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Bacterial Diterpene Synthases: New Opportunities for Mechanistic Enzymology and Engineered Biosynthesis

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Abstract

Diterpenoid biosynthesis has been extensively studied in plants and fungi, yet cloning and engineering diterpenoid pathways in these organisms remain challenging. Bacteria are emerging as prolific producers of diterpenoid natural products, and bacterial diterpene synthases are poised to make significant contributions to our understanding of terpenoid biosynthesis. Here we will first survey diterpenoid natural products of bacterial origin and briefly review their biosynthesis with emphasis on diterpene synthases (DTSs) that channel geranylgeranyl diphosphate to various diterpenoid scaffolds. We will then highlight differences of DTSs of bacterial and higher organism origins and discuss the challenges in discovering novel bacterial DTSs. We will conclude by discussing new opportunities for DTS mechanistic enzymology and applications of bacterial DTS in biocatalysis and metabolic pathway engineering.

Introduction

Terpenoids comprise the largest, structurally most diverse family of natural products and play important roles in all living organisms. Among the ~60,000 members known to date, ~12,000 are diterpenoids, most of which are produced in plants and fungi. Diterpenoids of bacterial origin are known but rare, however recent advances in genomics have revealed that the biosynthetic potential for terpenoids in bacteria, particularly in the actinimycetes, may be significantly underestimated [1,2].

Diterpenoid biosynthesis has been extensively studied in plants and fungi [3–6], yet cloning the respective genes and characterizing and engineering diterpenoid pathways in these higher organisms remain challenging [7,8]. Scattering of the biosynthetic genes on the genomic DNA of these higher organisms substantially increases the effort to clone all the genes encoding the complete biosynthetic machinery for a given diterpenoid natural product.

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In contrast, genes encoding secondary metabolite biosynthesis in actinomycetes are nearly always arranged on the bacterial chromosome as a cluster. Recent characterization of terpene synthases (TSs) from several actinomycete species demonstrated that these enzymes are not membrane-bound and can be overproduced with relative ease as soluble, functional recombinant proteins in heterologous hosts such as *E. coli* [9]. Diterpenoid biosynthesis in bacteria therefore may provide new opportunities to characterize these enzymes and to engineer their biosynthetic machinery for diterpenoid natural product structural diversity.

Diterpenoids are all derived from (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGDP). Diterpene synthases (DTSs), also known as diterpene cyclases, catalyze the critical step in diterpenoid biosynthesis by morphing GGDP into one of the many diterpenoid scaffolds, further transformations of which by the downstream enzymes afford the enormous structural diversity known for diterpenoid natural products. TSs in general, DTSs included, can display incredible fidelity, catalyzing multi-step cyclization reactions with exquisite regiochemical and stereochemical control [10] or display marked product promiscuity, with a single enzyme generating over fifty unique products from a single substrate [11]. It is the fidelity and promiscuity in this chemistry that has inspired a great interest in exploiting TSs for engineered biosynthesis of novel terpenoid natural products [7,8,12].

Here we will first survey diterpenoid natural products of bacterial origin and briefly review their biosynthesis with emphasis on DTSs that channel GGDP to various diterpenoid scaffolds. We will then highlight differences of DTSs of bacterial and higher organism origins and discuss the challenges in discovering novel bacterial DTSs. We will conclude by discussing new opportunities for DTS mechanistic enzymology and applications of bacterial DTS in biocatalysis and metabolic pathway engineering.

Bacterial diterpenoids

The discovery of gibberellins (GAs) from *Rhizobium phaseoli* in 1988, originally only known to fungi and plants, may represent the first report of bacterial diterpenoids [13,14]. It was followed by the discovery of verrucosan-2β-ol from *Chloroflexus aurantiacus* in 1993 [15] and isoagathenediol from *Rhodospirillum rubrum* in 1995 [16]. Since then, the list of bacterial diterpenoids has grown steadily, and Figure 1 summarizes the bacterial diterpenoids known to date. These include terpentecin from *Streptomyces griseolosporeus* MF730-N6 [17,18,19], the phenalinolactones from *Streptomyces* sp. Tu6071 [20], the brasilicardins from *Nocardia brasiliensis* IFM 0406 [21], viguiepinol and the oxaloterpins from *Streptomyces* sp. KO-3988 [22,23], cyclooctatin from *Streptomyces melanosporofaciens* MI614-43F2 [24], tuberculosinol and the isotuberculosinols from *Mycobacterium tuberculosis* H37Rv [25–29], platensimycin from *Streptomyces platensis* MA7327 [30–32], platencin from *Streptomyces platensis* MA7339 [33,34,35], the neoverrucosanes from *Saprospira grandis* [36,37], cyslabdan from *Streptomyces* sp. K04-0144 [38], the gifhornenolones from *Verrucosispora gifhornesis* YM28-088 [39], and JBIR-65 from *Actinomadura* sp. SpB081030SC-15 [40]. The actinomycetes have emerged as prolific producers of bacterial diterpenoids [1,2]. Bacterial producers of paclitaxel have also been reported, many of which were actinomycetes, however definitive evidence supporting their paclitaxel production remains elusive [41].

