



Platensimycin and Platencin Biosynthesis in *Streptomyces platensis*, Showcasing Discovery and Characterization of Novel Bacterial Diterpene Synthases

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Abstract

Diterpenoid natural products cover a vast chemical diversity and include many medicinally and industrially relevant compounds. All diterpenoids derive from a common substrate, (*E,E,E*)-geranylgeranyl diphosphate, which is cyclized into one of many scaffolds by a diterpene synthase (DTS). While diterpene biosynthesis has been extensively studied in plants and fungi, bacteria are now recognized for their production of unique diterpenoids and are likely to harbor an underexplored reservoir of new DTSs. Bacterial diterpenoid biosynthesis can be exploited for the discovery of new natural products, a better mechanistic understanding of DTSs, and the rational engineering of whole metabolic pathways. This chapter describes methods and protocols for identification and characterization of bacterial DTSs, based on our recent work with the DTSs involved in platensimycin and platencin biosynthesis.



1. INTRODUCTION

Terpenoids are ubiquitous compounds that play key metabolic roles in all forms of life. They are commonly the products of secondary metabolism in a variety of organisms and ultimately, comprise the largest, most structurally diverse family of natural products, with more than 60,000 known members. Terpenoids are defined by their biogenesis from five-carbon isoprene units and can be further categorized into classes according to the number of isoprene units forming their parent terpene scaffolds: hemiterpenoids (1 unit, C_5), monoterpenoids (2 units, C_{10}), sesquiterpenoids (3 units, C_{15}), diterpenoids (4 units, C_{20}), sesterterpenoids (5 units, C_{25}), and triterpenoids (6 units, C_{30}). The great potential for enzymatic derivatization of the parent scaffolds allows for the enormous, natural diversity of the terpenoid family of natural products.

Diterpenoid natural products include many medicinally and agriculturally relevant compounds that are of significant economic interest. A majority of the known diterpenoid compounds are produced in plants and fungi and much of the current knowledge on their biosynthesis has come from studies in these organisms (Bohlmann, Meyer-Gauen, & Croteau, 1998; Christianson, 2006, 2008; Peters, 2010; Tudzynski, 2005). Diterpenoid biosynthesis involves many of the common steps characteristic of terpene biosynthesis, including precursor generation via the mevalonate or methylerythritol phosphate pathways and subsequent oligomerization of isoprene monomers to long-chain polyprenyl diphosphates. Cyclization of the 20-carbon intermediate, (*E,E,E*) geranylgeranyl diphosphate (GGDP), by a diterpene synthase (DTS) is the critical step for generating diterpenoid structural diversity. This single step converts GGDP, a linear, achiral substrate, to one of many unique diterpene scaffolds with multiple chiral centers (Christianson, 2006; Peters, 2010). Terpene synthases (TSs), DTSs included, can be specific, catalyzing multistep cyclization reactions with a single regiochemical and stereochemical outcome (Felicetti & Cane, 2004), or they can be highly promiscuous, producing as many as 50 products from a single substrate (Steele, Crock, Bohlman, & Croteau, 1998). The ability to alter product profiles of TSs by substituting only a few amino acids has led to a significant interest in exploiting these enzymes for combinatorial biosynthesis (Greenhagen, O'Maille, Noel, & Chappell, 2006; O'Maille et al., 2008; Yoshikuni, Ferrin, & Keasling, 2006).

Classification of DTSs, like other TSs, groups enzymes according to the mechanism by which they initiate scaffold cyclization. Type I DTSs generate highly reactive carbocation intermediates via a heterolytic cleavage of the carbon—oxygen bond on a polyprenyl diphosphate, yielding inorganic pyrophosphate as a side product. Type II DTSs leave the carbon—oxygen bond intact and instead initiate the cyclization reaction via protonation of an olefin or epoxide ring. In both cases, side-chain residues in the active-site cavity guide the folding of the carbon scaffold and stabilize carbocation intermediates using steric and electrostatic forces (Christianson, 2006). The cyclization cascade ends when the carbocation is quenched, either through abstraction of a proton or by electrophilic attack by water. Because type II DTSs do not cleave the carbon—oxygen bond and thus yield terpene diphosphates, their products can serve as substrates of type I DTSs for further transformations. The biosynthesis of diterpenoids differs from that of smaller terpenoids in part by the comparatively high frequency of such two-step cyclizations.

Terpenoid biosynthesis plays a prominent role in the secondary metabolism of plants and some fungi, where it has been extensively studied during the past 50 years (Bohlmann et al., 1998; Christianson, 2006, 2008; Peters, 2010; Tudzynski, 2005). The past decade has seen a substantial increase in the number of characterized DTSs from bacteria (Fig. 8.1) (Smanski, Peterson, Huang, & Shen, 2012). Bacteria are now recognized for their substantial diterpenoid production and are likely to harbor a reservoir of as yet undiscovered DTSs; their downstream natural products can be exploited for drug discovery efforts (Dairi, 2005; Daum, Herrmann, Wilkinson, & Bechthold, 2009; Smanski et al., 2012). There are several advantages of studying diterpenoid biosynthesis in bacteria including: (i) the technical feasibility of working with bacterial enzymes facilitates mechanistic and structural studies, (ii) the presence of noncanonical catalytic motifs in bacterial DTSs promises to expand our understanding of the mechanistic requirements, and (iii) the opportunity to engineer whole biochemical pathways for the production of complex diterpenoid natural products.

