Improvement of Natamycin Production by Engineering of Phosphopantetheinyl Transferases in *Streptomyces chattanoogensis* L10

Hui Jiang,^{a,c} Yue-Yue Wang,^a Xin-Xin Ran,^a Wei-Ming Fan,^b Xin-Hang Jiang,^a Wen-Jun Guan,^{a,c} Yong-Quan Li^{a,c}

College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang, China^a; Zhejiang Zhenyuan Pharmaceutical Co. Ltd., Shaoxing, Zhejiang, China^b; Key Laboratory of Microbial Biochemistry and Metabolism Engineering of Zhejiang Province, Hangzhou, Zhejiang, China^c

Phosphopantetheinyl transferases (PPTases) are essential to the activities of type I/II polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) through converting acyl carrier proteins (ACPs) in PKSs and peptidyl carrier proteins (PCPs) in NRPSs from inactive apo-forms into active holo-forms, leading to biosynthesis of polyketides and nonribosomal peptides. The industrial natamycin (NTM) producer, *Streptomyces chattanoogensis* L10, contains two PPTases (SchPPT and SchACPS) and five PKSs. Biochemical characterization of these two PPTases shows that SchPPT catalyzes the phosphopantetheinylation of ACPs in both type I PKSs and type II PKSs, SchACPS catalyzes the phosphopantetheinylation of ACPs in type II PKSs and fatty acid synthases (FASs), and the specificity of SchPPT is possibly controlled by its C terminus. Inactivation of SchPPT in *S. chattanoogensis* L10 abolished production of NTM but not the spore pigment, while overexpression of the SchPPT gene not only increased NTM production by about 40% but also accelerated productions of both NTM and the spore pigment. Thus, we elucidated a comprehensive phosphopantetheinylation network of PKSs and improved polyketide production by engineering the cognate PPTase in bacteria.

Streptomyces species are some of the most prolific microbial groups of polyketide producers. Polyketides, some of which are used as medicines or veterinary agents, are biosynthesized by polyketide synthases (PKSs). Phosphopantetheinyl transferases (PPTases) are essential to the activities of type I/II PKSs, fatty acid synthases (FASs), and nonribosomal peptide synthetases (NRPSs) due to the posttranslational modification of acyl carrier proteins (ACPs) in PKSs/FASs and peptidyl carrier proteins (PCPs) in NRPSs from inactive apo-forms into active holo-forms (1, 2). Generally, each bacterium has two or more PPTases, including at least one holo-ACP synthase (ACPS)-type PPTase and at least one Sfp-type PPTase. However, as an exception, *Pseudomonas aeruginosa* harbors only one PPTase, PcpS (3, 4). Some PPTases are essential for viability of the host organism due to their roles in primary metabolism, while some PPTases appear to act only on carrier proteins within a particular secondary metabolite cluster (5–7). Thus, characterization of the phosphopantetheinylation network in bacteria will help us better understand polyketide biosynthetic pathways and further engineer polyketide productions.

The PPTases can be classified into three groups based on their structures. The group I PPTases (ACPS-type PPTases) are homotrimers with about 120 amino acids in each monomer. The group II PPTases (Sfp-type PPTases) are monomers of approximately 240 amino acids. The group III PPTases exist as domains of FASs and PKSs. Three conserved motifs, P1 (PxxP), P2 (GxD), and P3 [(F/W)(S/T/A)xKE(S/A)xxK], are identified according to sequence alignments. Both the Sfp-type PPTases and the group III PPTases contain all three motifs, while the ACPS-type PPTases contain only the P2 and P3 motifs (Fig. 1A) (6, 8–11). To the best of our knowledge, there has been no report of the study of the relationship of the structures and the specificities of PPTases.

Natamycin (NTM) (also known as pimaricin), which is widely used as an antifungal agent in both human therapy and the food industry, is a polyene macrolide biosynthesized by PKSs of several *Streptomyces* strains, such as *S. natalensis* (12), *S. gilvosporeus* (13), and an industrial producer, *S. chattanoogensis* L10 (14). Several

factors related to NTM biosynthesis have been well studied to improve NTM production, including the pathway-specific regulators (14–16), the gamma-butyrolactone regulatory system (17, 18), the pimaricin inducer (PI) factor system (19, 20), the P450 epoxidase (21), the phosphate regulation system (22), the pleiotropic regulatory system (23), and the exporter (24). We have previously cloned the *scn* PKS NTM biosynthetic gene cluster in *S. chattanoogensis* L10 (14, 23). However, it is still unknown how PPTases catalyze the phosphopantetheinylation of ACPs in *scn* PKS as well as how PPTases influence NTM production in *S. chattanoogensis* L10. Here, analysis of the whole genomic DNA of *S. chattanoogensis* L10 revealed four new PKSs and two PPTases, SchPPT and SchACPS. Characterization of these PPTases revealed that SchPPT catalyzed the phosphopantetheinylation of ACPs in both type I PKSs and type II PKSs, while SchACPS catalyzed the phosphopantetheinylation of ACPs in type II PKSs and FAS. Thus, we elucidated a comprehensive phosphopantetheinylation network of PKSs in *S. chattanoogensis* L10. NTM production was enhanced and accelerated through the overexpression of the SchPPT gene in *S. chattanoogensis* L10. Furthermore, we also characterized the structure-specificity relationship of SchPPT.

Received 13 January 2013 Accepted 15 March 2013

Published ahead of print 22 March 2013

Address correspondence to Wen-Jun Guan, guanwj@zju.edu.cn, or Yong-Quan Li, lyq@zju.edu.cn.

