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Aromatic Polyketides Produced by Bacterial Iterative Type I Polyketide Synthases

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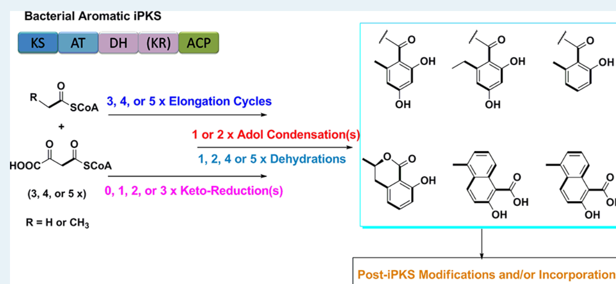
ABSTRACT: Polyketides comprise a large and highly diverse group of natural products produced by polyketide synthases (PKSs), and many of these compounds display remarkable biological activities. Although PKSs share a common mechanism in the assembly of polyketides from short carboxylic acid precursors, different types of PKSs have been classified according to their structures and modes of action. This review discusses a growing group of bacterial PKSs that are structurally type I but act in an iterative manner to produce aromatic polyketides. We summarize the genetic and biochemical features of these enzymes and compare them with other types of PKSs with an emphasis on the evolutionary relationship. We also discuss the different mechanisms for polyketide off-loading and the diverse post-PKS modifications on the resulting aromatic rings. Insights into bacterial iterative type I PKSs for aromatic polyketide formation and the relevant tailoring enzymes may guide rational bioengineering efforts to produce novel natural products.



KEYWORDS: *aromatic polyketides, polyketide synthases (PKSs), iterative type I PKSs (iPKSs), post-iPKS modifications, polyketide natural products*

INTRODUCTION

Polyketides represent a large group of natural products with highly diverse structures and biological activities.[1] These metabolites are found in bacteria, fungi, plants, and protists and constitute an important source of therapeutics, including antibiotics, immunosuppressants, antiparasitics, antitumorals, and cholesterol-lowering agents. Despite their vast structural and functional differences, polyketides share a common biosynthetic mechanism, as the carbon backbone arises from the programmed assembly of short chain acyl coenzyme A (CoA) precursors, and this process is catalyzed by polyketide synthases (PKSs).[1−3] The PKS chemistry is similar to that of fatty acid synthases (FASs): they share a common mechanism to perform the biosynthesis via a phosphopantetheinyl group, either from the CoA derivative or from the posttranslationally modified acyl carrier protein (ACP). The elongation of the polyketide chain requires the activity of the ketosynthase (KS), which catalyzes a decarboxylative Claisen condensation between the thioester-based substrates. In fatty acid biosynthesis, the β-keto group of the nascent polyketone chain is fully processed by stepwise ketoreductase (KR)-, dehydratase (DH)-, and enoyl reductase (ER)-catalyzed reactions to yield a saturated acyl chain. In contrast, PKSs use optional reductive steps to process the β-keto group, thus giving rise to a more complex pattern of functionalities. Several other factors, such as chain length control, the off-loading mechanism, and the selection of starter and extender building blocks, further expand the variability of the chemical structure. In addition, the resulting polyketide scaffold can be subjected to highly diverse post-PKS modifications for maturation. The great diversity in polyketide biosynthesis highlights the potential for pathway engineering to produce novel "unnatural" natural products using combinatorial biosynthesis and synthetic biology strategies, which have already been successfully implemented, particularly over the past decade.[4−9]

The striking similarities between FASs and PKSs suggest that they may have evolved from a common ancestor and diverged during evolution to constitute a branch point between primary and secondary metabolisms. Similar to FASs,[10,11] PKSs have been classified into different types according to their enzyme architectures and catalytic mechanisms.[1−3] Type I PKSs are multifunctional proteins consisting of linearly arranged and covalently fused domains for individual catalytic activities,[9,12] whereas type II PKSs are dissociable multienzyme complexes consisting of discrete and usually monofunctional proteins.[13] The chalcone synthase-like proteins are grouped as type III PKSs.[9,10] Unlike type I and type II PKSs, type III PKSs use CoA instead of ACP as an anchor for chain extension. PKSs are also categorized as iterative or noniterative based on whether they catalyze multiple rounds of elongation. Both type II and type III PKSs are iterative, whereas type I PKSs can be either iterative or noniterative. Despite some exceptions, the distribution of different PKSs appears to be origin-dependent.[1] Type II PKSs are found exclusively in bacteria, whereas type III

