA), which is uncommon in this group of organisms [37,38]. The *svp* gene encodes a 246-amino acid protein with a predicted molecular weight (MW) of 25 619 and an isoelectric point (pI) of 4.76. The deduced Svp protein contains all three conserved PPTase motifs (Fig. 1A) and shows significant similarities to the putative PPTases and their homologues of actinomycete origin, including LanZ6 (49% identity and 54% similarity), NshC (50% identity and 54% similarity), NysF (57% identity and 61% similarity), Rv2794c (49% identity and 58% similarity), SC5A7.23 (60% identity and 66% similarity), and the two unnamed proteins from *Streptomyces* sp. TH1 (56% identity and 62% similarity) and *M. leprae* (49% identity and 59% similarity). Interestingly, the homology between Svp and PPTases from other bacteria is significantly low, albeit apparent, such as the confirmed PPTases EntD (17% identity and 24% similarity) [18] and ACPS (18% identity and 30% similarity) from *E. coli* [39], Gsp from *B. brevis* (19% identity and 25% similarity) [25], and Sfp from *B. subtilis* (22% identity and 34% similarity) [20]. The third ORF is separated from *svp* by a 153-bp noncoding region. *orf3* starts with an ATG codon at nt 1358, preceded by a potential RBS (GAAGG), and ends with a TGA codon at nt 1121. The

deduced ORF3 product shows similarities to the N-terminal region of SC5H1.35c, a protein of unknown function from *S. coelicolor* (accession number CAB42960). The fourth ORF is separated from *orf3* by a 251-bp noncoding region. *orf4* starts with an ATG codon at nt 1610, preceded by a potential RBS (GGAGG), and ending out of the sequenced region. The deduced product of the incomplete *orf4*, the 50-amino acid N-terminus, contains a potential NAD/FAD binding motif, GXGX₂GX₃GX₆G [40], hence showing low similarities to diverse oxidoreductases.

2.3. Overexpression in *E. coli* and purification of the Svp protein

In order to test if *svp* encodes a functional PPTase, we overexpressed the *svp* gene in *E. coli* and purified the Svp protein to assay its catalytic competence on apo-ACPs or apo-PCPs. We amplified the *svp* gene, using PCR, from pBS17 and cloned it into the pQE-70 expression vector, yielding pBS18, in such a way that Svp will be produced as a fusion protein with a His₆-tagged C-terminus. Introduction of pBS18 into *E. coli* M15(pREP4) under the standard overexpression conditions recommended by the manufacturer resulted in overproduction of Svp predominantly in soluble form (>90%), which was readily purified by affinity chromatography on Ni-NTA agarose (Fig. 3). The purified Svp protein migrates as a single band with an apparent MW of 31 000 (calculated MW for His₆-tagged Svp is 26 830) upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and eluted with apparent MW of 31 600 upon gel filtration chromatography. The abnormal mobility of PPTases upon SDS–PAGE has been observed previously, such as Sfp migrating with an apparent MW of 32 000 (calculated MW of 26 135) [20] and Gsp migrating with an apparent MW of 30 000 (calculated MW of 27 856) [25].

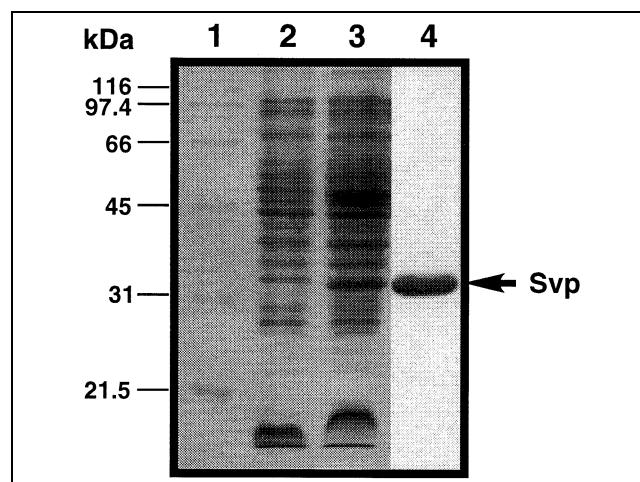


Fig. 3. Expression and purification of Svp from *E. coli* M15(pREP4)(pBS18). Lane 1, MW markers; 2, before IPTG induction; 3, after IPTG induction; 4, purified Svp.