H.J. and Y.-Y.W. contributed equally to this work.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00099-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.00099-13

FIG 1 Sequences and physical maps of PPTases. (A) Sequence alignment of SchPPT with group II PPTases (a) and SchACPS with group I PPTases (b). Conserved regions P1, P2, and P3 are indicated. Strains and GenBank accession numbers are as follows: SCO6673, *S. coelicolor* A3(2), accession number [NP_630748](#); SePptII, *Saccharopolyspora erythraea*, accession number [AAR92400](#); sfp, *Bacillus subtilis*, accession number [X63158](#); HuPPT, human PPTase; SpACPS, *Streptococcus pneumoniae*, accession number [AAG22706](#); BsACPS, *Bacillus subtilis* subsp. *subtilis* strain 168, accession number [CAB12269](#); SCO4744, *S. coelicolor* A3(2), accession number [NP_628902](#); EcACPS, *E. coli* K-12, accession number [P24224](#). (B) Structures of SchPPT, SchACPS, SchPPT-P2P3, and the SchPPT/SchACPS hybrid.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth, and culture conditions. Bacterial strains and plasmids used in the present study are listed in [Table 1](#), and primers are listed in Table S1 in the supplemental material. The spore preparation of *S. chattanoogaensis* L10 on YMG agar (0.4% yeast extract, 1% malt extract, 0.4% glucose, 2% agar, 0.2% CaCO₃ [pH 7.2]), the examination of NTM production in YEME with 4% glucose (0.3% yeast extract, 0.3% malt extract, 0.5% tryptone, 4% glucose), and DNA manipulations in *S. chattanoogaensis* L10 were carried out according to standard procedures (25). *Escherichia coli*-*Streptomyces* conjugation, fermentation of *S. chattanoogaensis* L10 and its recombinant strains, and quantification of NTM production were performed as described previously (11, 15, 20).

Genome sequencing and genome annotation. The nucleotide sequence of the *S. chattanoogaensis* L10 genome was determined by using a massively parallel pyrosequencing technology (Roche 454 GS FLX) (26). One hundred sixty contigs were assembled from 522,882 reads (average length of 437 bp) by using Newbler software of the 454 suite package, providing 25.3-fold coverage. The total size of the 160 contigs was 9.0 Mb, with a minimum length of 500 bp. The relationship among contigs was determined by using multiplex PCR. Gaps were filled by sequencing of PCR products. The final sequence assembly was performed by using the phred/phrap/consed package (<http://www.phrap.org/phredphrapconsed.html>), and the low-sequence-quality regions were resequenced.

Putative protein-coding sequences were determined by combining the prediction results of glimmer 3.02 (27) and the Z-Curve program (28). Functional annotation of coding DNA sequence (CDS) was performed by searching the NCBI nonredundant protein database and the KEGG protein database. Protein domain prediction and COG (29) assignment were performed by RPS-BLAST using the NCBI CDD library (30).

Production and purification of SchPPT, SchACPS, and ACPs. The nine ACP genes, and the SchPPT and SchACPS genes were amplified by PCR using primer pairs HJ0057/HJ0064, HJ0079/HJ0080, HJ0083/HJ0086, HJ0113/HJ0114, and WYY0013/WYY0018 from genomic DNA of *S. chattanoogaensis* L10 (see Table S1 in the supplemental material). The

resulting products were cloned into the pTA2 vector (Toyobo) directly and sequenced to confirm PCR fidelity. These genes were then digested with NdeI-HindIII and cloned into the same sites of pET28a (Novagen), yielding expression constructs ([Table 1](#)). Finally, these plasmids were introduced into *E. coli* BL21(DE3) to overproduce proteins as N-terminally His₆-tagged proteins.

The ACPs were overproduced under novel conditions, as described below. Introduction of expression plasmids into *E. coli* BL21(DE3) with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 4 to 6 h resulted in overproduction of proteins in a soluble form. SchACPS and SchPPT were overproduced in insoluble forms under the same conditions. Lowering the incubation temperature to 18°C and the IPTG concentration to 10 μM decreased the overall yield but improved solubility significantly.

Purification of these proteins by affinity chromatography on Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) was performed under standard conditions recommended by the manufacturer. The proteins were dialyzed against a solution containing 20 mM Tris-HCl (pH 8.0), 25 mM NaCl, 1 mM dithiothreitol (DTT), and 10% glycerol.

Construction of SchPPT-P2P3 and the SchPPT/SchACPS hybrid. The gene encoding the C-terminal 122 amino acids of SchPPT was amplified by PCR using primers HJ0111 and WYY0014 from pHJ0024, resulting in SchPPT-P2P3. The gene encoding the N-terminal 97 amino acids of SchPPT was amplified by PCR using primers WYY0013 and HJ0112a from pHJ0024, resulting in SchPPT-P1. The SchACPS gene was amplified by PCR using primers HJ0112b and WYY0016 from pHJ0025, resulting in SchACPS-2. The SchPPT/SchACPS gene was amplified by overlapping PCR using primers WYY0013 and WYY0016 from templates SchPPT-P1 and SchACPS-2. Cloning of the SchPPT-P2P3 and SchPPT/SchACPS genes into pET28a, expression of these two genes in *E. coli*, and purification of the two proteins were performed according to the procedures described above.