A number of strategies have been implemented in discovering new bacterial DTSs. While many natural product biosynthetic genes can be readily mined by PCR or genome-gazing, the low primary sequence conservation makes these approaches more difficult for bacterial DTSs (Smanski et al., 2012). Unlike in plants, where the terpene biosynthesis genes can be

Our current efforts to characterize platensimycin (PTM) and platencin (PTN) biosynthesis can serve as an example to discover new bacterial DTSs (Fig. 8.2; Smanski, Peterson, Rajski, & Shen, 2009; Smanski et al., 2011; Yu et al., 2010). PTM and PTN contain unique carbon scaffolds that can be traced back through stable-isotope feeding studies to a likely origin from *ent*-kaurene or *ent*-atiserene, respectively (Herath, Attygalle, & Singh, 2007, 2008; Wang et al., 2006, 2007). PTM and PTN gene clusters have been identified and cloned from multiple organisms and the DTSs have been characterized both *in vivo* and *in vitro* (Smanski et al., 2011). This work has helped to expand the sequence diversity associated with bacterial type I DTSs and has led to the identification of additional putative DTSs from published sequence databases. Comparing PTM and PTN biosynthetic gene clusters from different strains provides a snapshot into the natural evolution of new natural products, and preliminary efforts toward pathway engineering through the application of new DTSs have been successful (Smanski et al., 2011).

This chapter describes methods and protocols for *in vivo* and *in vitro* characterization of DTSs as exemplified by the *ent*-copalyl diphosphate (*ent*-CDP) synthases, PtmT2 and PtnT2, *ent*-kauran-16-ol synthase, PtmT3, and *ent*-atiserene synthases, PtmT1 and PtnT1, from the PTM and PTN biosynthetic machineries (Fig. 8.2). Included are methods and protocols useful for examining DTSs in their native host, in heterologous hosts, as well as *in vitro*. The specific methods described are suitable for the discovery and

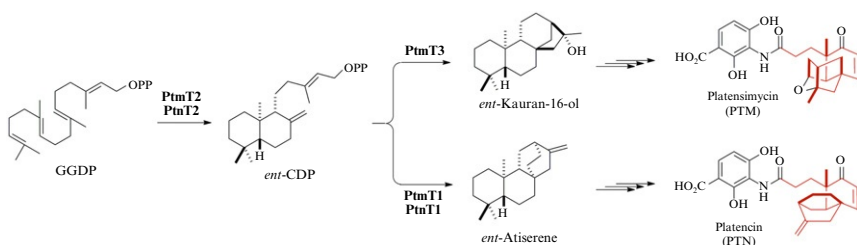


Figure 8.2 PTM and PTN biosynthesis in *S. platensis* MA7327 and MA7339 features a common biosynthetic pathway that diverges at the stage of diterpene cyclization, catalyzed by novel bacterial DTSs. Both MA7327 and MA7339 strains harbor the type II DTSs, PtmT2 and PtnT2 (*ent*-CDP synthases). MA7327 harbors two type I DTSs, PtmT3 (*ent*-kauran-16-ol synthase) and PtmT1 (*ent*-atiserene synthase), hence it is a PTM and PTN dual producer, while the MA7339 strain contains only one type I DTS, PtnT1 (*ent*-atiserene synthase), hence it is a PTN-specific producer (Smanski et al., 2011). The diterpene moieties of PTM and PTN are highlighted in red.

characterization of new DTSs from members of the genus *Streptomyces*, but can be applied to other prokaryotic phyla as well.



2. METHODS

2.1. *In vivo* confirmation of PtmT1 and PtmT3 as DTSs in PTM and PTN biosynthesis

In vivo manipulation of natural product-producing bacteria—constructing single-gene knockout mutants and determining how inactivation of various genes alters the production profile—has proven to be a valuable tool for determining the identities and roles of gene products within a biosynthetic pathway, including candidate DTSs. In the case of the PTM and PTN biosynthetic pathways from strains of *Streptomyces platensis* (Smanski et al., 2011; Wang et al., 2006, 2007), these *in vivo* methods contributed to the identification and functional assignments of two DTSs: PtmT3, responsible for an *ent*-kauran-16-ol intermediate in PTM production, and PtmT1 and PtnT1, responsible for an *ent*-atiserene intermediate in PTN production (Fig. 8.2). Following is a set of methods and protocols for identifying candidate bacterial DTSs and characterizing them in the native host.

2.1.1 Bioinformatic analyses to identify candidate DTSs

1. Identify candidate genes in a genomic region of interest using available open reading frame finders, including, *ORF Finder* from NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) or *StarORF* (<http://web.mit.edu/star/orf/>), or preferably, by creating a codon preference plot as described (Gribskov, Devereux, & Burgess, 1984).
2. Search for characterized homologues in public sequence databases using BLAST (<http://blast.ncbi.nlm.nih.gov/>). The Entrez Query feature is beneficial for narrowing the search results to include enzymes that have been validated with genetic or biochemical evidence. Detailed instructions on how to best use this feature are available online (http://www.ncbi.nlm.nih.gov/BLAST/blastcgitelp.shtml#entrez_query), and time spent learning to narrow BLAST search results effectively is well worth the effort.
3. Construct primary sequence alignment to aid in the identification of metal-binding active-site motifs. Freely available software packages or online tools, such as *BioEdit* (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and the *Biology Workbench* from the San Diego Supercomputer Center (<http://workbench.sdsc.edu/>), enable the facile construction of

ClustalW alignments from user-defined sequences. For bacterial type I DTSs, the canonical “D(D/E)xxD” metal-binding motif is typically found between residues 75 and 115 and the “NDX₂(S/T/G)X₃(E/D)” motif between residues 200 and 220. For bacterial type II DTSs, look for the canonical “DxDD” motif between residues 280 and 320. These active-site motifs are useful to guide annotations, but they are not present in all characterized bacterial DTS.

4. Assigning a particular chemical reaction to an identified DTS is difficult. If the diterpenoid is a submoiety of a larger natural product, functional predictions of neighboring genes with 20–30 kb of the DTS will provide additional clues to help assign a discrete chemical transformation.

2.1.2 Construction of a gene replacement or deletion on an isolated cosmid

The following steps describe the λ -RED-mediated PCR-targeted mutagenesis strategy for gene replacement (Gust, Challis, Fowler, Kieser, & Chater, 2003). For the following methods, it will be assumed that the genomic region containing the DTS has been isolated in a SuperCos 1-derived cosmid (Stratagene), and that the DTS is being replaced with an apramycin resistance cassette, *aac(3)IV*, although other vectors and resistance makers are available. These technologies have greatly improved the speed and efficiency of dissecting *Streptomyces* secondary metabolism *in vivo*.

