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Application of a Newly Identified and Characterized 18-*O*-Acyltransferase in Chemoenzymatic Synthesis of Selected Natural and Nonnatural Bioactive Derivatives of Phoslactomycins[▽]

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Phoslactomycins (PLMs) and related leustroducsins (LSNs) have been isolated from a variety of bacteria based on antifungal, anticancer, and other biological assays. *Streptomyces* sp. strain HK 803 produces five PLM analogs (PLM A and PLMs C to F) in which the C-18 hydroxyl substituent is esterified with a range of branched, short-alkyl-chain carboxylic acids. The proposed pathway intermediate, PLM G, in which the hydroxyl residue is not esterified has not been observed at any significant level in fermentation, and the only route to this potentially useful intermediate has been an enzymatic deacylation of other PLMs and LSNs. We report that deletion of *plmS*$_3$ from the PLM biosynthetic cluster gives rise to a mutant which accumulates the PLM G intermediate. The 921-bp *plmS*$_3$ open reading frame was cloned and expressed as an N-terminally polyhistidine-tagged protein in *Escherichia coli* and shown to be an 18-*O* acyltransferase, catalyzing conversion of PLM G to PLM A, PLM C, and PLM E using isobutyryl coenzyme A (CoA), 3-methylbutyryl-CoA, and cyclohexylcarbonyl-CoA, respectively. The efficiency of this process (k_{cat} of 28 ± 3 min^{-1} and K_m of 88 ± 16 μM) represents a one-step chemoenzymatic alternative to a multistep synthetic process for selective chemical esterification of the C-18 hydroxy residue of PLM G. PlmS$_3$ was shown to catalyze esterification of PLM G with CoA and *N*-acetylcysteamine thioesters of various saturated, unsaturated, and aromatic carboxylic acids and thus also to provide an efficient chemoenzymatic route to new PLM analogs.

Attachment of either short (C_2 to C_6) or medium (C_8 to C_{12}) acyl chains to both amine and alcohol moieties on polyketide and polypeptide natural products can represent a key step in generating the final biologically active molecule. This step is often, but not always, one of the later biosynthetic steps and is catalyzed by an acyltransferase. The corresponding gene is typically associated with the polyketide or polypeptide biosynthetic gene cluster. Despite the importance of this step, a relatively small number of these acyltransferases from actinomycetes have been identified, and very few have been fully characterized (2, 10, 15, 18).

Studies of biosynthetic processes where there is an acylation of a polyketide chain, have indicated that the enzymes have various degrees of promiscuity for the carboxylic acid substrates. For instance, genetic evidence has shown that *mdmB* and *acyA* encode 3-*O*-acyltransferases which transfer either acetyl or propionyl groups to position 3 in 16-membered macrolides such as midecamycin, spiramycin, carbomycin, and tylosin (3, 10). The *asm19* gene product has been identified as the 3-*O*-acyltransferase which catalyzes the attachment of the biologically essential acyl group in the macrocyclic ansamitocins (18). The *asm19* mutant accumulates an *N*-demethyl-4,5-desepoxymytansinol, indicating that acylation of the macrocycle precedes N methylation and epoxidation. *Escherichia coli* cell extracts containing a recombinant Asm19 protein have been shown to catalyze acylation of this mytansinol intermediate using a range of short straight- and branched-chain acyl coenzyme A (CoA) thioesters (C_2 to C_5). Finally, LovD, which catalyzes the acylation of the C-8 hydroxyl group of monacolin J to yield the natural product lovastatin, a pharmaceutically important fungal polyketide product produced by *Aspergillus nidulans*, has been characterized (30). This enzyme is able to utilize a wide range of different acyl donors activated as CoA, *N*-acetylcysteamine (NAC), or methyl thioglycolate esters and thus offers an economically attractive route for generating novel lovasotatin analogs for treatment of hypercholesterolemia.

One or multiple *O*-acyltransferases have been implied to be involved in the post-polyketide synthase tailoring steps leading to a series of natural products known as the phoslactomycins (PLMs) (Fig. 1) (6). These compounds (also known as leustroducsins [LSNs], phospholines, and phosphazomycins) have been isolated from various actinomycetes, and their structures are all identical with the exception of the acyl substituent at C-18 (4, 5, 12, 13, 20, 27, 28). *Streptomyces* sp. strain HK 803 produces at least five such acylated analogs (PLM A and PLMs C to F) (Fig. 1) as well as PLM B, in which the C-18 hydroxyl substituent is absent (4, 5, 27).

These natural products have been isolated based on their potent activity (as low as 0.008 μg/ml) against some phytopathogenic fungi (27, 28). The compounds also have relatively weak antitumor activity (50% inhibitory concentration of 2 to 3 μg/ml against L1210, P38,8, and El-4 cell lines) (19) which may arise from their activity as selective inhibitors of protein phosphatase 2A. (26). These natural products also show induction of a colony-stimulating factor (12) via NF-κB activation and thrombopoiesis (14). This array of promising biological

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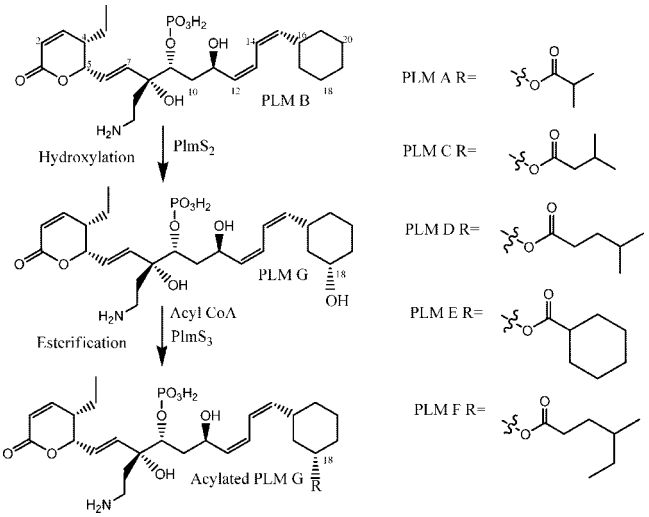