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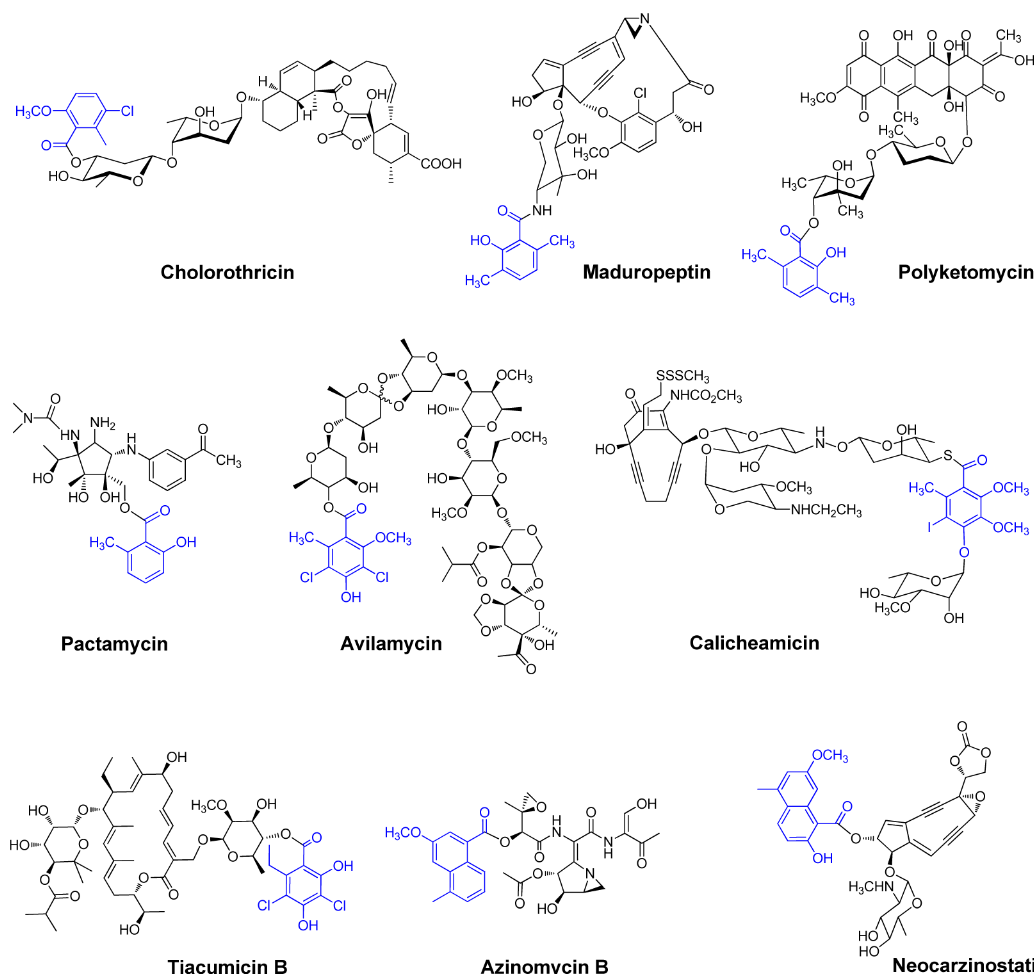

Figure 1. Representative natural products that contain bacterial iPKS-programmed aromatic polyketide moieties (highlighted in blue).

PKSs are primarily found in plants. Noniterative type I PKSs (often termed modular type I PKSs), the megasynthases as exemplified by the archetypal 6-deoxyerythronolide synthase (DEBS) in erythromycin biosynthesis, are found predominantly in bacteria and, very recently, in protozoans.[14,15] Iterative type I PKSs (iPKSs), defined as the PKSs that are structurally type I but act in an iterative manner, often serve as a hallmark for fungal polyketide biosynthesis.[16−18] The origin-dependent distribution of PKS suggest that these enzymes diverged into different classes very early, possibly before the divergence between FAS and PKS, as shown by some phylogenetic studies.[19]

Although iPKSs are mainly found in fungi, increasing evidence suggests that these enzymes are also widely distributed in bacteria and are essential for the biosynthesis of many bacterial metabolites (Figure 1). This review discusses a growing subclass of bacterial iPKSs that produce aromatic polyketides. We summarize the recent advances in the genetics and biochemistry of these enzymes and discuss the diverse strategies in the post-iPKS modifications. The commonality and specificity of bacterial aromatic iPKS compared with other PKSs, such as fungal iPKSs, are also highlighted. Because other subclasses of bacterial iPKSs and relevant FASs, including enediyne PKSs[20−22] and polyunsaturated fatty acid (PUFA) synthases,[23,24] have been well documented, they are not discussed in detail here.