Phosphopantetheinylation of ACPs *in vitro*. Each of the apo-ACPs was phosphopantetheinylated by SchPPT or SchACPS with coenzyme A

TABLE 1 List of strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference
Strains		
<i>S. chattanoogensis</i> L10	Industrial natamycin producer	20
sHJ002	Derived from L10; SchPPT:: <i>aac(3)IV</i> mutant	This study
sHJ003	Derived from L10; SchPPT overexpression mutant	This study
sHJ005	Derived from L10; <i>scw</i> ACP in-frame deletion mutant	This study
sHJ006	Derived from L10; <i>scw</i> PKS in-frame deletion mutant	This study
<i>E. coli</i> BW25113/pIJ790	Strain used for PCR-targeted mutagenesis	31
<i>E. coli</i> ET12567/pUZ8002	Methylation-deficient <i>E. coli</i> for conjugation	25
<i>E. coli</i> DH5α/BT340	Strain used for excising the DNA between two FRT sites	31
Plasmids		
pHJ0020	<i>scn</i> ACP0-1 cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0021	<i>scn</i> ACP0-2 cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0022	<i>scn</i> ACP7 cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0023	<i>scn</i> ACP10 cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0039	<i>scm</i> ACP2 cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0036	PKSIa ACP1 cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0038	<i>scw</i> ACP cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0049	PKSIc ACP gene cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0029	FAS ACP gene cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0024	SchPPT gene cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0025	SchACPS gene cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0048	SchPPT-P2P3 gene cloned as an NdeI-HindIII fragment into pET28a	This study
pHJ0050	SchPPT SchACPS genes cloned as an NdeI-HindIII fragment into pET28a	This study
pHY773	Derived from pIJ773; contains the disruption cassette P1-FRT- <i>aac(3)IV</i> -FRT-P2	Qin, unpublished
35E12	Cosmid pHAQ31 carrying the SchPPT gene	This study
pHJ0030	Derived from 35E12; the SchPPT gene was replaced with <i>aac(3)IV</i>	This study
09C1	Cosmid pHAQ31 carrying <i>scw</i> PKS	This study
pYY0030	Derived from pYY0028; <i>scw</i> ACP was in-frame deleted	This study
pYY0031	Derived from pYY0029; <i>scw</i> PKS was in-frame deleted	This study
pIJ8660	Site-specific integration vector carrying <i>ermEp*</i> , Φ31 <i>int</i> , and <i>attP</i>	32
pHJ0033	Derived from pIJ8660 with the SchPPT gene under the control of <i>ermEp*</i>	This study

^a FRT, FLP recombination target.

(CoA). A typical reaction mixture of 0.1 ml containing 100 mM Tris-HCl (pH 7.5), 1.25 mM MgCl₂, 2.5 mM tris(carboxyethyl)phosphine hydrochloride (TCEP), 200 μM ACP, 20 μM PPTase, and 2 mM CoA was incubated at 25°C for 30 min. The reactions were quenched by freezing reaction mixtures with dry ice.

HPLC analyses of ACPs. The ACPs produced from *E. coli* or from the phosphopantetheinylation reaction mixture were analyzed directly by high-performance liquid chromatography (HPLC) (LC-20AT; Shimadzu). HPLC separation was carried out on a C₁₈ column (Zorbax 300SB-C₁₈, 5 μm, 4.6 by 250 mm; Agilent). Solvent A consisted of 0.1% formic acid. Solvent B consisted of acetonitrile. The following binary gradient was used: a linear gradient from 0% to 75% solvent B from 0 to 15 min, a linear gradient from 75% to 100% solvent B from 15 to 16 min, 100% solvent B from 16 to 19 min, a linear gradient from 100% to 0% solvent B from 19 to 20 min, and 0% solvent B from 20 to 24 min, at a flow rate of 1 ml/min. UV detection was performed at 220 nm (SPD-20A; Shimadzu).

LC-MS analyses of ACPs. The ACPs produced from *E. coli* or from the phosphopantetheinylation reaction mixture were analyzed directly by liquid chromatography-mass spectrometry (LC-MS) (Agilent 1200, Thermo Finnigan LCQ Deca XP Max). LC separation was carried out on an Agilent SB-C₁₈ column (3.5 μm, 80 Å, 2.1 by 150 mm) at 35°C. Solvent A consisted of 0.1% formic acid. Solvent B consisted of acetonitrile. The following binary gradient was used: 5% solvent B from 0 to 5 min and a linear gradient to 75% solvent B from 5 to 45 min, followed by 3 min of isocratic elution of 75% solvent B and equilibration to initial conditions for 13 min at a flow rate of 0.2 ml/min. UV detection was performed at both 254 nm and 280 nm. MS with an electrospray ionization (ESI) source was per-

formed as follows: positive mode, source voltage of 2.5 kV, capillary voltage of 41 V, sheath gas flow of 45 arbitrary units, auxiliary/sweep gas flow of 5 arb, and capillary temperature of 330°C.

Construction of an SchPPT::*aac(3)IV* mutant. The SchPPT gene disruption mutant was constructed by using the PCR targeting system, as follows (31). First, a cosmid, 35E12, which harbors the SchPPT gene, was screened out by PCR amplification using primer pair HJ0077/HJ0078. Second, the disruption cassette *aac(3)IV* (apramycin resistance gene) was PCR amplified by using primer pair HJ0075/HJ0076 from pHY773 (Z. Qin, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China, unpublished results), with the resulting product carrying 59 ends with homology to the corresponding region of the SchPPT gene. The PCR product was then introduced into *E. coli* BW25113 carrying pIJ790/35E12, and transformed cells carrying mutagenized 35E12 were selected on LB agar containing apramycin. The mutagenized 35E12, in which the SchPPT gene was replaced with *aac(3)IV*, was designated pHJ0030 and confirmed by PCR analysis using primer pair HJ0077/HJ0078. Third, after conjugal transfer of pHJ0030 from *E. coli* ET12567 carrying pUZ8002 into *S. chattanoogensis* L10, exconjugants were obtained after selection for apramycin. Exconjugants were then inoculated onto YMG plates for two rounds of nonselective growth before selection by replica plating for thiostrepton-sensitive and apramycin-resistant colonies. The resulting strain, in which the SchPPT gene was replaced with *aac(3)IV*, was designated sHJ002 and confirmed by PCR analysis using primer pair HJ0077/HJ0078.

Construction of an SchPPT overexpression mutant. A site-specific integration vector, pIJ8660 (32), containing the *ermEp** promoter, Φ31 *int*, and *attP*, was used to construct an integration recombinant plasmid.