Bacterial DTSs

DTS classification follows other TSs. Type I TSs initiate a cyclization reaction via a heterolytic cleavage of the polyprenyl diphosphate, while type II TSs initiate the cyclization reaction via protonation of a double bond or an epoxide ring. In both cases, the resulting carbocation undergoes a cascade of cyclization, the fate of which is determined by a combination of steric and electrostatic forces within the active site cavity. The cyclization

cascade is ultimately terminated by abstraction of a proton or electrophilic attack by water (Figure 2A) [4,5]. Because type II TSs leave the diphosphate group intact, their products can serve as substrates for further cyclization by type I TSs. The high frequency with which such two-step cyclizations are employed differentiates diterpenoid biosynthesis from that of smaller terpenoids, which rarely implement a type II mechanism.

Terpentedienyl diphosphate synthase and terpentetriene synthase were the first two characterized bacterial DTSs, reported in 2001 for terpentecin biosynthesis from *S. griseolosporeus* MF730-N6 [18,19]. Since then, a total of 16 bacterial DTSs have been identified from various organisms, with genome sequencing efforts unveiling many more candidates whose functions as DTSs require experimental confirmation. Figure 2B summarizes the individual transformations catalyzed by these DTSs *en route* to their respective diterpenoid natural products, highlighting the remarkable catalytic landscape covered by bacterial DTSs.

Bacterial type II DTSs

Terpentedienyl diphosphate synthase (Cyc1) from *S. griseolosporeus* MF730-N6, the first bacterial type II DTS reported, converts GGDP to terpentedienyl diphosphate *en route* to terpentecin (Figure 2B). Although it displays only a moderate sequence similarity (<30%) to the N-terminal halves of characterized eukaryotic DTSs, the presence of a DXDD motif solidified its bioinformatics-based functional assignment [17,18], which was subsequently confirmed experimentally *in vitro* [17,19]. Other bacterial type II DTSs identified since include halimadienyl diphosphate synthase (Rv3377c) from *M. tuberculosis* [25,26,29] and *ent*-copalyl diphosphate (*ent*-CPP) synthases from *S.* sp. KO-3988 (ORF2) [22], *B. japonicum* (BjCPS) [42], *S. platensis* MA7327 (PtmT2) [43], and *S. platensis* MA7339 (PtnT2) [43], respectively (Figure 2B), all of which share the characteristic DXDD motif. The type II DTSs, reported for brasilicardin A (Bra4) and phenalinolactone A (Plat2) from *N. brasiliensis* IFM 0406 [21] and *S.* sp. Tu6071 [20], respectively, are atypical, with their signature DXDD motif replaced with a (E/D)SA(E/N) motif. Intriguingly, both clusters contain a separate gene homologous to eukaryotic squalene epoxidase, which is thought to convert GGDP to epoxy-GGDP. The latter would support a sterol-like cyclization reaction, where the non-canonical type II DTS Bra4 or PlaT2 would initiate the cyclization reaction by protonating an epoxy group rather than a double bond [20] (Figure 2B).

Bacterial type I DTSs

The staggering sequence diversity present in bacterial type I DTSs hinders sequence-gazing efforts but heightens our understanding of the minimal requirements for catalysis. Terpentetriene synthase (Cyc2) from *S. griseolosporeus* MF730-N6, the first bacterial type I DTS reported, was readily identified on the basis of its sequence homology to known bacterial type I TSs, and presence of the characteristic DDXXD and NSE/DTE motifs [18,19]. The other bacterial type I DTSs characterized since - including the tuberculosinol/isotuberculosinol synthase (Rv3378c) from *M. tuberculosis* [26,27,29], the cyclooctatenol synthase (CotB2) from *S. melanosporofaciens* [24], the pimaradiene synthase (ORF3) from *S.* sp. KO-3988 [22], and two *ent*-kaurene synthases (BjKS and PtmT3) from *B. japonicum* [42] and *S. platensis* MA7327 [43], respectively - however, have an average of just 13% sequence identity. No pairwise alignment displays greater than 20% sequence identity, despite the fact that the latter four enzymes act on a same substrate *ent*-CPP, and this is in contrast to plant and fungal type I DTSs that display an average of 28% and 25% pairwise identity, respectively. This sequence diversity is evident by the deep branching of bacterial type I DTSs, compared to plant and fungal enzymes, on a minimum-evolution phylogenetic tree (Figure 3A). Moreover, the canonical active site motifs have even been called into question with recent studies of bacterial type I DTSs. For example, the tuberculosinol/

isotuberculosinol synthase Rv3378c retains the DDXXD motif but lacks the conserved NSE/DTE motif [26,29]. The two *ent*-atiserene synthases (PtmT1 and PtnT1) from *S. platensis* MA7327 and MA7339, respectively, lack both the DDXXD and NSE/DTE motifs, and contain instead two atypical DXXXD motifs [43]. These findings expand our understanding of the structural elements required for catalysis and will guide future mechanistic investigations and DTS discovery efforts.