1. Design primers to amplify the antibiotic resistance cassette of choice that will replace the putative DTS. It is not important to replace the entire coding sequence, and in fact leaving the natural sequence at the 5' and 3' ends of the gene can help ensure normal expression of neighboring genes, which may require overlapping regulatory regions. Select the 39-bp homologous regions encoded in the oligonucleotide primers to be in-frame so that a markerless deletion construct can be made if necessary.
2. Introduce the DTS-containing cosmid into *Escherichia coli* BW25113/pIJ790 for λ -RED-mediated recombination. Note that this strain must be grown at 30 °C to maintain the plasmid. Plasmids are most efficiently introduced to this strain via electroporation.
3. Amplify the antibiotic resistance cassette from digested plasmid, pIJ773 or equivalent, by PCR, and purify the product by gel electrophoresis.
4. Introduce the PCR product by electroporation into *E. coli* BW25113/pIJ790 containing your cosmid, and select for resulting colonies with chloramphenicol, kanamycin, and apramycin resistance.

5. If markerless mutations are desired, proceed with steps from the λ -RED-mediated recombination protocol for removing the resistance marker by passing the modified cosmid through *E. coli* DH5 α /BT340, which expresses FLP-recombinase at 42 °C. Note that screening for a markerless *in vivo* mutation event is slightly more cumbersome than for an antibiotic gene replacement.

2.1.3 Replacement of the DTS gene in vivo with the antibiotic resistance cassette

1. Identify a method for introducing DNA to your strain of choice. For species in the genus *Streptomyces*, commonly used techniques include protoplast transformation, intergeneric conjugation, and mycelial electroporation (Kieser, Bibb, Buttner, Chater, & Hopwood, 2000). Intergeneric conjugation with the methylation deficient *E. coli* ET12567/pUZ8002 has been particularly robust for genetic manipulation of new strains in our laboratory, and this technique will be described in more detail as follows.
2. Purify the modified cosmid from *E. coli* BW25113/pIJ790 (see Section 2.1.2), and transfer it into the donor strain *E. coli* ET12567/pUZ8002. Because of the size of this construct, transformation efficiencies tend to be quite low, and electroporation is recommended. Grow the resultant *E. coli* donor strain at 37 °C overnight.
3. On the day of the gene replacement, inoculate 50 mL of LB, supplemented with 20 mM MgCl₂ and the required antibiotics, with 500 μ L of an overnight culture of the donor *E. coli* strain in step 2, and grow at 37 °C until OD₆₀₀ reaches 0.6.
4. For each gene replacement, quickly thaw a glycerol stock of $\sim 10^8$ spores isolated from your strain of interest. Pellet the spores in a microcentrifuge at low speed for 10 min, and resuspend the spores in 500 μ L of modified TSB medium (30 g/L tryptic soy broth, 100 g/L sucrose, 4 g/L glycine). Heat-shock the spores for 10 min at 50 °C, and let them recover at 28 °C for 2–3 h.
5. Upon the donor *E. coli* strain reaching OD₆₀₀ of 0.6 (3–4 h) (step 3), pellet the cells by centrifugation at high speed for 10 min, wash them twice with antibiotic-free LB supplemented with 20 mM MgCl₂, and resuspend the cells in 300 μ L of LB supplemented with 20 mM MgCl₂.
6. Pellet the heat-shocked recipient spores (step 4) by centrifugation at low speed for 10 min, and resuspend them gently with the 300 μ L suspension of donor *E. coli* (step 5).

7. Spread volumes of 10 μL , 50 μL , 100 μL , 150 μL of this suspension on a series of IWL-4 plates (37 g/L ISP4, 0.5 g/L yeast extract, 1 g/L tryptone) supplemented with 20 mM MgCl_2 (Liu and Shen, 2000). Better results are typically attained if these plates are made fresh on the morning of the experiment and allowed to dry in a sterile hood for 15 min with no lids. Allow spread plates to dry evenly before incubating at 30 °C overnight.
8. The following morning, dilute antibiotics into sterile water and soak 1 mL of the antibiotic dilution into each plate. The final antibiotic concentration will be 25 $\mu\text{g}/\text{mL}$ nalidixic acid (to select against the donor *E. coli*) and a second antibiotic to select for the particular resistance marker used for cosmid mutagenesis. Periodically rock or spread antibiotic solution during drying to ensure even coverage of the plate.
9. Incubate the plates for 5–7 days at 30 °C until small colonies of the mutant strain can be seen growing through the lawn of arrested growth.
10. Pick colonies and restreak on fresh plates with appropriate antibiotics.
11. For gene replacements, screen for double crossovers by replica plating resistant clones onto IWL-4 with 50 $\mu\text{g}/\text{mL}$ apramycin (assuming the apramycin resistance cassette replaced the DTS of interest) and with 50 $\mu\text{g}/\text{mL}$ apramycin and 50 $\mu\text{g}/\text{mL}$ kanamycin. Doubly resistant strains are single crossovers, while the loss of kanamycin resistance signifies that the gene of interest has been completely replaced. Figure 8.3A depicts the construction and selection of the ΔptmT3 mutant strain of *S. platensis* SB12008.

2.1.4 Southern analysis to verify the mutant strain genotype

While various PCR strategies can be used to quickly estimate the success of a gene replacement, the standard in the field is to confirm genetic mutations by Southern analysis.