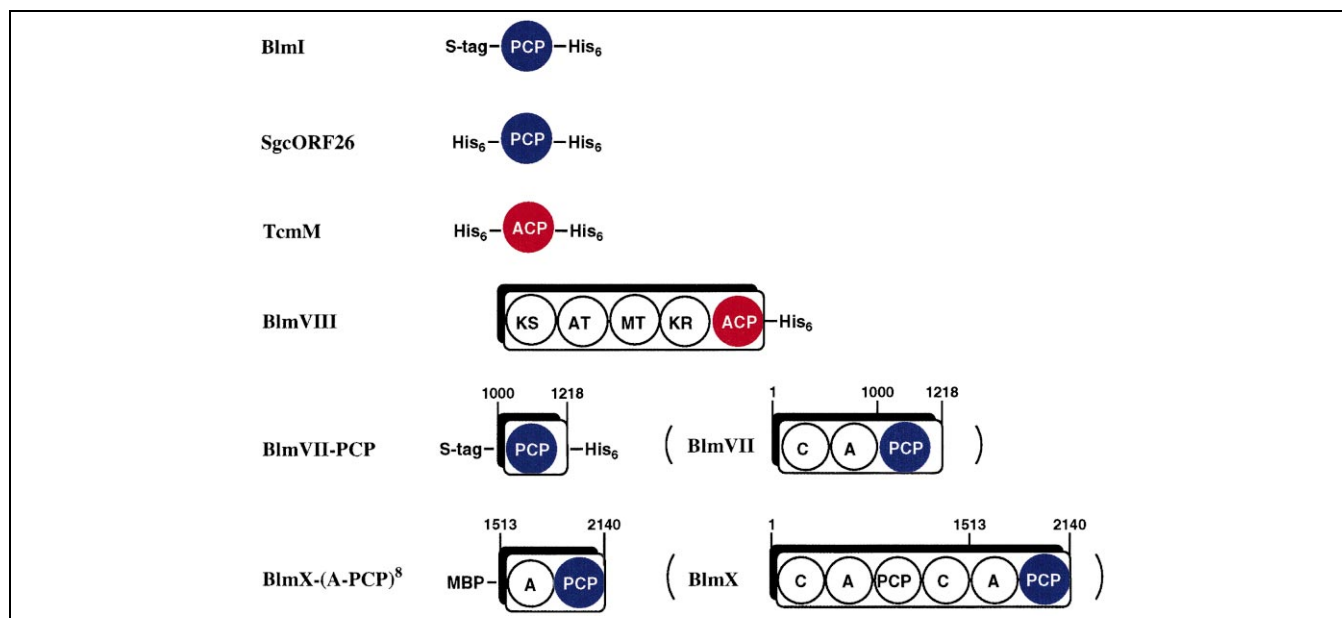


Fig. 4. ACPs (red) and PCPs (blue) used in this study. These carrier proteins are overproduced in *E. coli* as fusion proteins with various tags as indicated. BlmI, SgcORF26, TcmM are monofunctional type II proteins. BlmVIII is a five-domain type I PKS, and BlmVII and BlmX are three-domain and six-domain type I NRPSs, respectively. The numbers shown above the rectangles indicate amino acid residues. A, adenylation; AT, acyltransferase; C, condensation; KR, ketoreductase; KS, ketoacyl synthase; MT, methyltransferase.

2.4. Overexpression in *E. coli* and purification of apo-ACPs and apo-PCPs

To test Svp as a PPTase requires either apo-ACPs or apo-PCPs. We chose BlmI and SgcORF26 as examples of type II PCPs, the PCP domains of BlmVII and the second NRPS module (NRPS-8) of BlmX as examples of type I PCPs, TcmM as an example of type II ACP, and the BlmVIII PKS as an example of a type I ACP (Fig. 4). BlmI, BlmVII, BlmVIII, and BlmX are components of the Blm NRPS/PKS/NRPS megasynthetase from *S. verticillus* [28–31]. TcmM is the type II ACP of the well characterized Tcm PKS complex catalyzing the biosynthesis of tetracenomycin in *Streptomyces glaucescens* [41]. SgcORF26 is a type II PCP from the recently characterized gene cluster encoding the biosynthesis of the enediyne antitumor antibiotic C-1027 in *Streptomyces globisporus* [42]. We chose TcmM and SgcORF26 as substrates to test the specificity of the Svp PPTase for carrier proteins from other *Streptomyces* species.

We have previously reported the overproduction and purification of BlmI [29]. To overproduce BlmVII-PCP in *E. coli*, we determined the domain boundaries according to Marahiel and co-workers [43,44], amplified the *blmVII-PCP* fragment, using PCR, from pBS6 and cloned it into pQE-60 to yield pBS19, in which BlmVII-PCP would be produced as a fusion protein with a His₆ tag at its C-terminus. However, introduction of pBS19 into *E. coli* M15(pREP4) under the standard overexpression conditions recommended by the manufacturer yielded very poor BlmVII-PCP production, as judged by SDS-PAGE

(data not shown). We then moved the *blmVII-PCP* fragment from pBS19 into pET-29a to yield the second overexpression construct, pBS20, in which BlmVII-PCP was produced as a fusion protein with an S tag at the N-terminus and a His₆ tag at the C-terminus (Fig. 4). Introduction of pBS20 into *E. coli* BL21(DE-3) under the standard overexpression conditions recommended by the manufacturer resulted in good production of BlmVII-PCP with about 40% of the resultant protein in soluble form, which was purified by affinity chromatography on Ni-NTA agarose. BlmVII-PCP is significantly longer (219 amino acids) than a typical PCP domain (~90 amino acids) because we used the native C-terminus of BlmVII (Fig. 4). The latter has an additional 130-amino acid segment beyond the PCP domain, which shows no apparent sequence homology to any known proteins. The purified BlmVII-PCP migrates as a single band with an apparent MW of 36 000 (Fig. 5A), significantly larger than the calculated MW of BlmVII-PCP with both S and His tags of 26 758. Similar abnormal mobility of both ACP and PCP upon SDS-PAGE has been observed previously [2,44].

To overproduce BlmVIII in *E. coli*, the *blmVIII* gene was amplified, using PCR, from pBS6 and cloned into pQE-60 to yield pBS21, in which BlmVIII was produced as a fusion protein with the His₆ tag at the C-terminus (Fig. 4). While introduction of pBS21 into *E. coli* M15(pREP4) under the standard overexpression conditions recommended by the manufacturer yielded nearly completely insoluble protein, lowering the incubation temperature to 30°C and IPTG concentration to 200 µM improved the solubility of the resultant BlmVIII protein