Evolutionary relationship of bacterial and eukaryotic DTSs

Structures for a number of type I and type II TSs of bacterial, fungal, or plant origin are available [4,5], but no structure of a bacterial DTS is currently known. Nonetheless, structures of various known TSs coupled with detailed bioinformatic analyses of the bacterial variants now provide a clearer picture of DTS evolution. Bacterial type II DTSs are homologous to bacterial triterpene synthases for which the three-dimensional structure is known, including squalene hopene cyclase [44], and primary sequence alignments suggest a conservation of overall topology and active site location [45]. Thus, bacterial type II DTSs are hypothesized to contain the same βγ-didomain structure deriving from an ancient duplication of two $(\alpha/\alpha)_6$ barrels. On the contrary, bacterial type I DTSs are predicted to have a single α-domain "isoprenoid fold" based on their homology and overall primary sequence alignments to the bacterial type I sesquiterpene synthases, pentalenene synthase [9,46] and epi-isozizaene synthase [47]. Plant DTSs likely evolved from an early fusion of the bacterial type I and II enzymes to form bifunctional αβγ-tridomain DTSs that can catalyze both types of cyclization but in separate active sites. Such tridomain DTSs are still seen today, for example, in the bifunctional *ent*-kaurene synthase from the moss *Physcomitrella patens* [48] and the bifunctional abietadiene synthase from *Abies grandis* [49]. Monofunctional type I or type II DTSs found in plants commonly retain vestigial domains from ancestral enzymes that, while lacking active site motifs, likely remain for structural support [50–52]. The relevance of these evolutionary roots to current studies in DTS biochemistry and enzymology is many-fold. For example, bacterial DTSs, in particular type I DTSs, tend to be significantly smaller than their eukaryotic counterparts, perhaps to a degree that makes the bacterial enzymes inherently easier to manipulate for biochemical studies. The ancient roots of DTSs in prokaryotes also explain the greater sequence diversity observed in these enzymes (Figure 3). Further examination and utilization of bacterial DTSs in future studies could facilitate interrogating how sequence divergence in core catalytic motifs affects enzyme mechanism and product diversity in TSs.

Discovery of new bacterial DTSs

Challenges in discovering bacterial DTSs

The lack of sequence conservation in bacterial DTSs makes sequence-based approaches for their discovery difficult, albeit still possible [53]. Many of the bacterial DTSs characterized to date were identified by their clustering with more readily identifiable terpene biosynthetic genes. For example, screening genomic DNA of *S. griseolosporeus* MF730-N6 for mevalonate pathway genes led to the identification of terpentedienyl diphosphate synthase (Cyc1) and terpentetriene synthase (Cyc2) for terpentecin biosynthesis [18]. A similar strategy yielded the pimaradiene synthase (ORF3) for viguiepinol biosynthesis in *S.* sp. KO-3988 [54]. Other bacterial diterpene gene clusters have been identified only by screening for genes required for the biosynthesis of separate chemical moieties [20,43] or those involved in tailoring reactions [55]. The requirement for the common substrate GGDP can be exploited to identify bacterial DTSs. Previous studies have attempted to determine the chain-length determining factors that distinguish GGDP synthase from shorter- and longer-chain polyprenyl diphosphate synthases [56–59] and several key amino acid residues have been identified and verified *in vitro*. From these data, predictive algorithms could be

designed to selectively scan genomes for GGDP synthases, and such a strategy was used recently to clone the brasilicardin A gene cluster from *N. brasiliensis* [60].

Atypical DTSs in the platensimycin and platencin biosynthetic gene clusters

Platensimycin (PTM) and platencin (PTN) are composed of a substituted aminobenzoic acid and a diterpenoid-derived carboxylic acid, linked by an amide bond (Figure 1) [30–35]. The terpenoid moieties of PTM and PTN bear *ent*-kaurene and *ent*-atiserene scaffolds, respectively [61,62]. While *ent*-kaurene production has been studied in great detail in plants [63], fungi [64], and, more recently, bacteria [42], little is known about *ent*-atiserene production. Following the cloning of a PTM-PTN dual producing gene cluster from *S. platensis* MA7327, putative *ent*-CPP (Ptm2) and *ent*-kaurene synthases (Ptm3) were identified by sequence comparison with known DTSs. Comparison of the PTM-PTN gene cluster to a PTN-only producing gene cluster from *S. platensis* MA7339 ruled out the possibility that a promiscuous *ent*-kaurene synthase could provide both scaffolds. This led to the search and eventual discovery of PtmT1 and PtnT1 as the *ent*-atiserene synthases, novel type I DTSs that lack canonical active site motifs, for PTN biosynthesis [43]. A preliminary analysis of sequence databases has revealed other misannotated homologues, suggesting that the *ent*-atiserene synthase belongs to a previously unrecognized family of bacterial type I DTSs [43].

New opportunities for mechanistic enzymology and engineered biosynthesis

Mechanistic enzymology

Much work has been done in higher plants and fungi to attempt to fully understand the catalytic landscape of DTSs. Structural data point to the importance of precisely positioned amino acid side chains in the active site cavity that guide the folding of intermediates and stabilize carbocation intermediates. Mechanistic enzymology and structural biology of known bacterial DTSs, and of those yet to be discovered, will complement previous studies by providing a wealth of new and diverse sequences to populate the catalytic landscape (Figures 2 and 3). These studies will hopefully answer important questions such as how *ent*-kaurene synthases from *B. japonicum* and *S. platensis* can produce the same diterpene scaffold with such divergent primary sequences. Additionally, continued discovery and full characterization of non-canonical bacterial type I and type II DTSs will surely broaden our understanding of the mechanistic requirements for DTS reaction initiation.