1. Restriction-map the wild-type and mutant DNA sequences to find an appropriate single or double restriction digestion that is expected to yield unique banding patterns between the two samples. Finding a digest that yields unique fragments in the 2–5 kb range typically gives clean results.
2. Select a hybridization probe that should anneal to the uniquely sized digest fragments in both the wild-type and mutant genome. Probes in the 500–800 bp range are optimal, and care should be taken to design a probe that is unlikely to hybridize to multiple sites in the genome. For this reason, avoid probes that anneal completely within a gene expected to have multiple paralogues in the genome. An effective

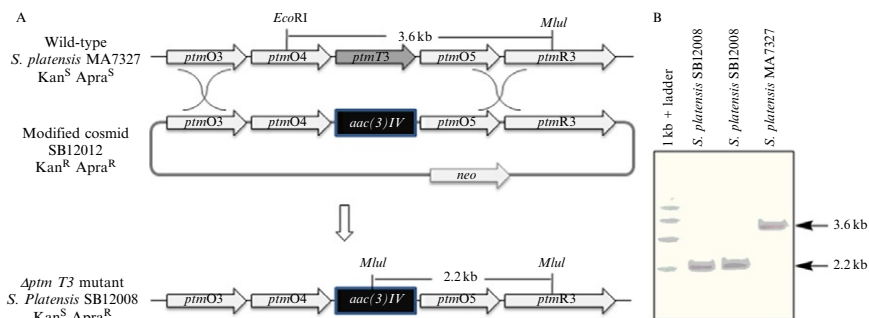


Figure 8.3 Genetic map and Southern analysis verifying the *in vivo* replacement of DTS gene *ptmT3* with the apramycin resistance (*aac(3)/IV*) cassette (Smsanski et al., 2011). (A) A mutated cosmid SB12012, generated by λ -RED-mediated PCR targeting, is introduced into the wild-type PTM and PTN dual producer *S. platensis* MA7327, and homologous recombination is selected for apramycin resistance to isolate the $\Delta ptmT3$ mutant of *S. platensis* SB12008. (B) Gene replacement is verified by digesting genomic DNA, isolated from the wild-type MA7327 and the $\Delta ptmT3$ mutants SB12008, with *EcoRI* and *MluI* and probing with a DNA fragment that anneals to the *ptmO5*–*ptmR3* junction.

strategy for good probe specificity is to select a sequence that spans between two neighboring genes and includes the intergenic sequence.

3. Amplify the probe sequence by PCR, and purify the resulting product by gel electrophoresis. Prepare the nonradioactive hybridization probe by labeling the PCR product with digoxigenin-dUTP using commercial kits such the DIG DNA Labeling Mix from Roche Diagnostics (Mannheim, Germany). Precipitate the labeled probe with LiCl, and store in TE buffer at -20°C . At the same time, a ladder probe should be made with the DNA ladder you will be using during gel electrophoreses.
4. Isolate genomic DNA from mutant and wild-type strains by standard techniques (Sambrook and Russel, 2001), and quantify by gel electrophoresis or spectroscopy to ensure that approximately equal amounts are used for digestion and Southern hybridization.
5. Completely digest 2 μg of chromosomal DNA in a 20- μL reaction using the restriction enzymes selected during step 2.
6. Electrophorese the DNA in a 0.8% agarose gel. Only include 1/10 the amount of DNA ladder typically needed to visualize the gel to avoid overexposure of the DNA ladder compared to the targeted genomic bands during visualization of the digoxigenin-labeled fragments.
7. Complete the Southern analysis as described in the manufacturer's protocols. Be sure that both the specific probe and the DNA ladder are

included in the hybridization solution. Used hybridization solution can be stored at -20°C and reused multiple times. Adjusting the time and temperature of the hybridization and wash steps can minimize non-specific binding of your probe. [Figure 8.3B](#) depicts a typical Southern analysis confirming the genotype of the $\Delta ptmT3$ mutant strain *S. platensis* SB12008 constructed in [Section 2.1.3](#).

2.1.5 HPLC analysis to determine the mutant strain chemotype

The following specific fermentation and analytical procedures are useful for characterizing DTS mutants in PTM and PTN biosynthetic pathways ([Fig. 8.2](#)). While the general methods are applicable to a wide range of producing strains or target molecules, the specific growth media or isolation and analytical protocols should be tailored to your molecule of interest.

1. Culture colonies of wild-type and mutant strains that were confirmed by Southern analysis in [Section 2.1.4](#) in R2YE liquid growth medium ([Kieser et al., 2000](#)) for 2–3 days until dense cultures are obtained. While antibiotic selection can be used for this growth period, it should be avoided during subsequent seed cultivation and fermentation, as the presence of antibiotics in the production medium may have unintended effects on secondary metabolite profile.
2. Prepare seed cultures by inoculating 50 mL of ISM-3 medium ([Smanski et al., 2009](#)) with 500 μL of the R2YE culture in step 1. Include sterile glass beads in the seed culture to aid in the dispersal of mycelial clumps if necessary. Incubate the culture at 30°C and 250 rpm for 40 h.
3. Prepare fermentation cultures by inoculating 50 mL PTNM medium ([Yu et al., 2010](#)) with 500 μL of the seed cultures. Supplement each 50-mL flask with 1.5 g of Amberlite XAD-16 resin to improve and facilitate PTM and PTN production and isolation. Incubate the culture at 30°C and 250 rpm for 10 days. Place unstoppered flasks containing H_2O in the shaker to help maintain high humidity and minimize losses due to evaporation during long fermentations.
4. Harvest cells and resin by centrifugation at high speed for 30 min, discard the supernatant, and wash cell/resin pellet twice with H_2O .
5. Extract the cell/resin pellet with 5 mL of acetone four times, combine the acetone extracts, concentrate in vacuum, and resuspend the residue in 1.5 mL of methanol.
6. Pellet any particulate debris in the methanol sample from step 5 by centrifugation at high speed for 10 min prior to HPLC analysis. Subject

50 μ L of the sample to HPLC analysis on an Apollo C18 column (5 μ m; 4.6 $\times$ 250 mm; Grace Davison Discovery Sciences, Deerfield, IL) with photodiode array detector. Elute the column at a flow rate of 1 mL/min with a 20-min gradient from 15% acetonitrile to 90% acetonitrile in 0.1% formic acid, followed by an additional 5 min at 90% acetonitrile in 0.1% formic acid. Figure 8.4 represents typical HPLC chromatograms of PTM and PTN profiles from various wild-type and mutant *S. platensis* strains.