FIG 2 PKSs in *S. chattanoogensis* L10. Shown is the gene organization of PKS gene clusters in *S. chattanoogensis* L10. (A) *scm* PKS; (B) *PKSIIa* PKS; (C) *scw* PKS; (D) *PKSIIc* PKS. AT, acyltransferase; TE, thioesterase; AMT, amidotransferase; KS, β -ketosynthase; DH, dehydratase; KR, ketoreductase; ACP, acyl carrier protein; MT, methyl transferase; Ox, oxidoreductase; ER, enoylreductase; GT, glycosyltransferase; DAc, deacetylase; DeH, dehydrogenase; Res, resistance transporter; Hyd, hydroxylase; Cyc, cyclase; CLF, chain length factor; Aro, aromatase.

The NdeI-NotI DNA fragment of the SchPPT gene was cloned from pHJ0024 into the same sites of pIJ8660, resulting in plasmid pHJ0033. pHJ0033 was transferred into *S. chattanoogensis* L10 via conjugal transfer from *E. coli* ET12567/pUZ8002 by using standard procedures. The resulting strain, in which SchPPT was expressed under the control of *ermEp**, was designated sHJ003 and confirmed by PCR analysis using primer pair Pri53/WYY0014.

Construction of Δ *scw* ACP and Δ *scw* PKS mutants. The *scw* ACP in-frame deletion mutant and the *scw* PKS in-frame deletion mutant were constructed by using the PCR targeting system, as follows (31). First, a cosmid, 09C1, which harbors the *scw* PKS gene cluster, was screened out by PCR amplification using primer pair WYY0027/WYY0028. Second, two disruption cassette *aac(3)IV* genes were individually PCR amplified by using primer pairs WYY0025/WYY0026 and WYY0029/WYY0026 from pHY773, with the resulting product carrying 59 ends with homology to the corresponding region of the *scw* ACP gene and the *scw* PKS gene cluster, respectively. Two PCR products were then individually introduced into *E. coli* BW25113 carrying pIJ790/09C1, and the transformed cells carrying mutagenized 09C1 were selected on LB agar containing apramycin. Two mutagenized 09C1 plasmids, in which the *scw* ACP gene and *scw* PKS gene cluster were replaced with *aac(3)IV*, were designated pYY0028 and pYY0029 and confirmed by PCR analysis using primer pairs WYY0027/WYY0028 and WYY0028/WYY0030, respectively. Third, pYY0028 and pYY0029 were individually transformed into *E. coli* DH5 α /BT340 in order to excise the *aac(3)IV* genes, resulting in pYY0030 and pYY0031, respectively (31). Finally, after conjugal transfer of pYY0030 and pYY0031 individually from *E. coli* ET12567/pUZ8002 into *S. chattanoogensis* L10, the single-crossover exconjugants were obtained after selection for thiostrepton. The single-crossover exconjugants were then inoculated onto YMG plates for two rounds of nonselective growth. The double-crossover exconjugants were obtained after selection for thiostrepton-sensitive colonies. The resulting mutants, in which *scw* ACP and *scw* PKS genes were in-frame deleted, were designated sHJ005 and sHJ006 and confirmed by PCR analysis using primer pairs WYY0027/WYY0028 and WYY0028/WYY0030, respectively.

Nucleotide sequence accession numbers. The SchPPT gene and its flanking sequences and the SchACPS gene and its flanking sequences were deposited in GenBank under accession numbers JQ283111 and JQ283112, respectively.

RESULTS AND DISCUSSION

Analysis of PPTase genes and PKS gene clusters in *S. chattanoogensis* L10. The whole genomic DNA of *S. chattanoogensis* L10 was recently sequenced to identify its PPTases. Analysis of the genomic sequence revealed two PPTase genes, the genes encoding SchPPT

and SchACPS. The GenBank accession numbers for the SchPPT gene with its flanking sequences and the SchACPS gene with its flanking sequences are JQ283111 and JQ283112, respectively. Both genes are not clustered with any secondary metabolite biosynthesis gene clusters, including the *scn* PKS gene cluster (23). Alignment of SchPPT and SchACPS with known PPTases showed that SchPPT contains three conserved motifs, PRWP, GVD, and ESAKESVYK, found in the Sfp-type PPTase family motifs P1 (PxxP), P2 (GxD), and P3 [(F/W)(S/T/A)xKE(S/A)xxK], while SchACPS contains just two conserved motifs, GID and FAAKEA VAK, found in the ACPS-type PPTase family motifs P2 and P3 (Fig. 1A) (10). (The underlined letters represent the conserved amino acids.) Thus, SchPPT and SchACPS belong to the Sfp-type PPTase group and the ACPS-type PPTase group, respectively.

Analysis of the genomic sequence also revealed four new PKS gene clusters in *S. chattanoogensis* L10, including the *scw* PKS, *scm* PKS, *PKSIIa*, and *PKSIIc* gene clusters. The *scw* PKS gene cluster contains homologous genes of all eight genes in *whiE* PKS gene clusters in several *Streptomyces* strains (33–37). The *whiE* PKS is a type II PKS, which is involved in structure-unknown spore pigment biosynthesis. Thus, we named our gene cluster the *scw* (*S. chattanoogensis whiE*) PKS gene cluster, which spans 8,276 bp of DNA sequence (Fig. 2).

The *scm* PKS gene cluster contains homologous genes of all eight genes in the migrastatin PKS gene cluster in *Streptomyces platensis* NRRL 18993 (38). The migrastatin PKS gene cluster is an AT-less type I PKS gene cluster, which is involved in migrastatin biosynthesis. Thus, we named our gene cluster the *scm* (*S. chattanoogensis migrastatin*) PKS gene cluster, which spans 49,863 bp of DNA sequence. The *PKSIIa* gene cluster consists of four type I PKS genes and seven accessory genes, spanning 48,808 bp of DNA sequence. The *PKSIIc* gene cluster consists of three type II PKS genes and seven accessory genes, spanning 4,841 bp of DNA sequence (Fig. 2).