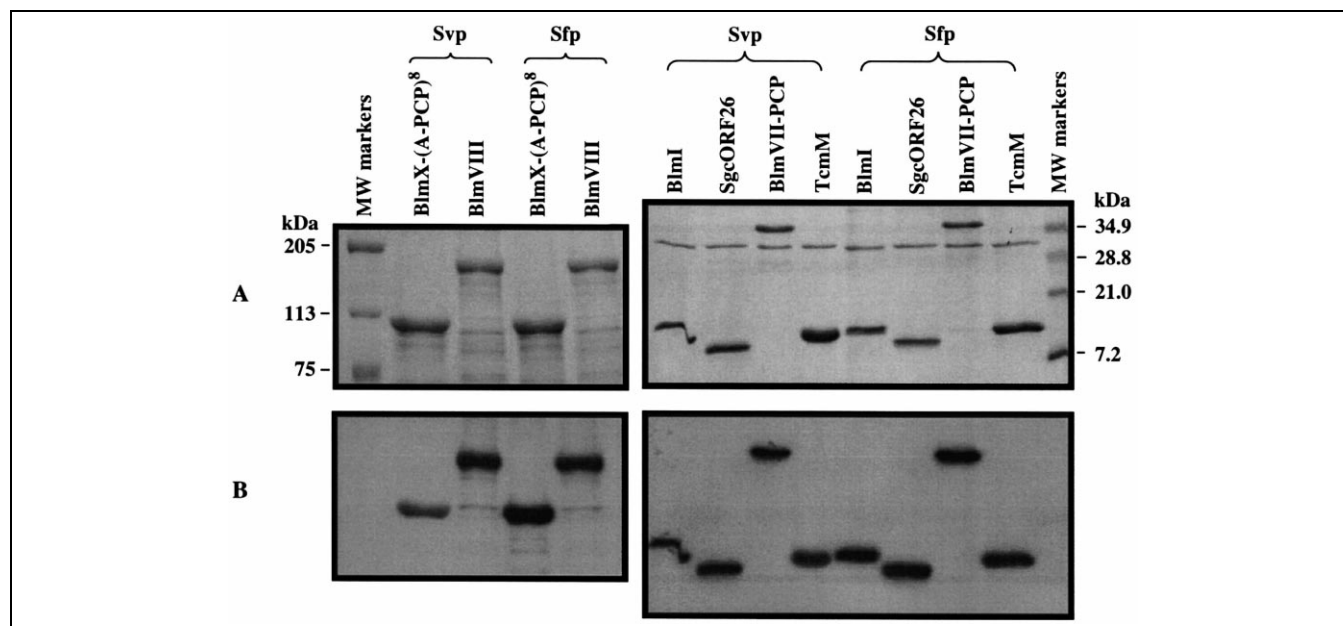


Fig. 5. In vitro 4'-phosphopantetheinylation of apo-PCPs and apo-ACPs by Svp in comparison to Sfp. Carrier protein and PPTase concentrations used in each assay (100 μ l) were: 11.6 μ M BlmI, 5.8 μ M BlmVII-PCP, 0.8 μ M BlmVIII, 1.5 μ M BlmX-(A-PCP)⁸, 11.6 μ M SgcORF26, or 11.6 μ M TcmM, and 1.5 μ M Svp or Sfp. (A) SDS-PAGE analysis on a 7.5% (left panel) and 15% gels (right panel). (B) Autoradiogram.

without significant loss of the overall yield. BlmVIII was subsequently purified by affinity chromatography on Ni-NTA agarose, and the purified BlmVIII protein migrates as a single band upon SDS-PAGE with an apparent MW of 195 000 (Fig. 5A), consistent with its calculated MW plus the His tag of 193 378.

Various attempts to overproduce soluble BlmX-PCP⁸ in *E. coli*, either as a PCP domain alone or in combination with other domains with or without the His₆ tag, were unsuccessful. We finally succeeded in overproducing BlmX-(A-PCP)⁸ as a fusion protein with the maltose binding protein (MBP) (Fig. 4). Boundaries for the A and PCP domains were similarly determined according to Marahiel and co-workers [43,44]. Thus, we amplified the *blmX*-(A-PCP)⁸ fragment, using PCR, from pBS6 and cloned it into pMAL-c2x to yield pBS22, in which the MBP will be fused to the N-terminus of BlmX-(A-PCP)⁸. Introduction of pBS22 into *E. coli* TB1 resulted in good production of BlmX-(A-PCP)⁸ with approximately 40% of the resultant protein in soluble form, which was subsequently purified by affinity chromatography on amylose resin. The purified BlmX-(A-PCP)⁸ protein migrates as one major band upon SDS-PAGE with an apparent MW of 105 000 (Fig. 5A), consistent with its calculated MW plus the MBP of 111 211.

To overproduce SgcORF26 in *E. coli*, the *sgc-orf26* gene was amplified, using PCR, from pBS1006 [42] and cloned into pET-29a to yield pBS23, in which SgcORF26 was produced as a fusion protein with a native N-terminus and a His₆-tagged C-terminus. No production of SgcORF26 could be detected, however, as judged by SDS-PAGE, upon introduction of pBS23 into *E. coli*

BL21(DE3) under various conditions examined. We subsequently moved the *sgc-orf26* gene from pBS23 into pET-28a to afford pBS24, in which SgcORF26 was produced as a fusion protein with His₆ tags at both the N- and C-termini (Fig. 4). Introduction of pBS24 into *E. coli* BL21(DE3), followed by fermentation at 30°C and induction with 100 μ M IPTG, resulted in the overproduction of SgcORF26 with about 15% of the resultant protein in soluble form, which was subsequently purified by affinity chromatography on Ni-NTA agarose. The purified SgcORF26 protein migrates as a single band upon SDS-PAGE with an apparent MW of 13 000 (Fig. 5A), consistent with its calculated MW plus the two His tags of 13 283.

Expression of *tcmM* in *E. coli* has been previously reported [41]. To facilitate purification, the *tcmM* gene was moved from pET22b-tcmhis₆ [45] into pET-28a to yield pBS25, in which TcmM was overproduced as a fusion protein with His₆ tags at both the N- and C-termini (Fig. 4). Introduction of pBS25 into *E. coli* BL21(DE3) under the standard expression conditions recommended by the manufacturer resulted in overproduction of soluble TcmM, which was readily purified by affinity chromatography on Ni-NTA agarose. The purified TcmM protein migrates as a single band upon SDS-PAGE with an apparent MW of 13 500 (Fig. 5A), consistent with its calculated MW plus the two His tags of 12 312.

2.5. In vitro 4'-phosphopantetheinylation of apo-ACPs and apo-PCPs with [³H-pantetheine]-CoA by Svp

To establish Svp as a PPTase, we tested whether it can

catalyze 4'-phosphopantetheinylation of an apo-ACP or apo-PCP into a holo-ACP or holo-PCP by transferring the P-pant moiety from CoA to the conserved serine residue of the carrier protein in vitro. Sfp, the PPTase from the surfactin biosynthetic gene cluster of *B. subtilis* that is known for its broad substrate specificity for both ACPs and PCPs [46], was chosen as a positive control. The Sfp PPTase was overproduced in *E. coli* MV1190 (pUC8-Sfp) and purified to near homogeneity as described previously [20]. Upon incubation of the purified apo-ACPs and apo-PCPs with [3 H]-pantetheine]-CoA in the presence of either the Svp or Sfp PPTase, we examined the covalent incorporation of the [3 H]P-pant moiety from CoA into holo-ACPs or holo-PCPs using autoradiographic analysis. The results are summarized in Fig. 5. As compared to Sfp, Svp is competent for the 4'-phosphopantetheinylation of both apo-ACPs and apo-PCPs by specifically transferring the P-pant group from CoA onto holo-carrier proteins, and no P-pant incorporation was observed in the absence of either the carrier proteins or the Svp or Sfp PPTase. Surprisingly, when [3 H]acetyl CoA was used as a substrate to test the specificity of Svp, the autoradiogram signals were very weak for either type I or type II ACPs/PCPs even after 2 weeks exposure; under identical conditions the