7. Compare the HPLC chromatograms between the wild-type and mutant strains, with authentic PTM and PTN as references, to determine the chemical phenotype. As depicted in Fig. 8.4, the $\Delta ptmT3$ mutation in *S. platensis* SB12008 completely abolished PTM production while leaving PTN production unperturbed, leading to the assignment of PtmT3 as an *ent*-kauran-16-ol synthase. Conversely, the $\Delta ptmT1$ mutation in *S. platensis* SB12007 completely abolished PTN production without effecting PTM levels, identifying PtmT1 as an *ent*-atiserene synthase (Smanski et al., 2011).

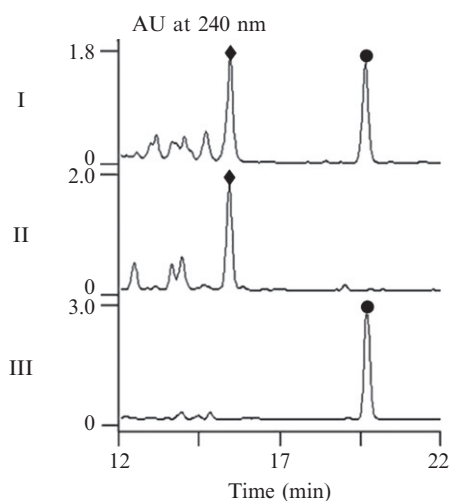


Figure 8.4 HPLC analysis following *in vivo* mutagenesis of DTSs involved in PTM and PTN biosynthesis in *S. platensis* wild-type and mutant strains. In fermentation conditions that lead to the production of both PTM and PTN in the wild-type strain MA7327 (I), the $\Delta ptmT1$ mutant SB12007 produces only PTM (II) and the $\Delta ptmT3$ mutant SB12008 produces only PTN (III). PTM (♦) and PTN (●).

2.2. *In vivo* confirmation of *ptmT3* encoding a DTS by heterologous expression

Heterologous expression is a well-proven method for confirming bioinformatic predictions of gene function. Conferring a new function to a heterologous host through the incorporation of nonnative DNA complements the *in vivo* mutagenesis experiments described above. The following set of protocols was used to convert *S. platensis* MA7339 from a PTN-specific producer to a PTM and PTN dual producer through the expression of the “PTM cassette,” including the PtmT3 *ent*-kauran-16-ol synthase (Smanski et al., 2011).

2.2.1 Construction of heterologous expression strain

1. Select a suitable host strain that will provide the substrate for the DTS in question. This can be a nonditerpene producer that has been engineered to produce the GGDP precursor or more advanced DTS substrates, such as *ent*-CDP (Cyr, Wilderman, Determan, & Peters, 2007). Alternatively, the host strain can be one known to produce the required substrate under certain growth conditions. The latter was the case for *S. platensis* MA7339, which was predicted to provide *ent*-CDP *en route* to PTN production (Fig. 8.2; Smanski et al., 2011; Yu et al., 2010).
2. Clone the candidate DTS into a stably maintained integrating shuttle vector under control of a strong promoter. Integrating plasmids, including pSET152, have the advantage of greater stability in the absence of antibiotic selection versus self-replicating plasmids (Kieser et al., 2000). A strong constitutively expressed promoter, such as *ErmE**, or an inducible promoter is important for ensuring proper transcription (Kieser et al., 2000). We have seen numerous examples in our lab of significantly altered transcription levels from native promoters when they are moved into heterologous hosts (Chen, Smanski, & Shen, 2010; Chen, Wendt-Pienkowski, & Shen, 2008; Feng et al., 2009; Yang et al., 2011).
3. Introduce the expression plasmid into the heterologous host strain of choice by intergenic conjugation, as described in Section 2.1.3. For expression of *ptmT3* in *S. platensis* MA7339, the expression construct pBS12603, in which the expression of the “PTM cassette” is under the control of *ErmE**, was introduced by conjugation to afford the recombinant strain *S. platensis* SB12604.
4. Ferment the recombinant strain in conditions known to elicit DTS precursor production. For *S. platensis* SB12604, conditions known for the wild-type *S. platensis* MA7339 to produce PTN, were utilized.

5. Analyze the fermentation of PTM and PTN by HPLC as described in Section 2.1.5. Figure 8.5 represents typical HPLC–MS chromatograms showing PTN production alone in the wild-type *S. platensis* MA7339 strain and PTM and PTN dual production in the recombinant *S. platensis* SB12604 strain (Smanski et al., 2011).

2.2.2 Structural validation of the diterpenoids produced in heterologous hosts

To ensure that new compounds produced by the heterologous hosts feature the anticipated diterpene scaffold, purify the new compounds from the

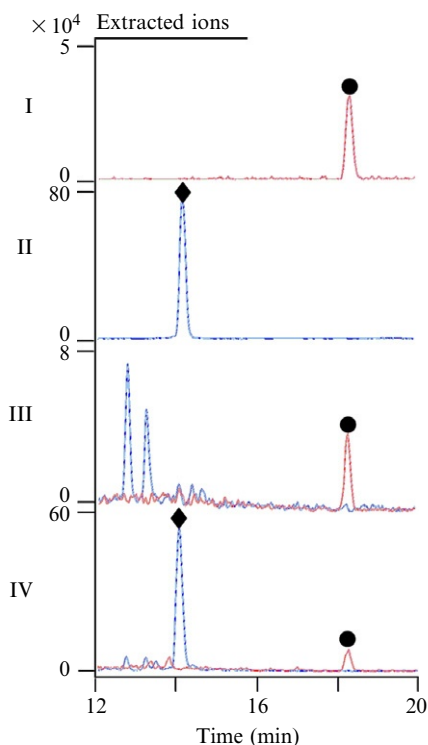


Figure 8.5 HPLC–MS analysis following heterologous expression of *ptmT3* involved in PTM biosynthesis in the PTN producer *S. platensis* MA7339 with mass detection for $[\text{PTM} + \text{H}]^+$ ion at m/z 442 in blue and $[\text{PTN} + \text{H}]^+$ ion at m/z 426 (Smanski et al., 2011). Authentic stands of PTM (I) and PTN (II) and the wild-type strain MA7339 that produces PTN only (III) and the recombinant strain SB12604 (i.e., MA7339 carrying the *ptmT3* expression plasmid pBS12603) that produces PTM and PTN (IV). PTM (◆) and PTN (●).

recombinant strains and establish their structures by a combination of mass and NMR spectroscopic analyses.