Phosphopantetheinylation of ACPs catalyzed by SchACPS and SchACPS *in vitro*. To characterize SchPPT and SchACPS *in vitro*, each protein was produced in *E. coli* and then purified to homogeneity (see Fig. S1 in the supplemental material). To obtain the substrates of SchPPT and SchACPS, nine ACPs were individually produced in *E. coli* and then purified to homogeneity (see Fig. S1 in the supplemental material). These ACPs are

TABLE 2 LC-MS analyses of ACPs

ACP	Mol wt ^a						
	Calculated		Found				
	Apo-form	Holo-form	From <i>E. coli</i>	With SchPPT	With SchACPS	With P2P3	With hybrid
<i>scn</i> ACP0-1	13,461	13,801	13,457	13,797	13,457	ND	ND
<i>scn</i> ACP0-2	14,434	14,774	14,430	14,768	14,428	14,430	14,428
<i>scn</i> ACP7	13,628	13,968	13,623	13,963	13,623	ND	ND
<i>scn</i> ACP10	12,685	13,025	12,682	13,022	12,682	ND	ND
<i>scm</i> ACP2	11,982	12,322	11,977	12,318	11,978	ND	ND
PKSIa ACP1	15,968	16,308	15,964	16,304	15,964	ND	ND
<i>scw</i> ACP	11,125	11,465	11,123	11,462	11,462	ND	ND
PKSIc ACP	11,181	11,521	11,177	11,518	11,517	ND	ND
FAS ACP	10,993	11,333	10,990/11,331 ^b	10,990/11,331 ^b	11,331	ND	ND

^a Shading indicates the apo-form, and boldface type indicates the holo-form. ND, not determined.

^b Both apo-proteins and holo-proteins were found.

scn ACP0-1 (the first ACP domain in the loading module of *scn* PKS), *scn* ACP0-2 (the second ACP domain in the loading module of *scn* PKS), *scn* ACP7 (the ACP domain in the seventh module of *scn* PKS), *scn* ACP10 (the ACP domain in the 10th module of *scn* PKS), *scm* ACP2 (the ACP domain in the second module of *scm* PKS), PKSIa ACP1 (the ACP domain in the first module of PKSIa), *scw* ACP (the ACP of *scw* PKS), PKSIc ACP (the ACP of PKSIc PKS), and FAS ACP (the ACP of FAS in *S. chattanoogensis* L10).

To determine the *in vitro* function of SchACPS and SchPPT, each of the nine ACPs was incubated with CoA in the presence of SchACPS or SchPPT. After incubation for 30 min at 25°C, reaction mixtures were analyzed by HPLC and LC-MS. Regarding the FAS ACP, HPLC data showed that the proteins produced from *E.*

coli were eluted as two peaks, a large peak with a retention time of 14.62 min and a small peak with a retention time of 13.58 min. LC-MS data showed that the large peak represented apo-proteins and that the small peak represented holo-proteins, indicating that *E. coli* ACPS could convert it from the apo-form to the holo-form incompletely (Table 2). After incubation with SchACPS, the peak of apo-proteins disappeared, and the peak of holo-proteins became larger by HPLC analysis, indicating that SchACPS converted the proteins from the apo-form into the holo-form completely. The ratio of the apo-form and the holo-form did not change after incubation with SchPPT, indicating that SchPPT could not catalyze the phosphopantetheinylation of FAS ACP (Table 2 and Fig. 3A).

FIG 3 HPLC analyses of FAS ACP (A) and PKSIc ACP (B). ▽, apo-form; ▼, holo-form. mAU, milli-absorbance units.

FIG 4 LC-MS analyses of *scw* ACP (A) and *scn* ACP0-2 (B).

Regarding the PKSIIc ACP, HPLC data showed that the proteins produced from *E. coli* were eluted as two peaks, a major peak with a retention time of 14.34 min and a trace peak at 13.70 min. LC-MS data showed that the major peak represented apo-proteins (Table 2). Although the trace peak was too small to be analyzed by LC-MS, it may represent holo-proteins, since it had a retention time identical to that of the holo-proteins, indicating that *E. coli* ACPS could hardly catalyze it. After incubation with SchACPS or SchPPT, the peak of apo-proteins became smaller, and the trace peak at a retention time of 13.70 min increased significantly. LC-MS data showed that the peak represented holo-proteins, indicating that both SchACPS and SchPPT converted the proteins from the apo-form into the holo-form (Table 2 and Fig. 3B).

Regarding *scw* ACP, HPLC data showed that the proteins were eluted as a single peak. LC-MS data showed that the peak represented apo-proteins (Table 2). After incubation with SchACPS or SchPPT, the molecular mass of *scw* ACP increased 340 Da compared to the molecular mass of its apo-form by LC-MS analysis, indicating that both SchPPT and SchACPS catalyzed the phosphopantetheinylation of *scw* ACP (Table 2 and Fig. 4A).

Regarding the four *scn* ACPs, *scm* ACP2, and PKSIIa ACPI, HPLC data showed that each of the proteins was eluted as a single peak. LC-MS data showed that these peaks represented apo-proteins (Table 2). After incubation with SchPPT, the molecular masses of these ACPs increased 340 Da compared to those of their apo-forms by LC-MS analysis (Table 2). However, the molecular masses of these ACPs did not change after incubation with SchACPS, indicating that SchPPT but not SchACPS converted these ACPs from the apo-form into the holo-form (Table 2 and Fig. 4B). Our *in vitro* results described above showed that

SchACPS catalyzed the phosphopantetheinylation of FAS ACP and ACPs in type II PKSs, while SchPPT catalyzed the phosphopantetheinylation of ACPs in both type I PKSs and type II PKSs (Fig. 5).