Exploiting bacterial DTSs to generate structural diversity

Since all diterpenoids derive from a common origin, GGDP, a degree of modularity exists among DTSs from different biosynthetic pathways. There is little evidence for protein-protein interactions between enzymes in a terpenoid biosynthetic pathway. This enables various type I and type II DTS to be mixed and matched to produce a variety of carbon scaffolds. For example, the PTN-producing strain, *S. platensis* MA7339, can be converted into a PTM-PTN dual producer by heterologously expressing the *ent*-kaurene synthase and other genes involved in PTM ether-ring formation, and this suggests that alternative *ent*-copalyl-derived diterpene scaffolds could be accepted into the PTM and PTN pathways (Figure 4A) [43]. The extent to which diverse diterpene scaffolds can replace native scaffolds in extant biosynthetic pathways depends on the ability of the downstream tailoring enzymes to accept the new scaffold. Structural similarities among different diterpene scaffolds may therefore dictate compatibility, as exemplified in the PTM and PTN biosynthesis. In order to fully explore the potential of diterpenoid biosynthetic pathways, we

must increase the number of characterized DTSs to begin to approximate the number of diterpenoid scaffolds found in Nature.

Engineered production of diterpenoid natural products in bacteria

Bacteria provide convenient hosts for engineered production of terpenoid natural products with important commercial value. This strategy has received considerable attention [7,8] and led to recent success when principles from the emerging field of synthetic biology were applied to produce precursors of the sesquiterpene antimalarial drug, artemisinin, in a bacterial host [65]. All indications suggest that diterpenoid pathways are equally amenable to such metabolic engineering efforts. This was recently demonstrated through construction of a modular DTS expression system in *E. coli*. Three different type II DTSs, affording *ent*-, *syn*-, and *normal*-CPPs, were first introduced into a recombinant *E. coli* strain that was engineered to produce GGDP. Several product-specific type I DTS were then added to afford recombinant strains that produce nine diterpenoids representing eight distinct scaffolds (Figure 4B) [66]. These diterpenoid scaffolds, alone, provide an entry point to the engineered production of over 2,000 known diterpenoid natural products.

Conclusions and prospective

Bacteria are emerging as prolific producers of diterpenoids, and bacterial DTSs are poised to make significant contributions to our current understanding of terpenoid biosynthesis. Advantages of studying diterpenoid biosynthesis in bacteria include (i) access to mechanistic and structural studies as facilitated by the technical feasibility of working with bacterial enzymes, (ii) expansion of mechanistic understanding through the characterization of novel enzymes with non-canonical catalytic motifs, and (iii) opportunities for whole pathway engineering to produce complex diterpenoid natural products. Recent findings have already challenged the paradigm of TS biochemistry and mechanistic enzymology from studies in higher organisms and promise to expand the boundaries of DTS catalytic landscape. Each new DTS characterized will either extend these boundaries further or fill in the gaps between existing sequences. The number of possible natural or unnatural diterpenoid scaffolds, accessible from the common substrate GGDP by DTSs, is staggering, and gaining access to these structures by engineered biosynthesis will greatly aid both drug discovery efforts and development of biotechnology applications.

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• of special interests

•• of outstanding interests

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Highlights

- Cloning and engineering diterpenoid pathways in plants and fungi remain challenging.
- Bacteria are emerging as prolific producers of diterpenoid natural products.
- Bacterial diterpene synthases are poised to make significant contributions to our understanding of terpenoid biosynthesis.
- Diterpenoid biosynthesis in bacteria provides new opportunities for pathway engineering to produce complex diterpenoid natural products.