FIG. 1. Proposed biosynthetic relationship between PLM products made by *Streptomyces* sp. strain HK 803. A cytochrome P450 monooxygenase ($PlmS_2$) catalyzes C-18 hydroxylation of PLM B to generate PLM G, which is subsequently 18-O acylated by $PlmS_3$.

activities has stimulated research into the field of PLMs for treatments of various diseases. Low yields and the presence of multiple acylated products from fermentations have provided a barrier to this work, and circuitous routes to obtaining individual compounds have been described. For instance, there have been no reports of any actinomycetes which produce PLM G (LSN H) (Fig. 1), in which the C-18 hydroxyl residue is present but not acylated, and this compound has been reported to be obtained only by cleavage of the acyl groups of a mixture of other PLMs and LSNs using porcine liver esterase (24). A multistep synthetic route to selectively acylate PLM G with 6-methyloctanoic acid (producing LSN B) has also been described (17).

Recently the entire 75-kbp Plm biosynthetic gene cluster has been cloned, sequenced, and analyzed (21) and has provided an opportunity to study the enzymatic hydroxylation and acylation processes which give rise to the range of PLM products. Deletion of the $plmS_2$ open reading frame (ORF), showing high sequence similarity to bacterial cytochrome P450 monooxygenases, has resulted in an NP1 mutant producing only PLM B (Fig. 1) (21). The $plmS_2$ ORF has been expressed as an N-terminally polyhistidine-tagged protein in *Streptomyces coelicolor*, and the purified protein has been shown to catalyze conversion of PLM B to PLM G (7). This work in conjunction with other studies (1, 23) has led to a proposal that the final two biosynthetic steps involve hydroxylation of PLM B (to give PLM G) and subsequent acylation with a broad range of acyl-CoA substrates (to give PLM A and PLMs C to F). The acylation step is required for potent antifungal activity of the PLMs. Initial analysis of the PLM biosynthetic gene cluster (21) did not reveal a candidate gene or genes whose products might be responsible for this acylation.

Here we identify the $plmS_3$ gene product as the singular acyltransferase in *Streptomyces* sp. strain HK 803 responsible for C-18 acylation of PLM G. Generation of a $plmS_3$ deletion mutant results in selective production of PLM G, supporting the proposed role of this gene product and providing the first direct fermentation method to access this intermediate. The 921-bp $plmS_3$ ORF was cloned and expressed as an N-terminally polyhistidine-tagged protein in *E. coli*, and the recombinant purified protein was shown to catalyze acylation of PLM G with isobutyryl-CoA, 3-methylbutyryl-CoA, and cyclohexylcarbonyl-CoA to give PLM A, PLM C, and PLM E, respectively. This efficient one-step enzymatic process offers an attractive alternative to the multistep synthetic process for selective acylation of PLM G. $PlmS_3$ was also shown to catalyze esterification of PLM G with CoA and NAC thioesters with a remarkably wide range of various saturated, unsaturated, and aromatic carboxylic acids and thus provides an efficient chemoenzymatic route to new PLM analogs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type *Streptomyces* sp. strain HK 803 and its deletion mutants NP8 and NP8/pMSG1′ were maintained on SY agar (1% soluble starch, 0.1% yeast extract, 0.1% N-Z amine type A), and the PLM production was performed as described previously (21). *Escherichia coli* cultures were grown at 37°C in Luria-Bertani medium supplemented with ampicillin or chloramphenicol (50 μg/ml) when necessary (22).

Targeted disruption of $plmS_2$-$plmS_3$. The $plmS_2$-$plmS_3$ genes are adjacent in the PLM biosynthetic gene cluster and were replaced with *aac(3)IV* (the apramycin resistance gene) using a PCR-targeted *Streptomyces* gene replacement method (8). The apramycin cassette for gene disruption from pIJ773 was amplified using the forward primer CCGTGCCGGGCCACCGGCGGATTGGAG AACGCAACCATG**ATTCCGGGGATCCGTCGACC** and reverse primer CCCG GACGACCGGGCCGGTTCAGCGTGCGGGAACGCTCA**TGTAGGCTGGA GCTGCTTC** (the pIJ773-homologous sequence is in bold). The resulting PCR product was used to replace $plmS_2$-$plmS_3$ first in a cosmid clone 3A11 (21) and then in *Streptomyces* sp. strain HK 803 following the established methodologies (8), using SY agar instead of MS agar (11). The allelic replacement of the $plmS_2$-$plmS_3$ genes in the NP8 mutant was confirmed by PCR amplification and sequencing.

Genetic complementation of the NP8 mutant. The recombinant conjugative plasmid pMSG1′ was generated from pMSG1 (Table 1) by recombinant allelic replacement of *aac(3)IV* with the spectinomycin resistance gene, *aadA*. The forward primer GATCGACTGATGTCATCAGCGGTGGAGTGCAATGTCG TG**ATGAGGGAAGCGGTGATCGC** and reverse primer GCCCCTCCAACGTC ATCTCGTTCTCCGCTCATGAGCTCA**TTATTTGCCGACTACCTTG** (pIJ778-homologous sequence is in bold) were used to amplify *aadA* from pIJ778, and the resulting PCR product was used to carry out the replacement using established methodology (9). pMSG1′, conferring spectinomycin resistance, was transferred by conjugation into the NP8 mutant using standard protocols (11).