Inactivation of SchPPT abolished the production of NTM but not the spore pigment. To determine the influence of the activity of SchPPT on NTM production *in vivo*, the SchPPT gene was replaced with the apramycin resistance gene *aac(3)IV* in *S. chattanoogaensis* L10 by using Redirect technology, as follows (31). Double-crossover mutants were screened for apramycin resistance and thiostrepton sensitivity. The resulting mutant, sHJ002, was confirmed by PCR using primers specific for both flanking regions of the apramycin resistance gene (see Fig. S2A in the supplemental material). sHJ002 was fermented in YEME liquid medium with 4% glucose in flasks by using *S. chattanoogaensis* L10 as a control. NTM production was monitored by HPLC analyses periodically. There was no NTM detected during the growth of sHJ002, confirming that SchPPT is essential for NTM production (Fig. 6A). Compared to the growth curve of *S. chattanoogaensis* L10, no obvious change was observed in that of sHJ002, confirming that SchPPT has no influence to strain growth (Fig. 6B).

To determine the roles of *scw* PKS and *scw* ACP genes in spore pigment production, the *scw* ACP gene and the *scw* PKS genes were in-frame deleted in *S. chattanoogaensis* L10, respectively, based on Redirect technology (31). Single-crossover mutants were screened for thiostrepton resistance. Double-crossover mutants were screened for thiostrepton sensitivity based on single-crossover mutants. The resulting Δ *scw* ACP mutant strain, sHJ005, and the resulting Δ *scw* PKS mutant strain, sHJ006, were confirmed by PCR using primers specific for both flanking regions of the *scw*

FIG 5 Phosphopantetheinylation network of PKSs and FAS catalyzed by SchPPT and SchACPS.

ACP gene and the *scw* PKS genes, respectively (see Fig. S2C and S2D in the supplemental material). sHJ005, sHJ006, and *S. chattanoogaensis* L10 were cultured in YMG solid medium. *S. chattanoogaensis* L10 produced spores with gray spore pigment after growth for 240 h. However, although sHJ005 and sHJ006 produced spores, both of them lost the ability to produce the spore pigment, confirming that *scw* ACP and *scw* PKS were essential for spore pigment production (see Fig. S3A in the supplemental material).

To determine the influence of the activity of SchPPT on spore pigment production *in vivo*, sHJ002 and *S. chattanoogaensis* L10 were cultured on YMG solid medium. Both *S. chattanoogaensis* L10 and sHJ002 produced spores with gray spore pigment after growth for 240 h, supporting the conclusion that the lack of activity of SchPPT of *scw* ACP in mutant strain sHJ002 could be complemented by that of SchACPS (see Fig. S3A in the supplemental material). Our *in vivo* results described above showed that inacti-

vation of SchPPT abolished the production of NTM but not the spore pigment, which is consistent with the *in vitro* results (Fig. 5).

Overexpression of SchPPT increased and accelerated polyketide production. To study the effect of the expression level of the SchPPT gene on NTM production, an SchPPT gene overexpression mutant was constructed. The SchPPT gene was cloned into an integration recombinant vector, pIJ8660 (32), to construct plasmid pHJ0033, in which the SchPPT gene was under the control of the *ermEp** promoter. pHJ0033 was introduced into *S. chattanoogaensis* L10, resulting in SchPPT gene overexpression mutant strain sHJ003. sHJ003 was confirmed by apramycin resistance and PCR using primers specific for amplification of the *ermEp**-SchPPT fusion gene (see Fig. S2B in the supplemental material). Three transformants in triplicate cultures were fermented in industrial YSG liquid medium in flasks by using *S. chattanoogaensis* L10 as a control. NTM production was monitored by HPLC analyses periodically. These three transformants pro-

FIG 6 NTM production of *S. chattanoogaensis* L10 and its recombinant strains. (A) NTM production of *S. chattanoogaensis* L10 and sHJ002 in YEME liquid medium. (B) Growth of *S. chattanoogaensis* L10 and sHJ002 in YEME. (C) NTM production of *S. chattanoogaensis* L10 and sHJ003 in YSG liquid medium. (D) HPLC analyses of NTM production of sHJ003 and *S. chattanoogaensis* L10 in YSG liquid medium. WT, wild type.

duced NTM with identical yields in the range of allowable error. The NTM yield of *S. chattanoogensis* L10 reached the highest level at 2.21 g/liter at 144 h. Notably, the NTM yield of sHJ003 reached the highest level at 3.12 g/liter at 120 h, while that of *S. chattanoogensis* L10 was 1.97 g/liter at the same time (Fig. 6C and D). Thus, sHJ003 not only increased NTM production by about 40% but also accelerated NTM production by 24 h. This high-yield and energy-saving strain may be beneficial for the industrial production of NTM.

To study the effect of the expression level of the SchPPT gene on spore pigment production, sHJ003, sHJ002, and *S. chattanoogensis* L10 were cultured on YMG solid medium. sHJ003, sHJ002, and *S. chattanoogensis* L10 started to produce spore pigment after 96 h, 144 h, and 220 h, respectively (see Fig. S3B in the supplemental material).

Relationship of structure and specificity of SchPPT. To characterize the relationship of the structure and the specificity of SchPPT, an N-terminally truncated SchPPT (SchPPT-P2P3) and an SchPPT/SchACPS hybrid were constructed. SchPPT-P2P3 contains the C-terminal 122 amino acids (D98 to R219) of SchPPT, which show high homology to SchACPS (Fig. 1A and B). The SchPPT/SchACPS hybrid fuses the N-terminal 97 amino acids (M1 to R97) of SchPPT at the N terminus, with the whole SchACPS at the C terminus (Fig. 1B). SchPPT-P2P3 and SchPPT/SchACPS were produced and purified from *E. coli* according to the procedures described above (see Fig. S1 in the supplemental material). *scn* ACP0-2 and FAS ACP were individually incubated with CoA in the presence of SchPPT-P2P3 or SchPPT/SchACPS.