1. Purify the compound of interest using your choice of methods. For PTM, PTN, and congeners, dissolve the crude extract (step 5, [Section 2.1.5](#)) in a nonpolar solvent, such as chloroform or 2% methanol in chloroform, adsorb it to a small amount of silica gel, load the adsorbed extract to a silica gel column, and develop column with increasing concentrations of methanol in chloroform. Follow the column chromatography by TLC or HPLC, and collect and combine the fractions containing PTM, PTN, and congeners. Subject the partially pure metabolites to semipreparative HPLC on a C18 reverse-phase column with a similar solvent gradient to that described in [Section 2.1.5](#), and repeat the semipreparative HPLC if necessary until the metabolites are pure.
2. Dissolve purified metabolites in an appropriate deuterated solvent, and analyze by ^1H NMR. For heterologous production of known compounds, a comparison of the ^1H NMR to authentic standards should be sufficient for structural confirmation. For new compounds, full structural elucidation with a combination of mass and ^1H , ^{13}C , and two-dimensional NMR analyses is required ([Smanski et al., 2009, 2011; Yu et al., 2010](#)).

2.3. *In vitro* characterization of PtmT2 and PtmT3 as DTSs in PTM biosynthesis

In vitro characterization of individual proteins complements the *in vivo* studies and provides direct evidence supporting the predicted activity and revealing the true catalytic function. Methods for *in vitro* characterization of both type I and type II DTSs are known ([Hamano et al., 2002; Hayashi, Matsuura, et al., 2008; Hayashi, Toyomasu, et al., 2008; Ikeda, Hayashi, Itoh, Seto, & Dairi, 2007; Kawaide, Imai, Sassa, & Kamiya, 1997; Morrone et al., 2009; Prsic, Xu, Wilderman, & Peters, 2004; Xu, Hillwig, Prsic, Coates, & Peters, 2004](#)). We describe methods for cloning, overexpression, purification, and functional characterization of PtmT2 (a type II DTS) and PtmT3 (a type I DTS) from the PTM biosynthetic machinery ([Fig. 8.2; Smanski et al., 2011](#)) to serve as models for *in vitro* characterization of newly discovered bacterial DTSs.

2.3.1 Expression, overproduction, and purification of PtmT2 and PtmT3 from *E. coli*

1. Prepare PCR primers for amplification of *ptmT2* and *ptmT3* from *S. platensis* MA7327 genomic DNA or cosmid DNA containing the

PTM and PTN dual biosynthetic gene cluster (Smanski et al., 2011), sequence the products to confirm PCR fidelity, and clone the PCR-amplified *ptmT2* and *ptmT3* into the suitable sites of pET28a (Novagen, Madison, WI) to afford the expression constructs, in which PtmT2 and PtmT3 will be overproduced as N-terminal His₆-tagged fusion proteins.

2. Introduce the expression constructs into *E. coli* BL21(DE3) by transformation, plate transformed cells on LB plates containing 50 µg/mL kanamycin, and pick a single colony to grow in 50 mL of LB containing 50 µg/mL kanamycin overnight at 37 °C.
3. Inoculate 500 mL of LB containing 50 µg/mL kanamycin in a 2-L Erlenmeyer flask with 5 mL of the overnight culture, and incubate the culture at 37 °C and 250 rpm until it reaches an OD₆₀₀ of 0.5.
4. Cool the culture to 18 °C, and induce *ptmT2* or *ptmT3* expression by adding IPTG to 0.1 mM. Continue the incubation at 18 °C and 250 rpm shaking for 12–24 h, and harvest the cells by centrifugation at 4 °C and 4150 rpm for 30 min.
5. Resuspend cells in threefold (w/v) lysis buffer (100 mM Tris (pH 8.0), 300 mM NaCl, 10% glycerol, 15 mM imidazole), add 1 mg/mL lysozyme, and incubate with gentle mixing at room temperature for 30 min.
6. Cool the cell slurry in an ice bath for 5 min, and lyse the cells by sonication on ice (medium power level output for 3 × 30 s cycles with 1 s pulses).
7. Centrifuge the lysate at 15,000 rpm for 30 min, and filter supernatant through in-line 0.8 µm and 0.45 µm HPF Millex-HV 25 mm syringe-driven filters prior to purification.
8. Purify the His₆-tagged PtmT2 and PtmT3 proteins by affinity chromatography on Ni-NTA resin. Use FPLC, such as an ÄKTA FPLC system (Amersham Pharmacia Biotech) with a HisTrap FF 5 mL column (GE Healthcare Life Sciences), to facilitate purification. Load the filtered supernatant onto the HisTrap FF column, wash the column with 10 column volumes Buffer A (50 mM Tris (pH 8.0), 150 mM NaCl, and 20 mM imidazole), elute the column with 50% Buffer B (50 mM Tris (pH 8.0), 100 mM NaCl, and 500 mM imidazole) at the flow rate of 2 mL/min, and collect 1.5 mL fractions.
9. Analyze the fractions using SDS-PAGE, pool fractions containing pure PtmT2 or PtmT3, concentrate to desired concentration (2 mg/mL) using a Vivaspin 20 (30,000 MWCO, Sartorius-Stedim), and store the purified proteins in 40% glycerol at –80 °C.

This procedure affords pure PtmT2 and PtmT3 as N-terminal His₆-tagged fusion proteins with an average final yield of ~40–50 mg/L of culture.

Figure 8.6 represents a typical SDS-PAGE analysis of the purified PtmT2 and PtmT3 proteins.