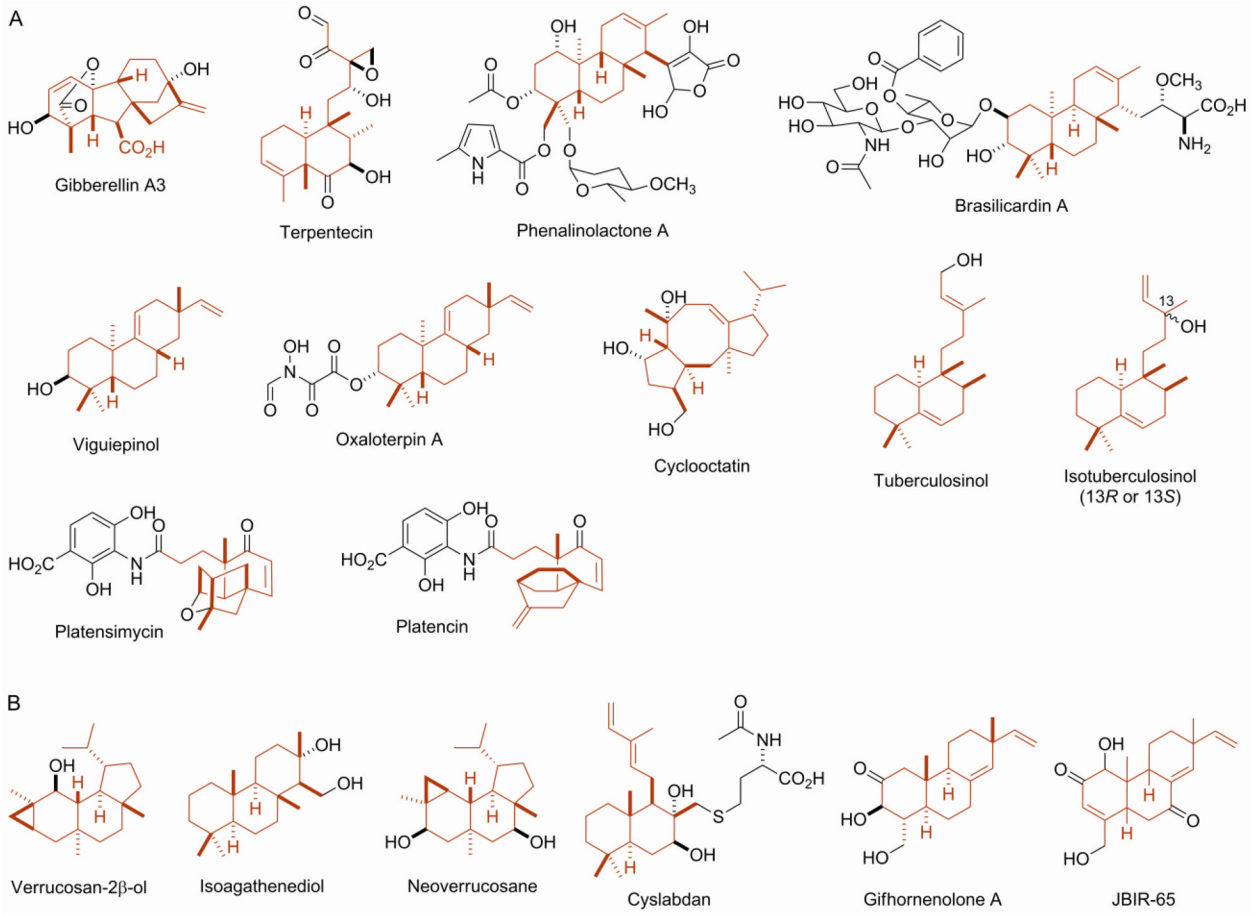

Figure 1.

Bacterial diterpenoid natural products with their diterpenoid carbon scaffolds highlighted in red: (A) the biosynthetic gene clusters for these natural products have been cloned and partially characterized and (B) biosynthesis for these natural products has not been studied.

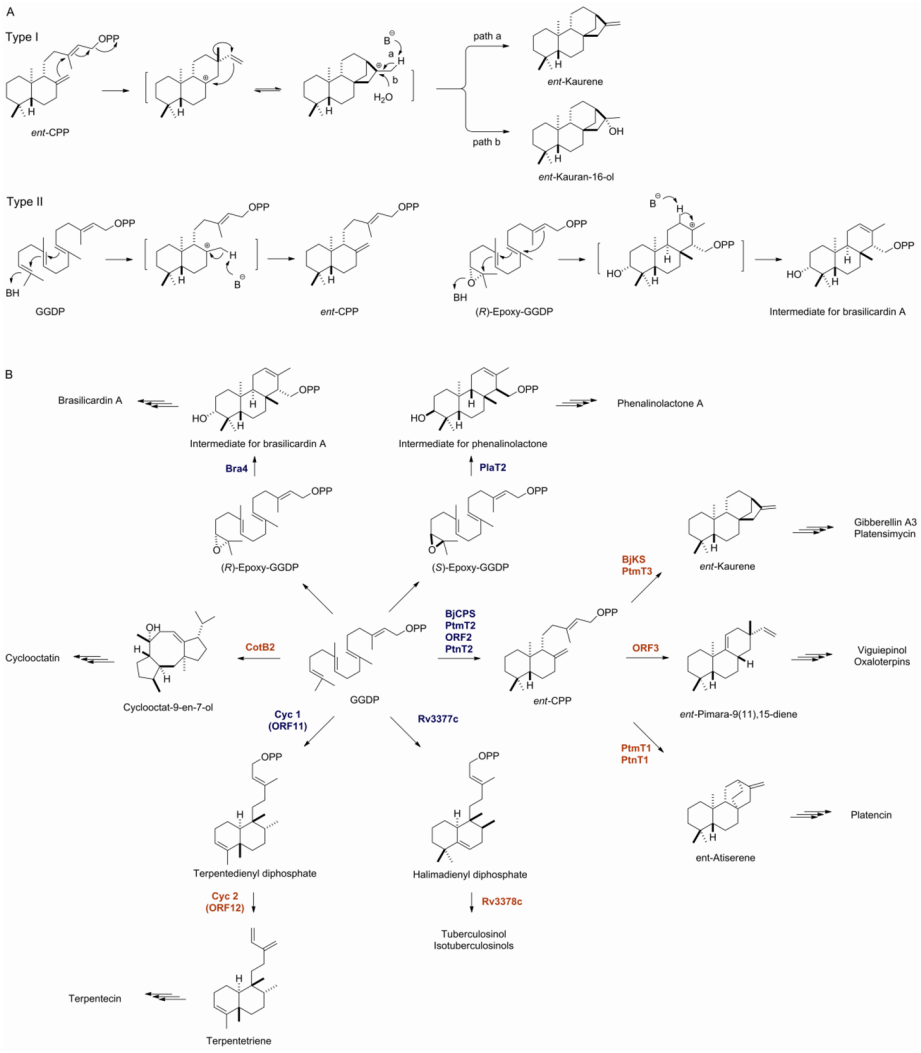

Figure 2.