Regarding *scn* ACP0-2, the reaction mixtures were analyzed by LC-MS. The molecular weights of *scn* ACP0-2 did not change after incubation with SchPPT-P2P3 or SchPPT/SchACPS, indicating that neither SchPPT-P2P3 nor SchPPT/SchACPS could catalyze the phosphopantetheinylation of it (Table 2 and Fig. 4B).

Regarding FAS ACP, the reaction mixtures were analyzed by HPLC. After incubation with SchPPT-P2P3, the ratio of the apo-form and the holo-form did not change, indicating that SchPPT-P2P3 could not catalyze FAS ACP. However, after incubation with SchPPT/SchACPS, the peak of apo-proteins disappeared, and the peak of holo-proteins became larger, indicating that SchPPT/SchACPS converted the proteins from the apo-form into the holo-form completely (Fig. 3A).

The different specificities of the ACPS-type PPTases and the Sfp-type PPTases should be due to their structural differences. The C-terminal halves of the Sfp-type PPTases show high homology with the whole ACPS-type PPTases (Fig. 1A) (1, 39). The N-terminal half and the C-terminal half of the Sfp-type PPTase form an intramolecular 2-fold pseudosymmetry, which raises a question: What is the role of the N-terminal half of the Sfp-type PPTase in the activity and the specificity of this group of PPTases (39)? SchPPT-P2P3 lost its ability to catalyze the phosphopantetheinylation of *scn* ACP and could not catalyze that of FAS ACP, suggesting that the N terminus is essential to the activity of the Sfp-type PPTases. The SchPPT/SchACPS hybrid catalyzed the phosphopantetheinylation of FAS ACP but not that of *scn* ACP, suggesting that the C terminus may control the specificity of the Sfp-type PPTases.

In summary, our data here characterized the phosphopantetheinylation network of PKSs in *S. chattanoogensis* L10, and NTM production could be significantly improved by engineering SchPPT. This is direct evidence that engineering of

polyketide production could be achieved through the manipulation of PPTases.

ACKNOWLEDGMENTS

We are grateful to the Chinese National Human Genome Center at Shanghai (CHGC) for help in genome sequencing and Huajun Zheng for assistance with initial analysis of the genome sequence.

This work was supported by National Basic Research Program of China (973 Program) grant 2012CB721005, National Key Technologies R&D Program of China grant 2011BAD23B05-2, National Natural Science Foundation of China grant 31200600, and Zhejiang Provincial Natural Science Foundation of China grants LY12C05005 and LZ12C01001.

REFERENCES

- Lambalot RH, Gehring AM, Flugel RS, Zuber P, LaCelle M, Marahiel MA, Reid R, Khosla C, Walsh CT. 1996. A new enzyme superfamily—the phosphopantetheinyl transferases. *Chem. Biol.* 3:923–936.
- Walsh CT, Gehring AM, Weinreb PH, Quadri LE, Flugel RS. 1997. Post-translational modification of polyketide and nonribosomal peptide synthases. *Curr. Opin. Chem. Biol.* 1:309–315.
- Barekzi N, Joshi S, Irwin S, Ontl T, Schweizer HP. 2004. Genetic characterization of *pcpS*, encoding the multifunctional phosphopantetheinyl transferase of *Pseudomonas aeruginosa*. *Microbiology* 150:795–803.
- Finking R, Solsbacher J, Konz D, Schobert M, Schafer A, Jahn D, Marahiel MA. 2002. Characterization of a new type of phosphopantetheinyl transferase for fatty acid and siderophore synthesis in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 277:50293–50302.
- Lu YW, San Roman AK, Gehring AM. 2008. Role of phosphopantetheinyl transferase genes in antibiotic production by *Streptomyces coelicolor*. *J. Bacteriol.* 190:6903–6908.
- Huang Y, Wendt-Pienkowski E, Shen B. 2006. A dedicated phosphopantetheinyl transferase for the fredericamycin polyketide synthase from *Streptomyces griseus*. *J. Biol. Chem.* 281:29660–29668.
- Gomez-Escribano JP, Bibb MJ. 2011. Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. *Microb. Biotechnol.* 4:207–215.
- Meiser P, Muller R. 2008. Two functionally redundant Sfp-type 4'-phosphopantetheinyl transferases differentially activate biosynthetic pathways in *Myxococcus xanthus*. *Chembiochem* 9:1549–1553.
- Weissman KJ, Hong H, Oliynyk M, Siskos AP, Leadlay PF. 2004. Identification of a phosphopantetheinyl transferase for erythromycin biosynthesis in *Saccharopolyspora erythraea*. *Chembiochem* 5:116–125.
- Sanchez C, Du L, Edwards DJ, Toney MD, Shen B. 2001. Cloning and characterization of a phosphopantetheinyl transferase from *Streptomyces verticillus* ATCC15003, the producer of the hybrid peptide-polyketide antitumor drug bleomycin. *Chem. Biol.* 8:725–738.
- Murugan E, Liang ZX. 2008. Evidence for a novel phosphopantetheinyl transferase domain in the polyketide synthase for enediyne biosynthesis. *FEBS Lett.* 582:1097–1103.
- el-Enshasy HA, Farid MA, el-Sayed SA. 2000. Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. *J. Basic Microbiol.* 40:333–342.
- Chen GQ, Lu FP, Du LX. 2008. Natamycin production by *Streptomyces gilvosporeus* based on statistical optimization. *J. Agric. Food Chem.* 56:5057–5061.
- Du YL, Chen SF, Cheng LY, Shen XL, Tian Y, Li YQ. 2009. Identification of a novel *Streptomyces chattanoogensis* L10 and enhancing its natamycin production by overexpressing positive regulator ScnRII. *J. Microbiol.* 47:506–513.
- Anton N, Santos-Aberturas J, Mendes MV, Guerra SM, Martin JF, Aparicio JF. 2007. PimM, a PAS domain positive regulator of pimarin biosynthesis in *Streptomyces natalensis*. *Microbiology* 153:3174–3183.
- Anton N, Mendes MV, Martin JF, Aparicio JF. 2004. Identification of PimR as a positive regulator of pimarin biosynthesis in *Streptomyces natalensis*. *J. Bacteriol.* 186:2567–2575.
- Lee KM, Lee CK, Choi SU, Park HR, Kitani S, Nihira T, Hwang YI. 2005. Cloning and in vivo functional analysis by disruption of a gene encoding the gamma-butyrolactone autoregulator receptor from *Streptomyces natalensis*. *Arch. Microbiol.* 184:249–257.
- Du YL, Shen XL, Yu P, Bai LQ, Li YQ. 2011. Gamma-butyrolactone regulatory system of *Streptomyces chattanoogensis* links nutrient utiliza-