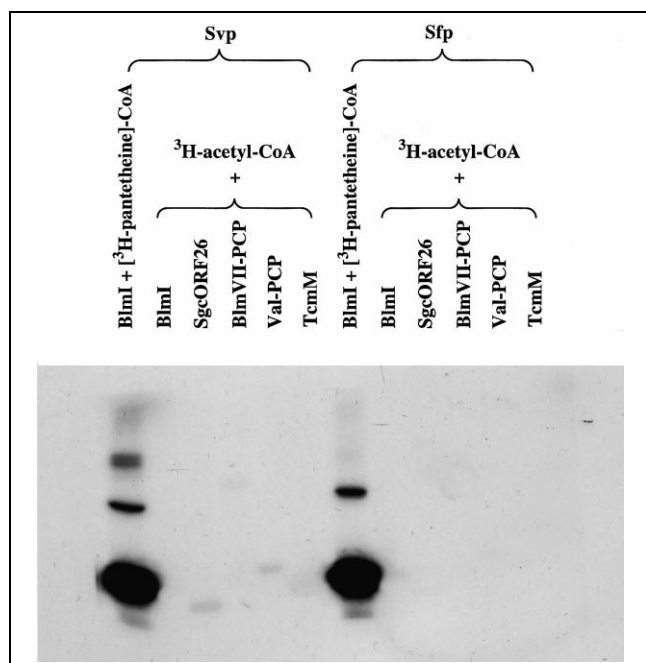


Fig. 6. In vitro 4'-phosphopantetheinylation of apo-PCPs and apo-ACPs using [3 H]-pantetheine]-CoA or [3 H]acetyl-CoA as a substrate by Svp in comparison of Sfp. Carrier protein, phosphopantetheinylation transferase, CoA or acetyl-CoA concentrations used in each assay (100 μ l) were: 11.6 μ M BlmI, 5.8 μ M BlmVII-PCP, 11.6 μ M SgcORF26, 11.6 μ M TcmM, or 5.8 μ M Val-PCP, 1.5 μ M Svp or Sfp, and 0.23 μ M [3 H]-pantetheine]-CoA (0.43 μ Ci) or [3 H]acetyl-CoA (1 μ Ci). Val-PCP is the PCP domain of the SvpS-2 NRPS module from the second gene cluster cloned from *S. verticillus* ATCC15003 that is also characterized by both NRPS and PKS genes (accession number AF340166).

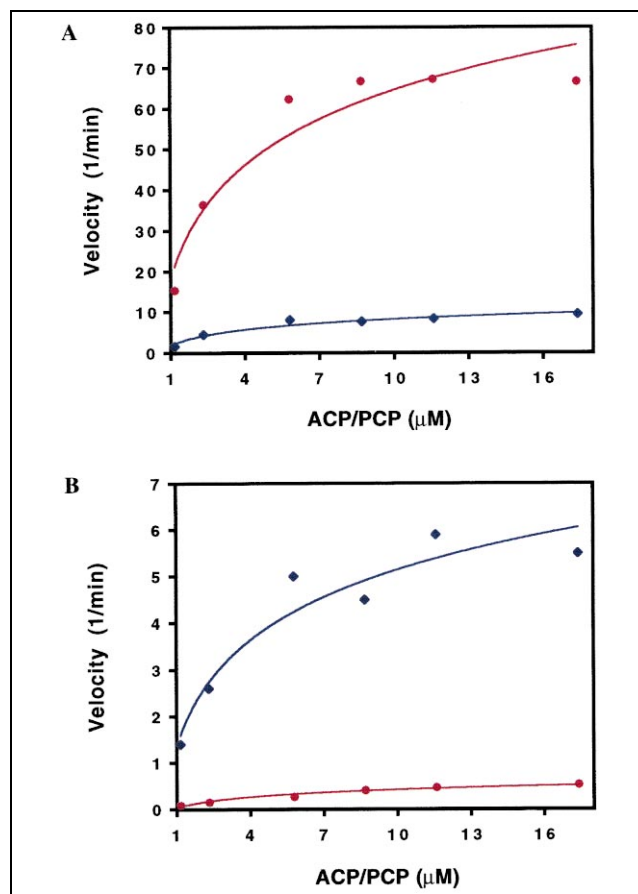


Fig. 7. Initial velocities of 4'-phosphopantetheinylation of apo-BlmI PCP (◆, blue) and apo-TcmM ACP (●, red) catalyzed by Svp (A) in comparison to Sfp (B) as a function of concentration of apo-BlmI PCP or apo-TcmM ACP.

positive control of BlmI with [3 H]-pantetheine]-CoA as a substrate gave very strong signal (Fig. 6). Both ACPs of *E. coli* [45] and Sfp of *B. subtilis* [46] have previously been shown by mass spectrometry to be able to utilize a range of CoA analogs with altered P-pant moieties as substrates, including acetyl CoA. The relaxed substrate specificity of Sfp was successfully exploited to load aminoacyl-phosphopantetheine to PCP for NRPS characterization [27]. However, under the same conditions Sfp also gave negligible signal on autoradiogram when [3 H]acetyl-CoA was used as a substrate (Fig. 6). These data suggest that the transfer of altered P-pant moieties, observed by mass spectrometry

Table 1

Catalytic efficiencies of Svp-catalyzed phosphopantetheinylation of apo-BlmI PCP and apo-TcmM ACP in comparison with those of Sfp

PPTase	Substrate	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{min}^{-1}$)
Svp	BlmI	3.9 ± 1.4	11 ± 1.3	2.8 ± 1.1
	TcmM	3.1 ± 1.1	86 ± 9.3	28 ± 10
Sfp	BlmI	3.7 ± 1.4	7.2 ± 0.85	1.9 ± 1.0
	TcmM	11 ± 2.7	0.89 ± 0.11	0.081 ± 0.022