2.3.2 Synthesis of GGDP from geranylgeraniol

In vitro characterization of DTSs requires GGDP as a substrate. Although GGDP is commercially available, its cost and lack of availability in larger quantities than 200 µg vials makes it unsuitable for studies of DTSs requiring significant amount of GGDP as a substrate. We describe a method to synthesize GGDP from its more readily available and cost-efficient alcohol derivative, geranylgeraniol (GGOH). Literature procedures for polyprenyl diphosphate synthesis are known (Cornforth & Popjak, 1969; Danilov, Druzhinina, Kalinchuk, Maltsev, & Shibaev, 1989; Davisson, Woodside, & Poulter, 1985; Keller & Thompson, 1993). We adapted our method from a protocol by Keller and Thompson (1993).

1. Transfer 500 mg (1.72 mmol) of neat GGOH (Sigma-Aldrich, St. Louis, MO) into a 50-mL polypropylene tube, and combine it with 2 mL trichloroacetonitrile.
2. Prepare a “TEAP” (triethylamine/phosphoric acid) solution by slowly adding 3.64 mL of solution A (2.5 mL of concentrated phosphoric acid

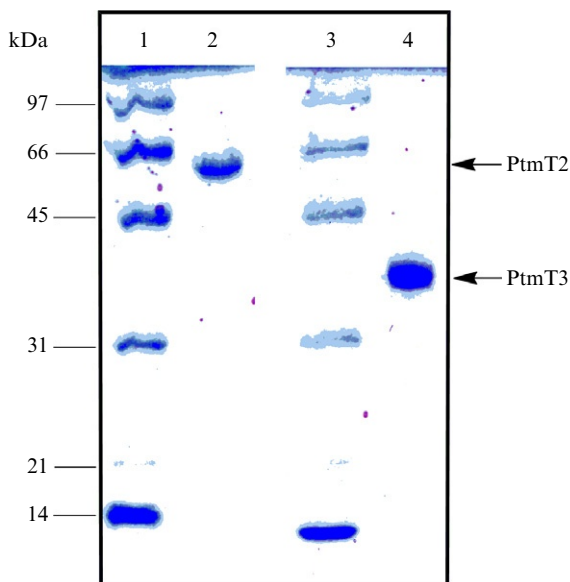


Figure 8.6 SDS-PAGE of purified PtmT2 and PtmT3 proteins: lanes 1 and 3, low-range protein MW standards; lane 2, PtmT2; and lane 4, PtmT3.

diluted into 9.4 mL of acetonitrile) to 6 mL of solution B (11 mL of triethylamine into 10 mL of acetonitrile) with constant stirring.

3. Add 2 mL of the TEAP solution to the tube containing GGOH with gently swirl, and incubate at 37 °C for 5 min. Add another 2 mL of the TEAP solution to the reaction mixture, and incubate at 37 °C for additional 5 min; repeat this step one more time. This reaction affords a mixture of geranylgeranyl mono-, di-, and triphosphate.
4. For a small-scale preparation, purify GGDP by preparative TLC plates following the literature procedure (Keller & Thompson, 1993).
5. For a larger scale preparation, purify GGDP by flash chromatography. A Buchi MPLC system (C-605/C-615) with a column (230 mm × 26 mm) packed with silica gel (230–400 mesh) or similar configuration capable of ~10 mL/min flow rate is recommended for the steps described below.
6. Equilibrate column at 10 mL/min using a mobile phase consisting of i-PrOH:NH₄OH:H₂O (6:2.5:0.5, v/v), load the reaction mixture (~8 mL) to the column, elute the column with the same mobile phase at a flow rate of 10 mL/min, and collect 10 mL fractions (~200).
7. Analyze for GGDP-containing fractions by TLC, with authentic GGDP standard as a reference, using Silica gel 60 plates developed in i-PrOH:NH₄OH:H₂O (6:3:1, v/v) and visualize by spraying anisaldehyde solution (90 mL ethanol, 5 mL *p*-anisaldehyde, and 5 mL sulfuric acid). Pool GGDP-containing fractions, concentrate to dryness, and dissolve dried material in a small volume of 25 mM NH₄HCO₃:CH₃OH (3:7, v/v) according to desired concentration.

With average yields of 30–40%, this method provides access to hundreds of milligrams of GGDP from a more cost-efficient, commercially available starting material.

2.3.3 Functional characterization of PtmT2 as an ent-CDP synthase

1. Run reactions in 500 µL of assay solution containing 50 mM Tris (pH 7), 1 mM MgCl₂, 5 mM 2-mercaptoethanol, and 10% glycerol. Use 5–10 µL of the substrate, GGDP (1 mg/mL solution in 25 mM NH₄HCO₃:CH₃OH, 3:7, v/v), per assay.
2. Initiate the reaction by adding 1–25 µL of purified PtmT2 (~2 mg/mL in 40% glycerol storage buffer), and allow to incubate at 30 °C for 1–24 h.
3. Terminate the reaction by extracting the assay mixtures with equal volumes of hexanes (keep aqueous layer for step 4). Pool the hexane extracts,

concentrate in vacuum, and set aside (store at -20°C) for GC–MS analysis. This initial hexane extraction does not extract the GGDP substrate, the predicted *ent*-CDP product, or other diphosphate-containing products from the aqueous layer; rather this step would isolate any nondiphosphate-containing diterpene products.

4. Add 10 U of calf intestinal alkaline phosphatase (CIAP, 10,000 U/mL, New England Biolabs, Ipswich, MA) to the aqueous layer, incubate at 37°C for 4 h to enzymatically cleave off the diphosphate moieties from any substrate or products.
5. Extract the CIAP-treated aqueous layer three times with equal volumes of hexanes, pool the hexane extracts, concentrate in vacuum, and store at -20°C for GC–MS analysis.
6. Resuspend samples in 100 μL hexanes before GC–MS analysis. Conduct GC–MS analysis on an Agilent Technologies 5973 N MSD (electron-ionization mode, 70 eV) with a 6890 Series Gas Chromatograph containing an HP-5 ms column [(5%-Phenyl)-methylpolysiloxane, 30 m $\times$ 0.25 mm ID $\times$ 25 μm film] or similar instrument. Inject 0.5–1 μL of the sample at 275°C in splitless mode with the following program for the column oven temperature: (i) isothermal at 40°C for 3 min, (ii) a temperature gradient at $20^{\circ}\text{C}/\text{min}$ to 300°C , and (iii) isothermal at 300°C for an additional 4 min. Collect mass spectral data from 50 to 500 m/z .
7. Confirm the identity of the products by comparing retention times and fragmentation patterns of samples with authentic standards or to those reported in the literature. Figure 8.7 represents a typical GC–MG chromatogram showing the PtmT2-catalyzed formation of *ent*-CDP from GGDP.