- tion, metabolism, and development. *Appl. Environ. Microbiol.* 77:8415–8426.
19. Recio E, Aparicio JF, Rumbero A, Martin JF. 2006. Glycerol, ethylene glycol and propanediol elicit pimarinin biosynthesis in the PI-factor-defective strain *Streptomyces natalensis* npi287 and increase polyene production in several wild-type actinomycetes. *Microbiology* 152:3147–3156.
 20. Recio E, Colinas A, Rumbero A, Aparicio JF, Martin JF. 2004. PI factor, a novel type quorum-sensing inducer elicits pimarinin production in *Streptomyces natalensis*. *J. Biol. Chem.* 279:41586–41593.
 21. Mendes MV, Recio E, Fouces R, Luiten R, Martin JF, Aparicio JF. 2001. Engineered biosynthesis of novel polyenes: a pimarinin derivative produced by targeted gene disruption in *Streptomyces natalensis*. *Chem. Biol.* 8:635–644.
 22. Mendes MV, Tunca S, Anton N, Recio E, Sola-Landa A, Aparicio JF, Martin JF. 2007. The two-component phoR-phoP system of *Streptomyces natalensis*: inactivation or deletion of phoP reduces the negative phosphate regulation of pimarinin biosynthesis. *Metab. Eng.* 9:217–227.
 23. Du YL, Li SZ, Zhou Z, Chen SF, Fan WM, Li YQ. 2011. The pleiotropic regulator AdpA is required for natamycin biosynthesis and morphological differentiation in *Streptomyces chattanoogensis*. *Microbiology* 157:1300–1311.
 24. Vicente CM, Santos-Aberturas J, Guerra SM, Payero TD, Martin JF, Aparicio JF. 2009. PimT, an amino acid exporter controls polyene production via secretion of the quorum sensing pimarinin-inducer PI-factor in *Streptomyces natalensis*. *Microb. Cell Fact.* 8:33. doi:10.1186/1475-2859-8-33.
 25. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, United Kingdom.
 26. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirase KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437:376–380.
 27. Delcher AL, Harmon D, Kasif S, White O, Salzberg SL. 1999. Improved microbial gene identification with GLIMMER. *Nucleic Acids Res.* 27:4636–4641.
 28. Guo FB, Ou HY, Zhang CT. 2003. ZCURVE: a new system for recognizing protein-coding genes in bacterial and archaeal genomes. *Nucleic Acids Res.* 31:1780–1789.
 29. Tatusov RL, Galperin MY, Natale DA, Koonin EV. 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* 28:33–36.
 30. Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH. 2007. CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res.* 35:D237–D240. doi:10.1093/nar/gkl951.
 31. Gust B, Challis GL, Fowler K, Kieser T, Chater KF. 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci. U. S. A.* 100:1541–1546.
 32. Sun J, Kelemen GH, Fernandez-Abalos JM, Bibb MJ. 1999. Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* 145(Part 9):2221–2227.
 33. Blanco G, Pereda A, Brian P, Mendez C, Chater KF, Salas JA. 1993. A hydroxylase-like gene product contributes to synthesis of a polyketide spore pigment in *Streptomyces halstedii*. *J. Bacteriol.* 175:8043–8048.
 34. Kelemen GH, Brian P, Flardh K, Chamberlin L, Chater KF, Buttner MJ. 1998. Developmental regulation of transcription of whiE, a locus specifying the polyketide spore pigment in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 180:2515–2521.
 35. Novakova R, Bistakova J, Kormanec J. 2004. Characterization of the polyketide spore pigment cluster whiESa in *Streptomyces aureofaciens* CCM3239. *Arch. Microbiol.* 182:388–395.
 36. Yu TW, Hopwood DA. 1995. Ectopic expression of the *Streptomyces coelicolor* whiE genes for polyketide spore pigment synthesis and their interaction with the act genes for actinorhodin biosynthesis. *Microbiology* 141(Part 11):2779–2791.
 37. Yu TW, Shen Y, McDaniel R, Floss HG, Khosla C, Hopwood DA, Moore BS. 1998. Engineered biosynthesis of novel polyketides from *Streptomyces* spore pigment polyketide synthases. *J. Am. Chem. Soc.* 120:7749–7759.
 38. Lim SK, Ju J, Zazopoulos E, Jiang H, Seo JW, Chen Y, Feng Z, Rajski SR, Farnet CM, Shen B. 2009. iso-Migrastatin, migrastatin, and dorrigocin production in *Streptomyces platensis* NRRL 18993 is governed by a single biosynthetic machinery featuring an acyltransferase-less type I polyketide synthase. *J. Biol. Chem.* 284:29746–29756.
 39. Reuter K, Mofid MR, Marahiel MA, Ficner R. 1999. Crystal structure of the surfactin synthetase-activating enzyme sfp: a prototype of the 4'-phosphopantetheinyl transferase superfamily. *EMBO J.* 18:6823–6831.