Bacterial iPKSs and Their Associated Aromatic Polyketide Products. Unlike the complex multicyclic aromatic scaffolds produced by type II PKSs (e.g., oxytetracycline[25]), polyketides produced by bacterial aromatic iPKSs are relatively simple and consist of a set of mono- or bicyclic aromatic products (Figure 2). These products include 6-methylsalicylic acid (6-MSA) in chlorothricin,[26,27] maduropeptin,[28] polyketomycin,[29] and pactamycin;[30,31] orsellinic acid (OSA) in avilamycin[32] and calicheamicin;[33] homoorsellinic acid (hOSA) in tiacumicin B;[34] 5-methyl-1-napthoic acid (5-methyl-1-NPA, NPA represents naphthoic acid) in azinomycin B;[35,36] 2-hydroxyl-5-methyl-1-naphthoic acid (2-hydroxyl-5-methyl-1-NPA) in neocarzinostatin;[37,38] and (*R*)-mellein that is encoded by an orphan PKS gene *SACE5532* (or *pks8*) from *Saccharopolyspora erythraea*.[39] Although most of these polyketides serve as the building blocks in the biosynthesis of certain natural products (Figure 1), whether (*R*)-mellein could be incorporated into unknown metabolites remains to be determined.

Architecture and Evolutionary Insights into Bacterial Aromatic iPKSs. Bacterial aromatic iPKSs share high homology with each other and are typically organized in a KS-AT-DH-(KR)-ACP order, except for the lack of KR domain in OSA synthases (Figure 2A). This organization is similar to that of modular type I PKSs but is distinct from that of other bacterial iPKSs, including enediyne PKSs and PUFA synthases (Figure 2A). Bacterial aromatic iPKS are also highly