Cloning of the $plmS_3$ gene. General DNA manipulations were performed following standard protocols (22). The $plmS_3$ ORF was PCR amplified using a forward primer (5′-CATATGGCCGACAGCCTTGCGGCC-3′) introducing a unique NdeI site at the 5′ end of the gene and a reverse primer (5′-CGCTCA GGTCCTAGGTCACCATGCGG-3′) introducing a unique BamHI site downstream from the TGA translational stop codon. DNA of cosmid clone 3A11 from a *Streptomyces* sp. strain HK 803 genomic library was used as a template (21). The 921-bp amplified DNA fragment was first cloned into the pCR2.1 TOPO TA vector (Invitrogen). The 921-bp NdeI-BamHI insert was further subcloned into the pET15b expression vector to generate pMSG4. The ORF was confirmed by DNA sequencing, and pMSG4 was transformed into *E. coli* BL21-Codon Plus(DE3) cells.

Expression and purification of N-terminally hexahistidine-tagged $PlmS_3$. *E. coli* BL21-Codon Plus(DE3)/pMSG4 transformants were inoculated into 30 ml LB medium containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol and were grown at 220 rpm and 37°C overnight. Each overnight culture was added to 600 ml of LB containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol, and the cultures were grown at 220 rpm and 37°C until an optical density at 600 nm of 0.6 was reached. The cultures were transferred to 25°C and were induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 h at 200 rpm. The cells were harvested by centrifugation. Cells from 1,200 ml culture were resuspended in 75 ml lysis buffer (50 mM sodium phosphate monobasic, 300 mM

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Streptomyces strains		
HK 803	Wild type; produces PLMs A through F	Riken, Japan
NP8	$plmS_2$-$plmS_3$ deletion mutant; Am^r; produces only PLM B	This work
NP8/pMSG1'	$plmS_3$ deletion mutant; Am^r Sp^r; produces only PLM G	This work
E. coli strains		
TG2	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) Δ(*srl-recA*)*306*::Tn*10*(Tet^r) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	22
BW 25113/pIJ790	Recombination host; Chl^r	8
ET12567/pUZ8002	Nonmethylating strain harboring nontransmissible pUZ8002 (plasmid capable of mobilizing *oriT*-containing vectors)	8
BL21-Codon Plus(DE3)	*E. coli* expression host; Chl^r	Stratagene
Plasmids		
pET15b	*E. coli* expression vector; Ap^r	Novagen
pMSG1	pGF200 with 0.7-kbp NdeI-BglI fragment replaced by 1.2-kbp NdeI-BamHI *plmS*2; Ap^r	7
pMSG1′	pMSG1 with *aac*(*3*)*IV* replaced by *aadA*; Sp^r	This work
pMSG4	pET15b with 921-bp NdeI-BamHI $plmS_3$ ORF; Ap^r	This work
pIJ773	Contains apramycin cassette for gene disruption; Ap^r Am^r	8
pIJ778	Contains streptomycin and spectinomycin cassette for gene disruption; Sp^r Am^r	8
Cosmid clone 3A11	Supercos 1 (Stratagene) derived; Ap^r Kan^r	21

[a] Ap^r, ampicillin resistance; Am^r, apramycin resistance; Chl^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Sp^r, spectinomycin resistance.

sodium chloride, and 10 mM imidazole, pH 8.0) containing lysozyme (0.5 mg/ml) and incubated on ice for 30 min. The cells were further ruptured by sonication, cell debris were removed by centrifugation, and the soluble fraction was applied to Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) equilibrated with lysis buffer. The column was washed with 75 ml of wash buffer (50 mM sodium phosphate monobasic, 300 mM sodium chloride, and 20 mM imidazole, pH 8.0), and the protein was eluted with buffer containing 300 mM imidazole. Fractions of 500 μl were collected, and an aliquot of 10 μl was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to check for the presence of $PlmS_3$. Desired fractions were pooled and dialyzed using a Slide-A-Lyser dialysis cassette (3,500-molecular-weight cutoff) against 100 mM potassium phosphate buffer, pH 7.0. The protein concentration was determined with the Bio-Rad protein assay dye reagent concentrate, using bovine serum albumin as a standard. Aliquots of protein were stored at −80°C.

PLM production and analysis. Production, purification, and analysis of PLM G and other PLM analogs using high-pressure liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) were carried out as described previously with some modifications (21). The mobile phases A (acetonitrile-water, 20:80) and B (acetonitrile-water, 80:20) both contained 0.05% formic acid, and gradients from 0% B to 60% B over either 40 or 70 min (at a flow rate of 1 ml/min) were used. PLM G was stored as a concentrated solution (5 mM) in methanol.

In vitro acylation of PLM G. Enzymatic conversion of PLM G into various PLM analogs was monitored with an HPLC assay. A standard assay was carried out by incubating the Ni-NTA affinity-purified $PlmS_3$ (40 μg) in 100 mM potassium phosphate buffer (500 μl; pH 7.0) with PLM G (22 μM), acyl-CoA, or various NAC thioesters (500 μM) at 30°C for 1.5 h. The acyl-CoA substrates were purchased from Sigma-Aldrich, whereas NAC thioesters of various carboxylic acids were synthesized as described previously (1). Simultaneous control experiments in the absence of either the enzyme or substrate were conducted. The enzymatic reaction was stopped by addition of 125 μl acetonitrile (20%), and the reaction mixture was filtered. HPLC analysis of an aliquot under conditions previously described (21) allowed the loss of the PLM G peak and the formation of an acylated PLM product peak to be monitored. The mass of the new peak was determined by LC-MS.