Bacterial diterpene synthases (DTSs): (A) Mechanisms of type I and type II DTSs and (B) pathways for bacterial diterpenoid natural product biosynthesis, highlighting known bacterial type I (blue) and type II DTSs (red) that convert GGDP to diverse diterpenoid scaffolds *en route* to the final natural products. See Figure 1 legend for structures of the diterpenoid natural products. See Figure 3 legend for accession numbers of the type I and type II DTSs. DTSs, diterpene synthases; *ent*-CPP, *ent*-copalyl diphosphate; GGDP, geranylgeranyl diphosphate.

Figure 3A

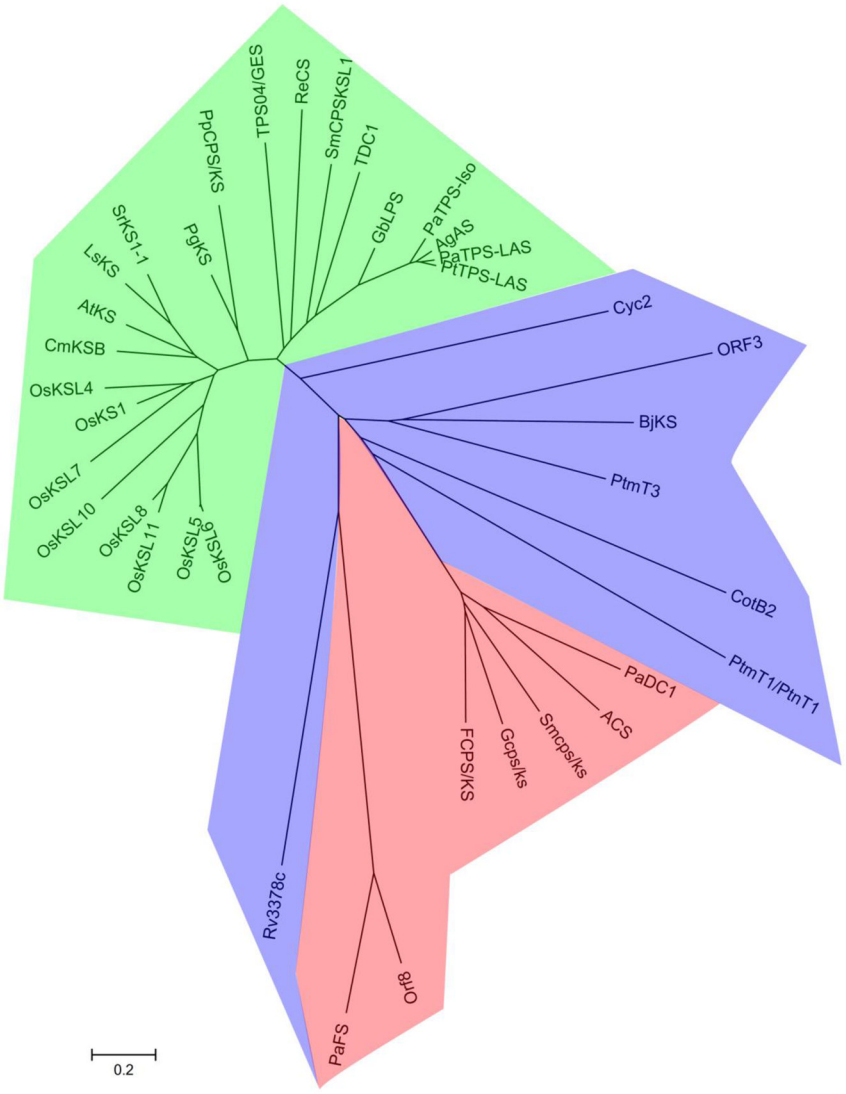

Figure 3B

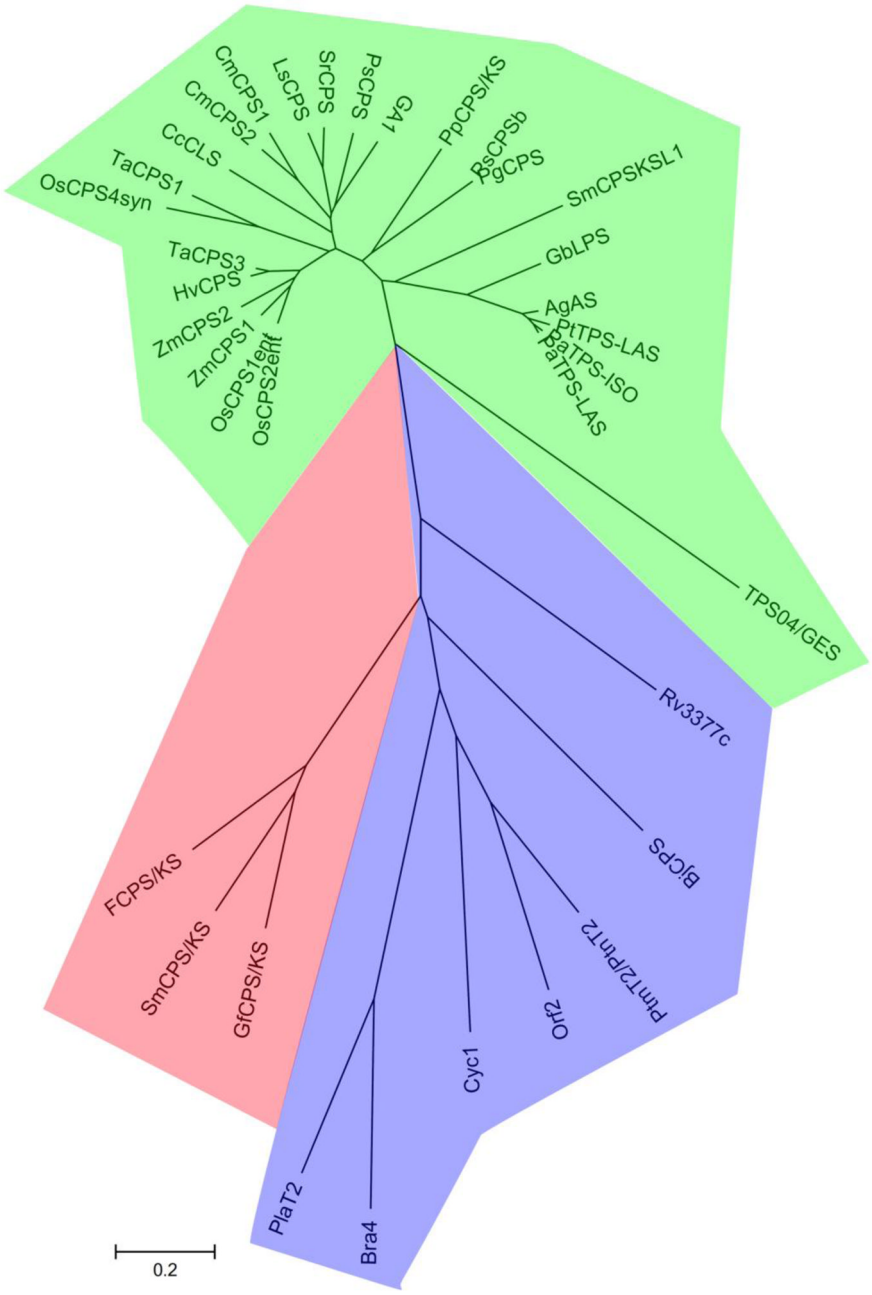

Figure 3.

Minimum evolution trees of primary amino acid sequences from plant (green), fungal (red), and bacterial (blue) type I (A) and type II DTSs (B). The branch lengths illustrate the extent of sequence diversity found in bacterial DTSs compared with plant or fungal enzymes. The trees were constructed in Mega5.0 with a ClustalW-generated primary sequence alignment. Shown in parentheses are accession numbers. *Bacterial type I DTSs*: Cyc2 (BAB39207), ORF3 (BAD86798), BjKS (BAC47415), PtmT3 (ACO31279), CotB2 (BAI44338), PtmT1 (ACO31274), PtnT1 (ADD83014), Rv3378 (P_217895). *Fungal type I DTSs:* PaDC1 (BAG30961), ACS (BAB62102), Smcps/ks (CAP07655), Gfcps/ks (Q9UVY5), FCPS/KS (BAA22426), Orf8 (bsc8) (BAI44849), PaFS (BAF45925). *Plant type I DTSs*: OsKSL6