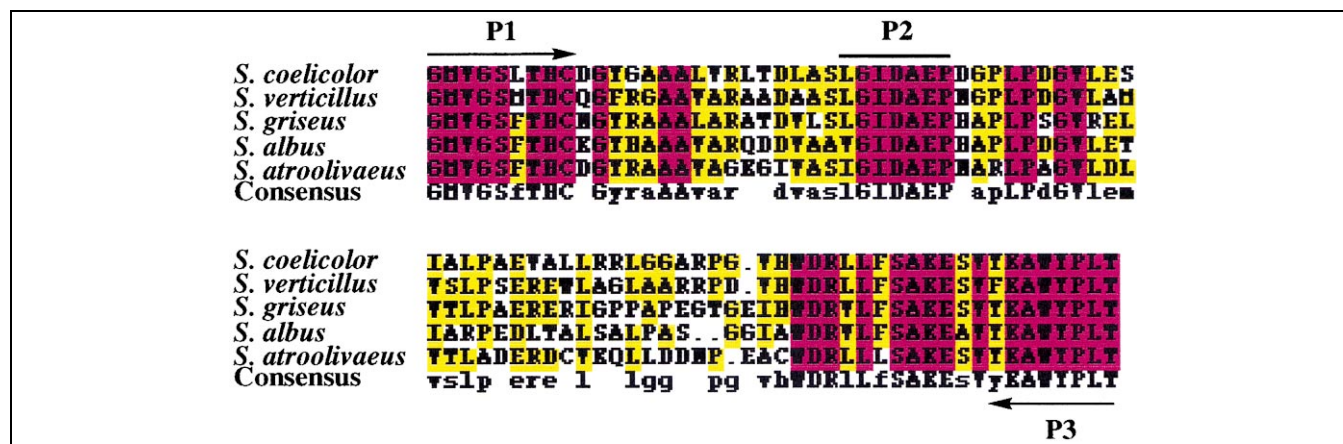


Fig. 8. Alignment of the deduced amino acid sequences of the cloned PPTase fragments from different *Streptomyces* species. These fragments were cloned by PCR using the P1/P3-2 pair of primers designed according to the conserved P1 and P3 motifs of PPTases from actinomycetes. The conserved P2 motif is present in all cloned PPTase fragments.

in the early studies [27,45,46], probably represents a minor activity of the PPTases.

2.6. Kinetics of apo-TcmM ACP and apo-BlmI PCP as substrates for Svp and Sfp

Initial rate assays were used to determine the kinetic parameters for P-pant transfer from CoA to both apo-TcmM and apo-BlmI, using both Svp and Sfp as catalysts. Saturation kinetics for the carrier proteins were observed (Fig. 7). As shown in Table 1, the K_m values for BlmI with either Svp or Sfp are identical ($\sim 4 \mu\text{M}$) within experimental error, while the k_{cat} values are very similar. In contrast, the two PPTases show significantly different K_m values and very different k_{cat} values for TcmM. These two kinetic parameters combine to provide the catalytic efficiency, k_{cat}/K_m . With BlmI as substrate, k_{cat}/K_m for both Svp and Sfp is identical within experimental error. With TcmM as substrate, k_{cat}/K_m is very different for the two enzymes, with Svp being a 346-fold better catalyst.

2.7. Cloning of PPTase gene fragments from other peptide or polyketide natural product-producing *Streptomyces* species

To test the general utility of the PCR approach for identifying and cloning PPTase genes, PCR reactions were performed using the P-1/P3-2 pair of primers on chromosomal DNAs prepared from five different *Streptomyces* species. *S. coelicolor* A3(2) was chosen as a positive control since it is known to contain a Sfp-like sequence (SC5A7.23, accession number CAA19952). *Streptomyces atroolivaceus* and *Streptomyces albus* JA3495 produce leinamycin [47] and oxazolomycin [48], respectively, both of which are hybrid peptide–polyketide metabolites. *Streptomyces griseus* DSM40695 produces the macrotetrolides that are polyketides [49]. Distinctive products with the

expected size of approximately 250 bp were readily amplified from the chromosomal DNAs of all five organisms. These fragments were cloned and sequenced. The deduced amino acid sequences are characterized by the conserved P2 motif and show high homology among each other as well as to putative and known PPTases such as Svp (Fig. 8). Therefore, it is evident that these fragments encode parts of PPTases, and this approach provides a novel access to PPTase genes for peptide and polyketide biosynthesis.

3. Discussion

The antitumor drug BLM is a natural hybrid peptide–polyketide metabolite, the biosynthesis of which provides an excellent opportunity to investigate intermodular communication between NRPS and PKS modules. At the onset of this project, it seemed that bacterial NRPS gene clusters generally included, or were located in the proximity of, a gene encoding the pathway-specific PPTase [18,20,21]. However, sequence analysis of the *blm* cluster had failed to reveal an associated PPTase gene [30]. Since neither NRPS nor PKS will be functional unless its carrier protein is fully modified by the attachment of the P-pant group to the conserved serine residue, we decided to search for a PPTase from *S. verticillus* that presumably would be responsible for the modification of both ACP and PCPs of the Blm NRPS/PKS/NRPS megasynthetase. Although sequence conservation among PPTases from different organisms, limited to two poorly defined motifs, P2 and P3 (Fig. 1A), has been previously recognized [9,10], initial attempts to amplify a PPTase from *S. verticillus* by PCR, using degenerate primers designed according to the P2 and P3 motifs, were unsuccessful. We then used a sequence comparison strategy, taking into account the following two considerations. First, we biased our search

towards a PCP-specific PPTase since the *blm* locus encodes at least 10 NRPS modules but only one PKS module [30]. Second, we used the confirmed PCP-specific PPTases to search the database for homologues only from the closely related *Streptomyces* and *Mycobacterium* genera. In this way, we identified a new motif, P1, in addition to the P2 and P3 motifs (Fig. 1A). Realizing that these motifs consist of only a few amino acids, we took advantage of the recently developed CODEHOP strategy of PCR primer design [36]. PCR amplification using CODEHOP-designed primers yielded a specific DNA fragment of the expected size from *S. verticillus* genomic DNA, leading to the cloning of the *svp* gene. The *svp* gene is not clustered with any of the NRPS or PKS gene clusters known in *S. verticillus*. The general utility of this PCR approach for identifying and cloning PPTase genes was demonstrated in the four additional *Streptomyces* species tested. Given the current interests in peptide and polyketide biosynthesis and the inability to identify a PPTase associated with the specific NRPS or PKS clusters, our approach should be directly applicable to the cloning of a PPTase gene from organisms known to produce peptide, polyketide or hybrid peptide–polyketide metabolites, in particular from the high G+C content actinomycetes.