For kinetic determination of PLM A synthesis, the Ni-NTA affinity-purified $PlmS_3$ (6.8 μg) was incubated with isobutyryl-CoA (500 μM) and various concentrations of PLM G (from a 4.3 mM stock solution) in 100 mM potassium phosphate buffer (pH 7.0) in a reaction volume of 500 μl at 30°C for 15 min. The reaction rate was linear during this assay, and the peak area of the PLM A determined by HPLC analysis was used to calculate the rates of product formation. One unit of the enzyme activity was defined as the amount of enzyme yielding 1 nmol of product per min under these assay conditions.

Determination of the relative efficiency of the acylation reaction with various acyl thioester substrates (100 μM) was carried out using the Ni-NTA affinity-purified $PlmS_3$ (6.9 μg) with PLM G (22 μM) and acyl-CoA/NAC in 100 mM potassium phosphate buffer (pH 7.0) in a reaction volume of 500 μl at 30°C for 15 min. The samples were analyzed by HPLC as stated above, using the loss of PLM G to determine the percentage of PLM G acylation with each substrate.

Deacylation of PLM A and PLMs C to F. Deacylation of a mixture of natural PLMs by $PlmS_3$ was monitored by HPLC analysis, looking at both loss of substrate and formation of the PLM G product. For kinetic assays, the enzyme (44 μg) was incubated with various concentrations of PLM A in 100 mM potassium phosphate buffer (pH 7.0) in a reaction volume of 500 μl at 30°C for 2 h.

Thioesterase activity of $PlmS_3$. The thioesterase activity of $PlmS_3$ with acyl-CoA thioesters was determined spectrophotometrically by measuring an increase in A_{412}, reflecting formation of 5-thio-2-nitrobenzoate as a result of reaction of the SH group of the released CoA-SH product with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (9). For kinetic assays, the reaction mixture (500 μl) contained 100 mM potassium phosphate buffer (pH 7.0), DTNB (0.5 mM), $PlmS_3$ (14.6 μg), and various concentrations of isobutyryl-CoA. The samples were incubated at 30°C for 30 min, and the absorbance was measured at 412 nm. The nanomoles of CoA-SH product formed were calculated using a standard curve for reaction of various concentrations of CoA-SH with DTNB.

RESULTS

In-frame deletion of $plmS_2$-$plmS_3$ in *Streptomyces* sp. strain HK 803. The $plmS_3$ ORF, adjacent to $plmS_2$ in the *plm* biosynthetic gene cluster, encodes a protein with 25 to 30% amino acid identity to several putative hydrolytic enzymes (proteases, lipases, and esterases) which share a common alpha/beta hydrolase fold. A multiple alignment of $PlmS_3$ and these proteins revealed that it contained the highly conserved serine (Ser106)-aspartate (Asp229)-histidine (His257) catalytic triad associated with these proteins. Only very low amino acid sequence similarity was observed between $PlmS_3$ and other acyltransferases involved in post-polyketide synthase tailoring reactions.

Targeted disruption of the $plmS_2$ gene has previously resulted in an NP1 mutant which produces only PLM B (21). A strain, NP8, in which both the adjacent $plmS_2$ and $plmS_3$ genes were replaced with the apramycin resistance gene was gener-

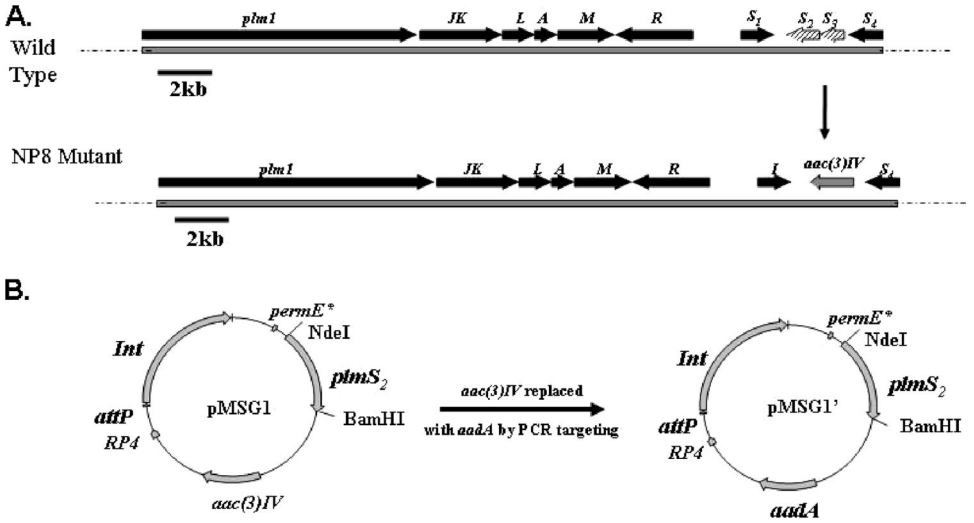


FIG. 2. Genetic strategy for generation of the NP8/pMSG1′ derivative of *Streptomyces* sp. strain HK 803. (A) Replacement of $plmS_2$-$plmS_3$ by *aac(3)IV* using a PCR targeting method. (B) Replacement of *aac(3)IV* in the $plmS_2$ expression plasmid (pMSG1) by *aadA* to generate pMSG1′.

ated from *Streptomyces* sp. strain HK 803. The NP8 mutant, like the NP1 mutant, produced only PLM B, consistent with the proposal that $PlmS_2$ and $PlmS_3$ catalyze the final two steps in PLMs biosynthesis (Fig. 1).