(ABH10733), OsKSL5 (ABH10732), OsKSL11 (AAZ76733), OsKSL8 (BAD34478), OsKSL10 (ABH10735), OsKSL7 (ABH10734), OsKS1 (AAQ72559), OsKSL4 (AAU05906), CmKSB (AAB39482), AtKS (AAC39443), LsKS (BAB12441), SrKS1-1 (AF097310_1), PgKS (ADB55708), PpCPS/KS (BAF61135), TPS04/GES (NP_564772), RcCS (XP_002513340), SmCPSKSL1 (AEK75338), TDC1 (AAC49310), GbLPS (AAL09965), PaTPS-Iso (AAS47690), AgAS (Q38710), PaTPS-LAS (AAS47691), PtTPS (AAX07435). *Bacterial type II DTSs*: Rv3377c (NP_217894), BjCPS (BAC47414), PtmT2 (ACO31276), PtnT2 (ADD83015), Orf2 (BAD86797), Cyc1 (BAB39206), Bra4 (BAG16278), PlaT2 (ABB69743). *Fungal type II DTSs*: FCPS/KS (BAA22426), SmCPS/KS (CAP07655), GfCPS/KS (Q9UVY5). *Plant type II DTSs:* OsCPS1ent (BAD42449), OsCPS2ent (Q6ET36), ZmCPS1 (AAT49065), ZmCPS2 (ADB55709), HvCPS (BAH56560), TaCPS3 (AAT70083), OsCPS4syn (NP_0010521), TaCPS1 (BAH56558), CcCLS (ADJ93862), CmCPS1 (AAD04292), CmCPS2 (AAD04923), LsCPS (BAB12440), SrCPS (AAB87091), PsCPS (AAB58822), GA1 (AAA53632), PpCPS/KS (BAF61135), PsCPSb (ADB55709), PgCPS (ADB55707), GbLPS (AAL09965), AgAS (Q38710), PtTPS-LAS (AAX07435), PaTPS-ISO (AAS47690), PaTPS-LAS (AAS47691), SmCPSKSL1 (AEK75338), TPS04/GES (NP_564772). DTSs, diterpene synthases.