PPTases characterized to date exhibit high specificity towards the carrier protein substrates, leading to the proposal that each P-pant-requiring pathway has its own posttranslational modifying PPTase activity [9]. Sfp, the only PPTase known prior to our work to accept ACPs, ArCP, and PCPs from various organisms, has been called a ‘promiscuous’ PPTase whose relaxed carrier protein specificity has been viewed as an exception to the aforementioned hypothesis. We overexpressed and purified Svp and demonstrated it to be a PPTase that is also able to accept a wide variety of apo-carrier proteins as substrates, including both type I and type II ACPs and PCPs from either *S. verticillus* or other *Streptomyces* species. The kinetic analyses presented here show that the catalytic efficiencies of Svp and Sfp are very similar for apo-BlmI as a substrate, but, strikingly, Svp is a 346-fold better catalyst than Sfp with apo-TcmM as a substrate. These results not only confirmed that *svp* encodes a PPTase, but also prompted us (1) to reconsider the early assumption that each P-pant-requiring pathway has its own PPTase activity and (2) to re-examine the previous classification of PPTases into either NRPS-type or FAS/PKS-type according to their carrier protein specificity [10].

Very recently, the X-ray crystal structure of Sfp has been solved [50]. Instead of classifying PPTases according to their apparent carrier protein specificity, the PPTase superfamily was re-classified on a structural basis into two subfamilies: Sfp-type PPTases that are monomers of about 230 amino acids and ACPS-type PPTases that are homodimers with the subunit of about half the size of the Sfp-type enzymes [50]. Interestingly, the structure of Sfp exhibits an intramolecular two-fold pseudosymmetry, sug-

gesting a similar fold and dimerization mode to the homodimeric ACPS-type PPTases [50]. However, the even more recent elucidation of the structure of AcpS from *B. subtilis* and the characterization of AcpS from *Streptococcus pneumoniae* show that, at least in the group of low G+C Gram-positive bacteria, ACPS enzymes exist as homotrimers [51,52]. Each trimer contains three active sites, one half of which comes from one molecule of AcpS and the other half from a second AcpS molecule in the trimer. The active site in AcpS is only formed when two AcpS molecules dimerize, but the enzyme remains inactive until trimer formation [51]. These features have led us to the following speculation on the structural and functional evolution of PPTases. The small ACPS-type oligomeric enzyme might be considered the ‘ancestral’ form of PPTase, now conserved in most bacterial fatty acid biosynthesis pathways. In one evolutionary route, after a gene fusion event, this discrete ACPS-like protein became a domain in fungal multifunctional FAS. It is notable that even in this new context, the PPTase seems to conserve its oligomeric (dimeric) nature [9,12,14]. Therefore, the ACPS-type PPTases, both discrete proteins and FAS domains, are more dedicated enzymes, generally involved in fatty acid biosynthesis, and, hence, are essential for survival. Following a different evolutionary path, after a DNA duplication event and further accumulation of mutations, the oligomeric ACPS-like subunit became a monomeric PPTase of the Sfp type, twice the size of its ACPS-type ancestor. The Sfp-type enzymes are involved in functions not essential under normal conditions, such as biosynthesis of secondary metabolites. Therefore they are ‘free’ to develop a broader substrate specificity such as Sfp and Svp, potentially serving several pathways. Our findings that Svp is a monomeric, 246-amino acid PPTase with a broad substrate specificity and is probably involved in BLM biosynthesis support these speculations.

We further propose that Svp may be the only PPTase responsible for secondary metabolite biosynthesis in *S. verticillus*. This is consistent with the fact that (1) PCR using degenerate primers designed according to known PPTases yielded only one single product, as evidenced by the fact that eight randomly selected clones yielded an identical sequence; (2) DNA probing of the genomic library using the PCR-amplified probe resulted in only one single *svp* locus, in spite of exercising great care not to miss any possible weak hybridizing signal from multiple PPTase loci; and (3) the broad substrate specificity of Svp towards both ACPs and PCPs so that no additional PPTase is needed for the other NRPS and PKS clusters.

Nonribosomal peptide, polyketide, and hybrid peptide–polyketide natural products encompass a wide array of clinically important drugs, and genetic engineering of NRPS and PKS has proved to be extremely powerful in expanding the repertoire of natural product libraries, hence potentially leading to the discovery of new drugs [32,53]. Since neither NRPS nor PKS will be functional

unless its carrier proteins are fully modified with the P-pant group, Sfp has been by far the most important PPTase currently used to produce active NRPS and PKS for these studies. The Svp PPTase reported here could be easily overproduced in and purified from *E. coli* and showed as broad substrate specificity as Sfp if not broader, exhibiting an excellent catalytic efficiency toward both ACPs and PCPs. Given the fact that most of the current efforts on combinatorial NRPS and PKS biosynthesis are carried out in *Streptomyces* or closely related actinomycetes, Svp should be of great utility, either in vitro or in vivo by co-expression with the NRPS or PKS genes, to produce functional enzymes. Moreover, since Svp can phosphopantetheinylate efficiently both ACPs and PCPs, it should be particularly useful in engineering biosynthesis of hybrid peptide–polyketide natural products [32,53]. The latter family includes many important drugs, such as BLM, rapamycin, pristnamycin, and epothilone.

4. Significance

PPTases have been classified according to their carrier protein specificity, and, in organisms containing multiple P-pant-requiring pathways, each pathway has been suggested to have its own PPTase activity. We developed a PCR method for cloning PPTase genes from actinomycetes and cloned a PPTase gene, *svp*, from *S. verticillus*, the producer of the antitumor drug bleomycin. The *svp* gene was mapped to an independent locus not clustered with any of the known NRPS or PKS clusters. Svp is a PPTase capable of modifying both type I and type II ACPs and PCPs from either *S. verticillus* or other *Streptomyces* species. As compared to Sfp, the only ‘promiscuous’ PPTase known previously, Svp displays a similar catalytic efficiency (k_{cat}/K_m) for the BlmI PCP but a 346-fold increase in catalytic efficiency for the TcmM ACP. Our results favor the structural based re-classification of PPTases into two subfamilies: ACPS-type and Sfp-type. ACPS-type PPTases are generally involved in fatty acid biosynthesis and, hence, are essential for survival. Sfp-type PPTases, to which Svp belongs, are generally responsible for secondary metabolite biosynthesis, hence non-essential, and may evolve new or broader specificities towards their carrier protein substrates. Since neither NRPS nor PKS will be functional unless its carrier proteins are fully modified with the P-pant group, the development of a PCR method for cloning PPTase genes, and the availability of Svp, in addition to Sfp, should facilitate future endeavors in engineered biosynthesis of peptide, polyketide, and, in particular, hybrid peptide–polyketide natural products.