Selective production of PLM G by the NP8/pMSG1′ strain. Genetic complementation of a $plmS_2$ deletion strain, NP2 (an NP1 derivative) by pMSG1 (a recombinant conjugative plasmid expressing the $plmS_2$ gene under the control of the *ermE** promoter) has previously resulted in an NP2/pMSG1 strain in which production of PLM A and PLMs C to F is restored (7). Plasmid pMSG1′ was generated from pMSG1 by facile allelic replacement of *aac(3)IV* by *aadA*, and the resulting spectinomycin resistance-conferring plasmid was transferred into the NP8 derivative strain by conjugation (Fig. 2). HPLC analysis of the fermentation products of the resulting strain, NP8/pMSG1′, revealed neither PLM B nor any acylated PLM products (PLM A and PLMs C to F). Rather, a new peak with a much shorter retention time was observed (Fig. 3). This new product coeluted with a PLM G standard (previously generated by $PlmS_2$-catalyzed hydroxylation of PLM B [7]). LC-MS analysis of the fermentation broth confirmed the expected mass (529 Da) for PLM G, and nuclear magnetic resonance analyses of the purified product were consistent with the PLM G structure. As $plmS_3$ is the only *plm* biosynthetic gene absent in NP8/pMSG1′, the selective production of a PLM G product clearly implicates $PlmS_3$ as an 18-*O*-acyltransferase which catalyzes the final step in the biosynthesis of the PLMs.

Production of PLM G by NP8/pMSG1′ was typically about 6 mg/liter under standard fermentation conditions and was com-

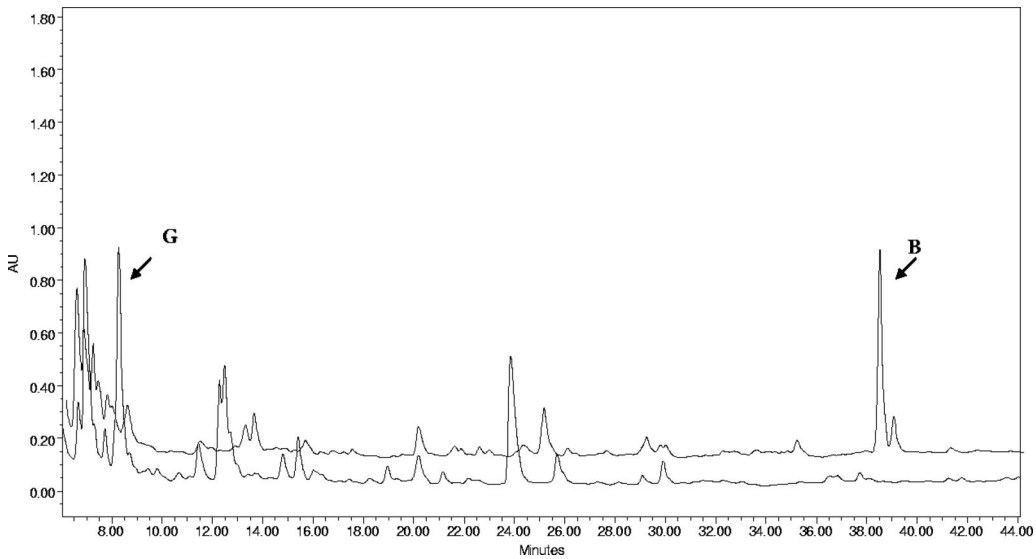


FIG. 3. HPLC analysis demonstrating PLM B production by the NP8 derivative of *Streptomyces* sp. strain HK 803 (top trace) and PLM G production by NP8/pMSG1′ ($plmS_3$ expression plasmid) (bottom trace).

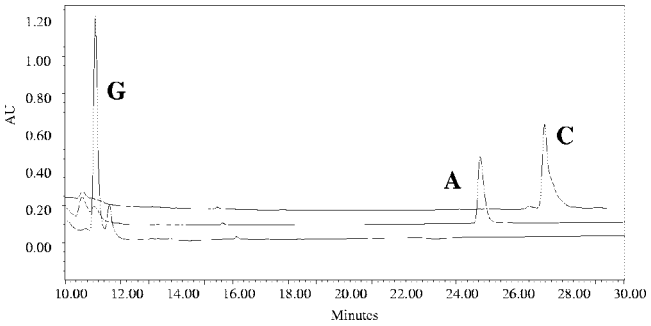


FIG. 4. HPLC chromatogram demonstrating $PlmS_3$-catalyzed esterification of PLM G (bottom trace) to yield PLM A (middle trace) and PLM C using isobutyryl-CoA and isovaleryl-CoA, respectively.

parable to the yields of PLM B produced by NP8 or NP2 mutants. This mutant was used to obtain pure PLM G as a substrate for in vitro acylation assays with $PlmS_3$.

Heterologous expression of $PlmS_3$. The 921-bp *plmS*$_3$ ORF was cloned into prokaryotic expression vector pET15b, and $PlmS_3$ was expressed as a soluble N-terminally polyhistidine-tagged protein in *Escherichia coli* BL21-Codon Plus(DE3) cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of *E. coli* BL21(DE3)/pMSG4 cell extracts grown under IPTG induction conditions revealed the presence of relatively high levels of a 36-kDa protein (consistent with the predicted masses of $PlmS_3$ and the 2-kDa hexahistidine tag). This expressed protein was absent in similarly prepared extracts of a control culture of *E. coli*/pET15b. $PlmS_3$ was purified as a His-tagged protein by affinity chromatography on an Ni-NTA agarose column to more than 50% purity. The typical yields of purified $PlmS_3$ were 1 to 1.5 mg/liter.