2.3.4 Functional characterization of PtmT3 as an *ent*-kauran-16-ol synthase

PtmT3 is predicted to use *ent*-CDP as a substrate. Since *ent*-CDP is not commercially available, functional characterization of PtmT3, or other DTSs that utilize *ent*-CDP as a substrate, requires synthesis of *ent*-CDP. Methods for *ent*-CDP synthesis are known (Cavender, 1977; Nakano & Djerassi, 1961). We also exploited PtmT2 to convert GGDP into *ent*-CDP *in situ* in a coupled reaction to assay PtmT3 as an *ent*-kauran-16-ol synthase directly.

1. For assays utilizing *ent*-CDP as a substrate, run reactions in 500 μL of assay solution containing 50 mM Tris (pH 7), 1 mM MgCl_2 , 5 mM 2-mercaptoethanol, and 10% glycerol. Add *ent*-CDP to the assay

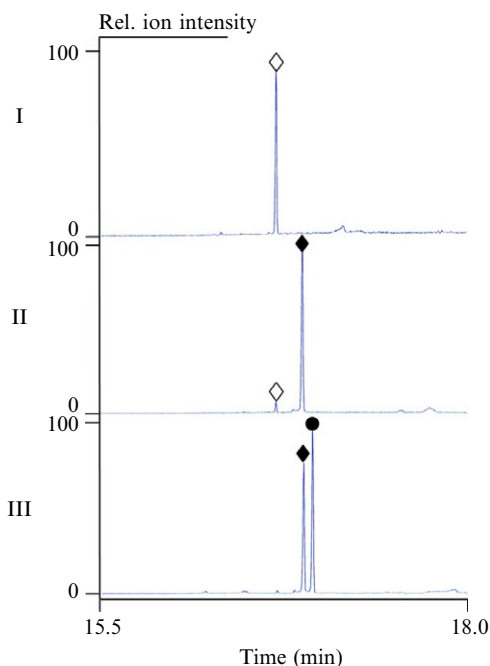


Figure 8.7 GC–MS analysis following *in vitro* assays of PtmT2 as an *ent*-CDP synthase and PtmT3 as an *ent*-kauran-16-ol synthase with mass detection for M^+ ion at m/z 290 for GGDP and *ent*-CDP and the $[M - H_2O]^+$ ion at m/z 272 for *ent*-kauran-16-ol: (I) GGDP standard; (II) PtmT2 catalyzed formation of *ent*-CDP from GGDP; and (III) PtmT3-catalyzed formation of *ent*-kauran-16-ol from *ent*-CDP. GGDP, ($\diamond$); *ent*-CDP ($\blacklozenge$); *ent*-kauran-16-ol.

solution to a final concentration of 1–50 μM . Initiate the reaction by adding 1–25 μL of PtmT3 (~ 2 mg/mL in 40% glycerol storage buffer), and incubate at 30 $^{\circ}C$ for 1–24 h.

- For assays exploiting PtmT2 to generate *ent*-CDP *in situ* as a substrate, run reactions in 500 μL of assay solution containing 50 mM Tris (pH 7), 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 10% glycerol. Add GGDP to the assay solution to a final concentration of 50 μM . Initiate the reaction by adding 25 μL of PtmT2 (~ 2 mg/mL in 40% glycerol storage buffer) followed by addition of 1–25 μL PtmT3 (~ 2 mg/mL in 40% glycerol storage buffer), and incubate at 30 $^{\circ}C$ for 1–24 h.
- Follow steps 3–7 in [Section 2.3.3](#) to terminate the reactions, extract the products, and analyze and determine their identity by GC–MS analysis. [Figure 8.7](#) represents a typical GC–MS chromatogram showing the PtmT3-catalyzed formation of *ent*-kauran-16-ol from *ent*-CDP.



3. CONCLUSIONS

Bacterial DTSs offer the opportunity to broaden our understanding of terpene biosynthesis and can be utilized in future metabolic pathway engineering for high value compounds (Smanski et al., 2012). Realizing the full potential of bacterial DTSs will require a focused and interdisciplinary effort, drawing on the expertise of natural products chemists, mechanistic biochemists, microbiologists, bioinformaticists, structural biologists, and more. Sequence databases already contain numerous uncharacterized DTSs that can be mined to yield useful biochemical data. Also, microorganisms from underexplored niches have proven to be a rich source for novel chemistry, and efforts to characterize the diterpene production from these organisms should be increased. The extreme sequence diversity in TSs in general and bacterial DTSs in particular hampers efforts to predict biochemical function from primary sequence information. A grand challenge to future natural product chemists and biologists will be to fully characterize the catalytic landscape of bacterial DTSs. This will improve not only our ability to predict function from structure but also allow future researchers to precisely design new DTSs to act as biocatalysts for engineering new biochemical pathways for drug discovery.

The protocols provided here describe robust current methodologies to find and characterize bacterial DTSs. This is an exciting time for research in natural product biosynthesis, as current tools in chemistry, molecular biology, and bioinformatics allow enzymes with unique chemistries to be identified and functionally characterized with an incredible efficiency. Bacterial diterpenoid biosynthesis represents an underexploited resource for new biochemistry and chemical diversity. Nature's ability to generate new structures with incredible biological activities through terpenoid biosynthesis is staggering, and it is our hope that the coming decade will bring an increased commitment to understand and utilize this incredible resource in confronting future challenges in medicine, agriculture, and industry.

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