5. Materials and methods

5.1. General procedures

E. coli DH5 α [54], *E. coli* BL21(DE-3) (Novagen, Madison, WI, USA), *E. coli* M15(pREP4) (Qiagen, Santa Clarita, CA, USA), *E. coli* TB1 (New England Biolabs, Beverly, MA, USA), *S. albus* JA 3453 [48], *S. atroolivaceus* [47], *S. coelicolor* A3(2) [55], *S. griseus* DSM DSM40695 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) [49], *S. verticillus* ATCC15003 (American Type Culture Collection, Rockville, MD, USA) [30] were used in this work. *E. coli* MV1190 (pUC8-Sfp) [25], pBS6 [30], pBS1006 [42], pET22b-tcmhis₆ [45], and pANT841 [56] were described previously, and pET28a and pET-29a (Novagen), pGEM-T (Promega, Madison, WI, USA), pMAL-c2x (New England Biolabs), and pQE60 and pQE70 (Qiagen) were from commercial sources.

Plasmid preparation was carried out using commercial kits (Qiagen). *Streptomyces* chromosomal DNA was isolated according to literature protocols [54,55]. Restriction enzymes and other molecular biology reagents were from commercial sources, and digestion and ligation followed standard methods [54]. For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA).

Automated DNA sequencing was carried out on an ABI Prism 377 DNA Sequencer (Perkin-Elmer/ABI, Foster City, CA, USA). This service was provided by Davis Sequencing Inc. (Davis, CA, USA). Data were analyzed by the ABI Prism Sequencing 2.1.1 software and the Genetics Computer Group (GCG) program (Madison, WI, USA).

5.2. PCR cloning of PPTase genes and isolation and sequencing of the *svp* locus

The primers were designed according to the conserved motifs found in putative PCP-type PPTases from actinomycetes (Fig. 1) by the CODEHOP strategy [36]. The reaction mixture contained 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 5% dimethyl sulfoxide, 0.25 mM of each of the four dNTPs, 100 pmol of each primer, 2.5 U *Taq* DNA polymerase (Promega), and 50 ng of *Streptomyces* chromosomal DNA, in a final volume of 50 μ l. A touchdown PCR program was used, which consists of (1) denaturation at 95°C for 5 min, (2) 10 cycles of 40 s at 95°C, 1 min at 60–51°C (decreasing 1°C every cycle) and 20 s at 72°C, (3) 30 cycles of 40 s at 95°C, 1 min at 50°C and 20 s at 72°C, and (4) 7 min at 72°C. The 250-bp products, amplified with the P1/P3-2 primers were gel-purified, cloned into pGEM-T, and sequenced. To clone the *svp* gene, the PCR product was cloned into pGEM-T to yield pBS26 and used as a probe to screen the *S. verticillus* genomic library [30]. Among the 10 000 colonies analyzed, 25 clones were true positive by Southern analysis and hybridized to the same 4.7-kb *Bam*HI fragment. One of the cosmid clones, pBS27, was chosen, from which the 4.7-kb *Bam*HI fragment was cloned into pANT841 to yield pBS17.

The 1761-bp DNA sequence reported in this work was determined from pBS17.

5.3. Overexpression in *E. coli* and purification of Svp, BlmI, BlmVII-PCP, BlmVIII, BlmX-(A-PCP)⁸, SgcORF26, and TcmM

The *svp* gene was amplified by PCR from pBS17 using a forward primer 5'-TATAATGCATGCTCGCCGCCCTCCCC-3' (the *Sph*I site is underlined) and a reverse primer 5'-TTAAGA-TCTCGGGACGGCGGTCCGGTC-3' (the *Bgl*II site is underlined). The resultant PCR product was digested and cloned into the *Sph*I-*Bgl*II sites of pQE-70 to yield pBS18. The latter was introduced into *E. coli* M15(pREP4) under standard expression conditions, in which Svp will be produced with an Ile-to-Leu mutation at the second amino acid, due to the introduction of the *Sph*I site, and an Arg-Ser-His₆ tag at the C-terminus.

Overexpression and purification of BlmI was carried out as described previously [29]. To overproduce BlmVII-PCP, the C-terminal 657-bp fragment of *blmVII* was amplified by PCR from pBS6 using a forward primer 5'-ATATCCATGGCG-GCCTTGCCCGACCCGGCC-3' (the *Nco*I site is underlined) and a reverse primer 5'-GGAAGATCTTTCGGGACGCGGG-CACGGCTG-3' (the *Bgl*II site is underlined). The resultant PCR product was digested and cloned into the *Nco*I-*Bgl*II sites of pQE-60 to yield pBS19 for overexpression in *E. coli* M15(pREP4). Due to poor production, *blmVII*-PCP was subsequently moved as an *Nco*I-*Hind*III fragment from pBS19 and cloned into the same sites of pET-29a to afford pBS20 for expression in *E. coli* BL21(DE3). In the latter construct, BlmVII-PCP will be produced as a fusion protein with an S tag at the N-terminus and a His₆ tag at the C-terminus.

The *blmVIII* gene was amplified by PCR from cosmid pBS6 using a forward primer 5'-TGAATTCCATGGGCCATGCCG-ACGCGGGCGAC-3' (the *Nco*I site is underlined) and a reverse primer 5'-ATGGATCCCAGCACCACTCTTCCTGGC-3' (the *Bam*HI site is underlined). The resultant PCR product was digested and cloned into the *Nco*I-*Bgl*II sites of pQE60, and a 3.6-kb *Kpn*I/*Bgl*II fragment from pBS6 was subsequently used to replace the corresponding region of the PCR-amplified *blmVIII* gene to eliminate any possible PCR error, yielding pBS21. Introduction of pBS21 into *E. coli* BL21(DE3) under standard expression conditions resulted in production of BlmVIII as a fusion proteins with a His₆ tag at the C-terminus.

To overproduce BlmX-(A-PCP)⁸, the C-terminal 1881-bp fragment of *blmX* was amplified by PCR from pBS6 using a forward primer 5'-TAGAATTCGCTCCGACCGCGAC-3' (the *Eco*RI site is underlined) and a reverse primer 5'-TATTCTCGAGT-CATTCGGCGGCACCTC-3' (the *Xho*I site is underlined). The resultant PCR product was digested and cloned into the *Eco*RI-*Sal*I sites of pMAL-c2x to yield pBS22. Introduction of pBS22 into *E. coli* TB1 under standard expression conditions resulted in production of BlmX-(A-PCP)⁸ as a fusion protein with the MBP at the N-terminus.