In vitro enzymatic conversions by recombinant $PlmS_3$. Commercially available isobutyryl-CoA and isovaleryl-CoA were incubated separately with the purified PLM G and Ni-NTA-purified $PlmS_3$ in a potassium phosphate buffer (pH 7) at 30°C for 1.5 h. An HPLC assay which allowed the conversion of the PLM G substrate to either PLM A or PLM C clearly demonstrated the predicted acyltransferase activity of $PlmS_3$ (Fig. 4). Coinjection of PLM A and PLM C standards and LC-MS analyses further confirmed that the predicted acylated PLMs were generated by $PlmS_3$ incubations. The esterification of PLM G was $PlmS_3$ dependent; no reaction in the absence of the enzyme was observed. The use of HEPES buffer instead of phosphate buffer did not alter the levels of PLM G acylation by $PlmS_3$.

In addition to PLM G acylation activity, the recombinant $PlmS_3$ exhibited the ability to hydrolyze both acyl thioesters and the acylated PLM products (PLM A and PLMs C to F). Prolonged incubation of a mixture of PLMs (isolated from the wild-type *Streptomyces* sp. strain HK 803 strain) with $PlmS_3$ at 30°C resulted in conversion to PLM G (Fig. 5). The mixture also contained PLM B, in which the C-18 hydroxyl is absent, and this was predictably unchanged during the incubation. The recombinant $PlmS_3$ also exhibited acyl-CoA thioesterase activity, releasing the free CoA-SH which was detected using a spectrometric DTNB assay (measuring an increase in A_{412} due to the formation of 5-thio-2-nitrobenzoate). This activity was observed with a range of short branched-chain acyl-CoA thioesters.

Kinetic parameters of the reactions catalyzed by His-tagged-$PlmS_3$. Kinetic parameters for the acyltransferase activities of $PlmS_3$ were established using an HPLC assay to monitor the acylation of PLM G with 500 μM isobutyryl-CoA (generation of PLM A). Kinetic parameters for the deacylation of PLM A by $PlmS_3$ were assayed by HPLC, and those for isobutyryl-CoA hydrolysis were assayed using a DTNB spectrophotometric assay. As shown in Table 2 the PLM G acylation reaction (apparent k_{cat} of 28 min^{-1}) is significantly faster than the deacylation of the resulting PLM A product (apparent k_{cat} of 0.5 min^{-1}). K_m values for PLM G (acylation) and PLM

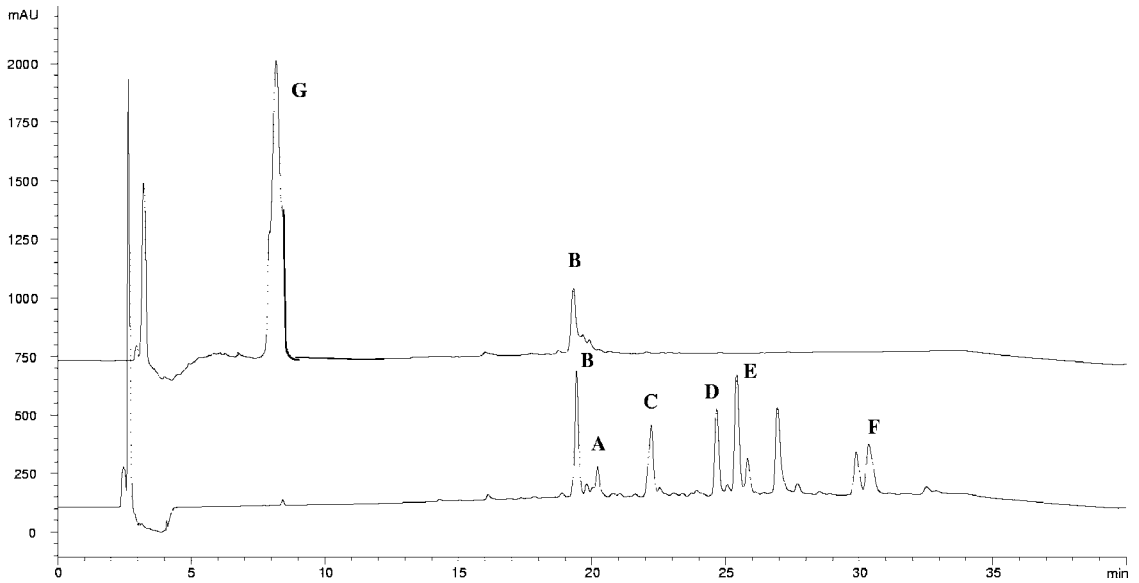


FIG. 5. HPLC assay demonstrating $PlmS_3$-catalyzed generation of PLM G (top trace) by hydrolysis of a mixture of acylated PLMs (bottom trace). PLM B, which does not contain an acylated C-18-hydroxyl substituent, is present at the beginning and end of the incubation.

TABLE 2. Apparent kinetic parameters of the catalytic activities exhibited by $PlmS_3$

Catalytic activity	Substrate	Mean ± SD		
		K_m(μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM min^{-1})
Acyltransferase	PLM G	88 ± 16	28 ± 3	0.32 ± 0.04
Deacylation	PLM A	92 ± 10	0.5 ± 0.05	0.005 ± 0.0005
Thioesterase	Isobutyryl-CoA	320 ± 50	3 ± 0.3	0.01 ± 0.014

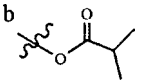
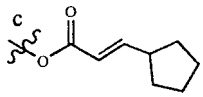
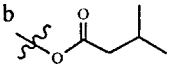
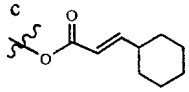
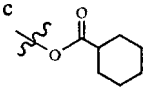
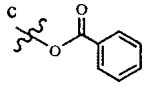
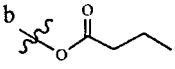
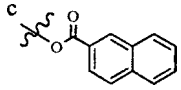
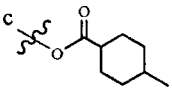
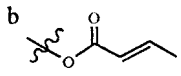
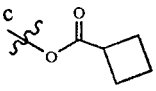
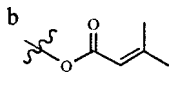
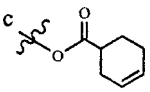
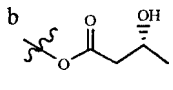
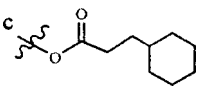
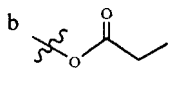
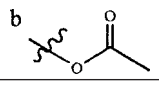
A (hydrolysis) were very similar. The rate of isobutyryl-CoA hydrolysis by $PlmS_3$ was also low (apparent k_{cat} of 3 min^{-1}) relative to the acyl transfer reaction.