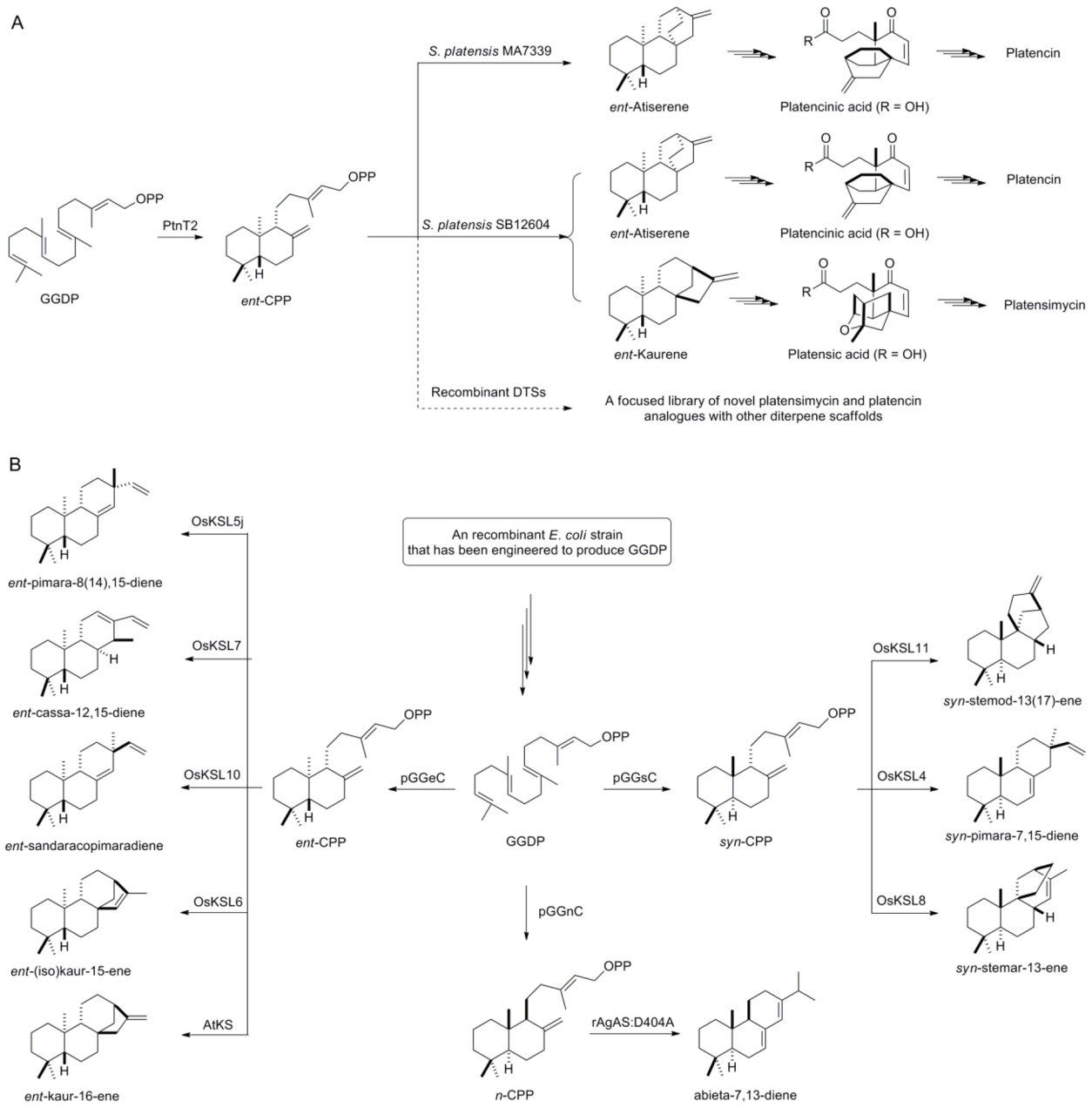

Figure 4.

(A) Modularity of DTS biochemistry demonstrated by conversion of the platencin-producing strain, *S. platensis* MA7339, into a platensimycin and platencin dual-producing strain, *S. platensis* SB12604, by genetic engineering [43], and a proposal of producing new analogues by heterologous expression of additional type I DTSs in *S. platensis* MA7339. (B) Utility of DTS modularity demonstrated through expression of various type I and type II DTSs in a recombinant *E. coli* strain that has been engineered to produce GGDP to yield eight different diterpene scaffolds, which provide an entry point to engineered production of thousands of diterpenoid natural products. DTSs, diterpene synthases; *ent*-CPP, *ent*-copalyl diphosphate; *n*-CPP, *normal*-copalyl diphosphate; *syn*-CPP, *syn*-copalyl diphosphate; GGDP, geranylgeranyl diphosphate; pGGeC, pGGnC, and pGGsC, three engineered type II DTSs that convert GGDP to *ent*-CPP, *n*-CPP, and *syn*-CPP, respectively; AtKS,

rAgAS:D404A, OsKSL4, OsKSL5j, OsKSL6, OsKSL7, OsKSL8, OsKSL10, and OsKSL11, 11 engineered type II DTSs that convert *ent*-CPP, *n*-CPP, or *syn*-CPP to the nine diterpenoid scaffolds, respectively [66].