From pBS1006, *sgc-orf26* was amplified by PCR using a forward primer 5'-GGGAATTCATATGTCCACCGTTTCCGA-

CAC-3' (the *Nde*I site is underlined) and a reverse primer 5'-GGCAAGCTTCTGCGTTCCGGAGCCCTC-3' (the *Hind*III site is underlined). The resultant PCR product was digested and cloned into the *Nde*I-*Hind*III sites of pET29a to give pBS23, in which SgcORF26 would be produced as a fusion protein with a native N-terminus and a His₆ tag at the C-terminus. To construct pBS24, *sgc-orf26* was moved as an *Nde*I/*Hind*III fragment from pBS23 and cloned into the same sites of pET28a. The latter resulted in the production of SgcORF26 as a fusion protein with His₆ tags at both N- and C-termini. Both pBS23 and pBS24 were introduced into *E. coli* BL21(DE3) under standard expression conditions.

To construct pBS25, *tcmM* was moved as an *Nde*I-*Xho*I fragment from pET22b-tcmhis₆ and cloned into the same sites of pET-28a. Introduction of pBS25 into *E. coli* BL21(DE3) under standard expression conditions resulted in production of TcmM as a fusion protein with His₆ tags at both N- and C-termini.

Purification of His-tagged proteins by affinity chromatography on Ni-NTA resin was carried out as previously described [29]. Purification of the MBP fusion protein by affinity chromatography on amylose resin was carried out as recommended by the manufacturer.

5.4. In vitro phosphopantetheinylation of apo-ACPs and apo-PCPs

For in vitro phosphopantetheinylation, the reaction mixtures contained 0.8–11.6 μM apo-PCP or apo-ACP, 1.5 μM Svp or Sfp, 0.225 μM [³H-pantetheine]-CoA (0.43 μCi, 40 Ci/mmol), 5 mM dithiothreitol (DTT), 10 mM MgCl₂, 75 mM Tris-HCl, pH 8.0, in a final volume of 100 μl. For assays using [³H-pantetheine]-CoA and [³H]acetyl-CoA to compare the substrate specificity between Svp and Sfp, 50 mM sodium phosphate buffer, pH 6.5 was used. The assays were initiated by addition of [³H-pantetheine]-CoA, allowed to incubate at 37°C for 1 h, and terminated by addition of 900 μl of cold 10% trichloroacetic acid (TCA). All procedures are essentially the same as previously described [27]. The samples were resolved by SDS-PAGE on a 15% (for BlmI, SgcORF26, BlmVII-PCP, TcmM) or a 7.5% gel (for BlmX-(A-PCP)⁸ and BlmVIII). The gels were soaked in Amplifier (Amersham, Arlington Heights, IL, USA) for 20 min, dried between two sheets of cellulose membrane (KOH Development Inc., Ann Arbor, MI, USA), and visualized by autoradiography on X-ray films (Fuji Medical Systems, Stanford, CT, USA).

5.5. Kinetics of Svp and Sfp with BlmI and TcmM

The pH profile of Svp was determined in 75 mM MES/sodium acetate (for pH 4.5–6.5) and 75 mM Tris-HCl (for pH 7.0–9.5) buffers. Svp exhibits an optimal activity around pH 6.5–7.0. Kinetic studies were carried out at pH 8.0 to avoid substrate inhibition and precipitation of the carrier proteins at high concentrations. Full time courses for P-pant transfer were used to determine the time frame in which initial rate conditions (<10% conversion) were maintained. For time courses, the reactions contained 11.6 μM BlmI or TcmM, 15 nM Svp or Sfp, 11.6 μM [³H-pantetheine]-CoA (0.43 μCi, 373 mCi/mmol), 5 mM

DTT, 10 mM MgCl₂, 75 mM Tris–HCl, pH 8.0, in a final volume of 800 µl. All reactions were initiated by addition of [³H-pantetheine]-CoA and allowed to incubate at 37°C. An aliquot of 100 µl was taken at 1, 2, 5, 10, 20, 30, 60 min and transferred into a tube containing 50 µg bovine serum albumin (BSA) on ice, to which 900 µl of cold 10% TCA was added immediately to terminate the reaction. Precipitated proteins were isolated, washed, and suspended in 100 µl of 1 M Tris–HCl, pH 8.0. All procedures are essentially the same as previously described [46]. The protein samples were then mixed with 7 ml of scintillation fluid (ScintiSafe Gel, Fisher, Pittsburgh, PA, USA) and counted on a Beckman LS-6800 scintillation counter to determine the radioactivity.

The time courses described above showed that initial rate conditions are obtained up to at least 5 min of reaction. The initial rate reactions contained 1.16–17.4 µM apo-BlmI or apo-TcmM, 15 nM Svp or Sfp, 11.6 µM [³H-pantetheine]-CoA (0.43 µCi, 373 mCi/mmol), 5 mM DTT, 10 mM MgCl₂, 75 mM Tris–HCl, pH 8.0, in a final volume of 100 µl. The reactions were initiated by addition of [³H-pantetheine]-CoA, incubated at 37°C for 5 min, and terminated by addition of 900 µl of cold 10% TCA and 50 µg of BSA. Radioactivities associated with the precipitated proteins were similarly determined by scintillation counting. Experiments in which CoA was varied (0.34–112 µM) at fixed apo-BlmI or apo-TcmM concentration (29 µM) yielded *K_m* values of 1.6 ± 0.7 µM (Svp/BlmI), 0.62 ± 0.11 µM (Sfp/BlmI), and 1.5 ± 0.5 µM (Sfp/TcmM). The reported value for the latter is 0.7 µM [46]. Thus, the CoA concentration used (11.6 µM) in the initial rate experiments with apo-BlmI and apo-TcmM is saturating. The initial rate data were fitted directly to the Michaelis–Menten equation using the nonlinear regression available in Kaleidagraph (ver. 3.09) (Synergy Software, Reading, PA, USA).

5.6. Accession number

The nucleotide sequence of the *svp* locus from *S. verticillus* ATCC15003 has been deposited into GenBank under accession number AF210311.

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