Acyl side chain specificity and chemoenzymatic synthesis of new PLM analogs. The absence of a single gene, *plmS*$_3$, led to a mutant that was blocked in formation of all acylated PLMs. This observation and other properties of the recombinant enzyme (slow hydrolysis of all natural acylated PLMs) were consistent with a biosynthetic role of this enzyme in catalyzing acylation of PLM G with a range of acyl-CoA thioesters. This role indicates promiscuity with regard to the acyl thioester substrate, and we tested the extent of this using CoA-SH and NAC thioesters. As shown in Table 3, $PlmS_3$ has a preference for generating PLM products with short, branched and aromatic side chains at C-18. Under a given set of conditions, comparable levels of new and known PLMs were generated using branched and cyclic acyl thioesters (Table 3, left). The use of aromatic, unsaturated, or even hydroxylated substrates was also possible (Table 3, right), even though the amounts of these PLM products were less under these same conditions. The use of more $PlmS_3$ or prolonged incubation allowed us to increase the conversion rate and obtain good quantities of these new PLM products. Two products with identical *m*/*z* values were observed using DL-3-hydroxybutyryl-CoAs, indicating that both enantiomers were used as substrates by $PlmS_3$, giving rise to two diastereomeric products. Long-chain acyl-CoAs were not substrates, indicating that strains which produce LSNs with longer acyl side chains must have a $PlmS_3$ homolog with different substrate specificity. Thioesters of heterocyclic carboxylic acids were also not substrates for $PlmS_3$.

DISCUSSION

The accumulation of PLM G by the NP8/pMSG1′ strain, together with the observation of $PlmS_3$-catalyzed acylation of PLM G using various acyl thioester substrates, has provided unequivocal evidence that this enzyme alone is the 18-*O*-acyltransferase proposed to catalyze the final step in the PLM

TABLE 3. Generation of 14 novel PLM compounds and 3 known PLM compounds by $PlmS_3$-catalyzed acylation of PLM G

PLM side chain	Mass of new peak $[M + H]^+$	% Acylation of PLM G[a]	PLM side chain	Mass of new peak $[M + H]^+$	% Acylation of PLM G
	600.13	83.8		652.13	63.3
	614.13	84.3		666.20	53.9
	640.20	95.3		634.13	35.6
	600.20	80.2		684.20	37.2
	670.20	91.6		598.13	24
	612.27	88.2		612.13	4.6
	638.13	77.7		616.07	11.4
	668.33	79.0		586.07	30.4
				572.07	1.5

[a] Calculated by determining loss of PLM G peak relative to control over a standard 500-ml assay mixture ($PlmS_3$ [6.8 μg], PLM G [22 μM], or acyl-CoA [b] or NAC derivative [c] [100 μM] at 30°C for 15 min in 100 mM potassium phosphate buffer at pH 7.0).

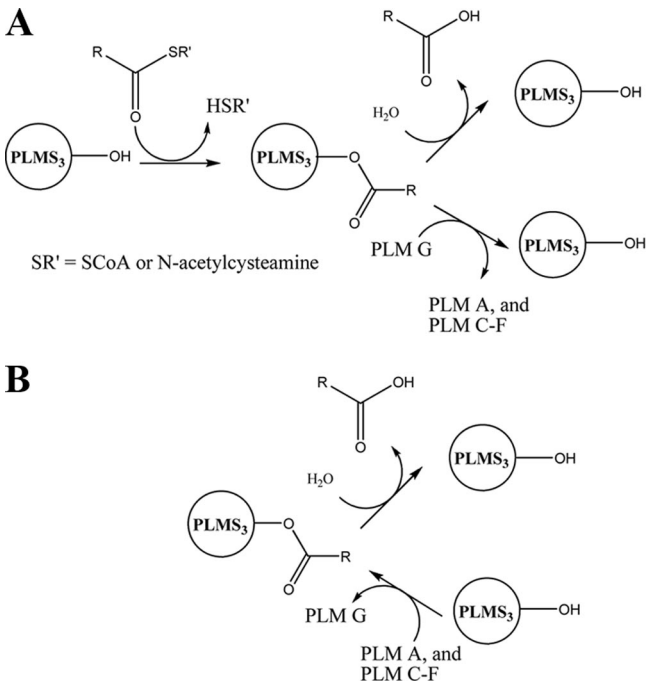


FIG. 6. Proposed role for a common acyl-enzyme intermediate in the reactions catalyzed by $PlmS_3$.

biosynthetic pathway. The yields of PLM G produced by this mutant are comparable to those for PLM B production by the NP8 mutant (5 to 10 mg/liter), and thus deletion of *plmS*$_3$ in PLM-producing strains provides for a direct and effective fermentation route to access this PLM intermediate. Previously there have been only less-direct routes to PLM G, i.e., enzymatic hydroxylation of purified PLM B using the recombinant $PlmS_2$ P450 monooxygenase (7) or enzyme-catalyzed deacylation of a mixture of acylated PLMs/LSNs using pig liver esterase (24).

$PlmS_3$ was most efficient in the ability to catalyze acylation of PLM G with the proposed physiological substrates, such as isobutyryl-CoA. Nonetheless, it also can catalyze hydrolysis of either acyl-CoA thioesters or a mixture of PLM A and PLMs C to F (providing PLM G). The predicted amino acid sequence revealed homology to the alpha/beta hydrolase fold superfamily of enzymes and the canonical catalytic triad associated with this group of enzymes. We envision that the acylation reaction proceeds via a ping-pong mechanism (Fig. 6A) in which the first step is formation of an acyl-enzyme intermediate using the conserved nucleophilic serine and the appropriate acyl-CoA substrate (releasing CoA as the first product). Binding of the second substrate, PLM G, then permits the acyl transfer generating the final PLM product and restoration of $PlmS_3$ to its initial state. LovD, which catalyzes acylation of monacolin J to generate lovastatin, has also been proposed to proceed via such a ping-pong mechanism, and this has been supported by mutational and kinetic studies (30). In the case of $PlmS_3$, the slow acyl-CoA hydrolysis reaction presumably involves the same initial formation of the acyl-enzyme intermediate. In this case the acyl group is released by reaction with water rather than the C-18 hydroxyl group of PLM G (Fig. 6A). The reversibility of the $PlmS_3$-catalyzed reaction also means that the same hydrolytically susceptible acyl-enzyme intermediate can be generated by reacting an acylated PLM with PLM S_3 (Fig. 6B), leading to the observed $PlmS_3$-catalyzed deacylation (generation of PLM G) of PLM A and PLMs C to F. The hydrolytic activity of $PlmS_3$ was significantly smaller than the acyltransferase activity, suggesting that in the active site PLM G can position more optimally than water for reaction with the acyl-enzyme intermediate. $PlmS_3$ represents an alternate to pig liver esterase (24) for catalyzing formation of PLM G by hydrolysis of acylated PLMs.

The enzyme $PlmS_3$ has significant promiscuity with respect to the acyl group that can be transferred to PLM G, suggesting a rather nonspecific acyl binding pocket in the enzyme. The ability of $PlmS_3$ to transfer this acyl group to an alcohol, other than that at C-18 of PLM G, has not been evaluated due to a lack of appropriate PLM G analogs. *Streptomyces* sp. strain HK 803 and *Streptomyces nigrescens* SC273 have both been shown to produce five different acylated PLM products (4, 23). The structures of these suggest isobutyryl-CoA (PLM A), 3-methylbutyryl-CoA (PLM C), 4-methylbutyryl-CoA (PLM D), cyclohexylcarbonyl-CoA (PLM E), and 4-methylhexanoyl-CoA (PLM F) as the $PlmS_3$ substrates. These acyl-CoAs are presumably provided by primary metabolic processes such as branched-chain amino acid catabolism and fatty acid biosynthesis/degradation. The exception is the cyclohexylcarbonyl-CoA which is generated by the PLM biosynthetic process and used to both initiate biosynthesis of the PLM core structure and convert PLM G to PLM E (21, 23). The demonstrated ability of the recombinant $PlmS_3$ to preferentially use branched-chain acyl-CoA substrates is consistent with the range of PLM products generated in a fermentation. It is predicted that the ratio of these products is determined by the relative pools of acyl-CoA precursors and the substrate specificity of $PlmS_3$. Furthermore, there may be lower levels of other naturally occurring acylated PLM products which have not yet been identified from these strains. These PLMs may be generated by $PlmS_3$-catalyzed acylation of PLM G with other branched-chain acyl-CoAs or straight-chain acyl-CoA substrates (butyryl-CoA and crotonyl-CoA are both known primary metabolites [16] and substrates for this enzyme).

The promiscuity of $PlmS_3$ toward acyl-CoA substrates and the resulting mixture of fermentation products create issues with generating adequate amounts of a specific acylated product. For instance, LSN B (PLM G acylated with a 6-methyloctanoic acid) has a number of interesting and potentially useful biological activities (17, 24, 25). The natural product is produced as a mixture of LSNs in *Streptomyces platensis* SANK 60191 and could be obtained only at low levels (<10 mg) by large-scale fermentation (60 liters) and considerable purification efforts. An alternative approach has been to isolate the mixture of LSNs, convert them to PLM G using an enzymatic hydrolysis, and then carry out chemical esterification of the C-18 by the Yamaguchi method (17). This 11-step process occurs in less than 2% overall yield and requires temporary removal of the C-9 phosphate monoester (by enzymatic hydrolysis) and a series of protection and deprotection steps with the other alcohol groups of PLM G. A one-step, potentially quantitative enzymatically catalyzed acylation of PLM G (using $PlmS_3$ or the presumed homolog from the LSN B producer *S.*

platensis) represents an attractive chemoenzymatic approach to generating specific acylated known PLMs.

Finally, $PlmS_3$ offers a facile route to access new acylated PLMs. To date, all biological studies have focused on the known naturally occurring C-18-acylated PLMs. An initial analyses demonstrated comparable protein phosphatase 2A-inhibitory activity (29) and antifungal activity (5). However, we have shown recently that the acyl group is required for PLM antifungal activity against *Rhodotorula glutinis* and, in this case at least, plays an important role (7). $PlmS_3$ can catalyze the attachment of a broader range of acyl substrates (including both aromatic and hydroxycarboxylic acids) than those found in the isolated PLMs. This enzymatic esterification, alongside established chemical methods for N acylation of PLM G (24) and other methods to alter the product of the PLM biosynthetic process, now offers new possibilities to generate libraries of PLM analogs which can be tested and evaluated for a range of biological activities and potential pharmaceutical or agricultural applications.

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