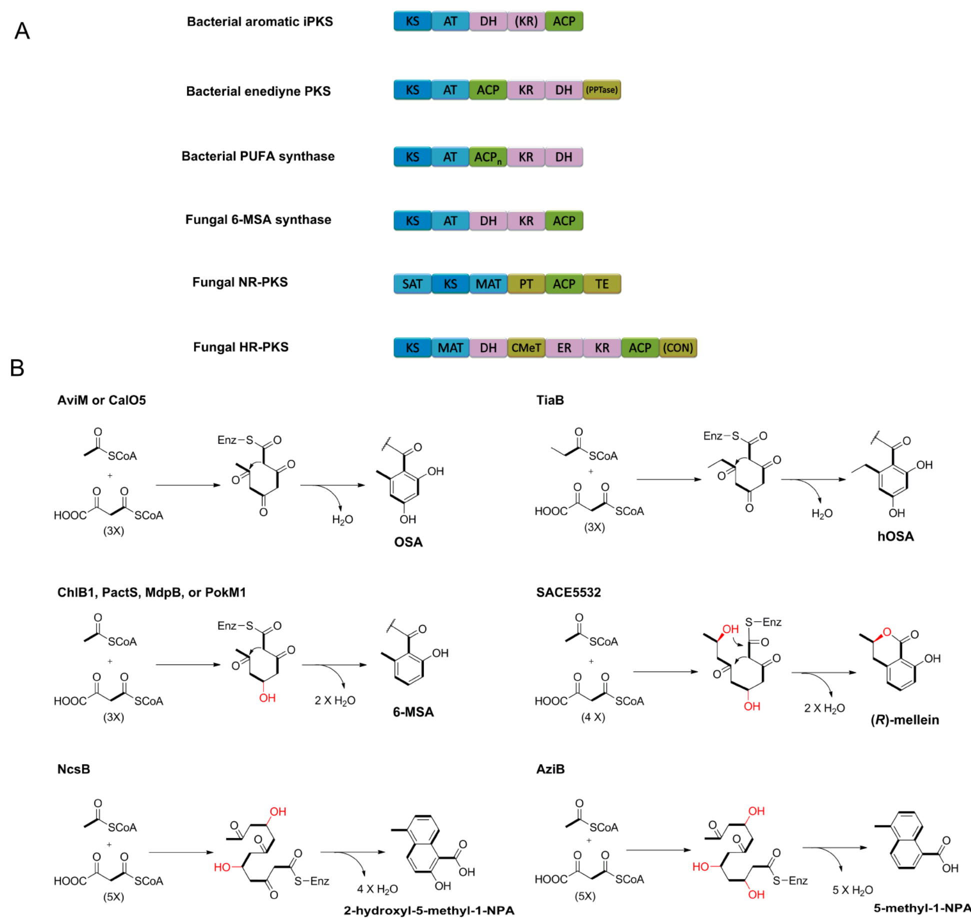

Figure 2. Architecture comparison of iterative type I PKSs and FAS, and aromatic polyketide formation catalyzed by bacterial iPKSs. (A) Domain organizations of iterative type I PKSs and FAS, including aromatic iPKS, enediyne PKS and PUFA synthase in bacteria, and typical NR-PKS and HR-PKS in fungi. KS, β-ketoacyl-ACP synthase (ketosynthase); AT, acyltransferase; DH, dehydratase; KR, ketoreductase; ACP, acyl carrier protein; ACP*n*, multiple ACPs; PPTase, phosphopantetheinyl transferase; SAT, starter unit-ACP transacylase; MAT, malonyl CoA−ACP transacylase; PT, product template; TE, thiolesterase; *C*-MeT, *C*-methyltransferase; ER, enoyl reductase; and C, condensation domain. (B) Bacterial aromatic iPKS-catalyzed reactions. The products include OSA produced by AviM or CalO5; hOSA produced byTiaB; 6-MSA produced by ChlB1, PactS, MdpB, or PokM1; (*R*)-mellein produced by SACE5532; 2-hydroxyl-5-methyl-1-NPA produced by NcsB; and 5-methyl-1-NPA produced by AziB. The selective keto-reduction patterns are highlighted in red.

homologous to fungal 6-MSA synthases,[17] in both amino acid similarity and domain organization (Figure 2A). Fungal iPKSs can also synthesize the OSA moiety. The fungal OSA synthases, however, are a diverse group of enzymes with variable architecture and catalytic outcomes and are distinct from bacterial OSA synthases.[40] This suggests a convergent evolution of PKSs to produce similar aromatic polyketides. Fungal OSA synthases belong to the family of nonreducing PKSs (NR-PKSs), which, in addition to the KS, AT and ACP domains, contain an extended N-terminal domain that functions as a starter unit-ACP transacylase (SAT) for priming polyketide biosynthesis and a central product template (PT) domain that is involved in the control of intermediate cyclization (Figure 2A).[16,41−43] Another large family of fungal PKSs is the highly reducing PKSs (HR-PKSs), as exemplified by LovB and LovF in lovastatin biosynthesis.[44] Fungal HR-PKSs are also modular PKSs, and some of them contain an additional *C*-methyltransferase (CMeT) domain[18] (Figure 2A).

Several phylogenetic studies have investigated the evolutionary process and catalytic diversity of PKSs.[19,45−47] Remarkably, in the KS phylogenetic tree, fungal 6-MSA synthases nest within the clade containing bacterial aromatic iPKSs and are far separated from the clade containing other fungal PKSs.[46,47] This result suggests that, unlike most fungal iPKSs, fungal 6-MSA synthases may possibly have originated from bacteria via an ancient horizontal gene transfer process, which may serve as a mechanism to enhance the biosynthetic capabilities of fungi.[47] Further studies are needed to validate this proposal. The KS phylogeny of various PKSs, including 10 currently characterized bacterial aromatic iPKSs, is shown in Figure 3A. Bacterial aromatic iPKSs form a group that is sister to the fungal 6-MSA synthase group. These two groups of enzymes make up a large clade that is sister to modular PKSs but are phylogenetically distant from the clade containing two other types of bacterial iPKSs, that is, enediyne PKSs and PUFA synthases, and the clade containing fungal NR-PKSs and HR-PKSs (Figure 3A). The phylogenetic analysis based on the full-length enzymes, including fungal 6-MAS synthases and bacterial aromatic iPKSs, also shows that bacterial iPKS and fungal 6-MSA synthases cluster into different clades (Figure 3B). The phylogenetic difference between bacterial aromatic iPKSs and other PKSs has facilitated the development of a rapid PCR approach to specifically access the genes encoding these enzymes. This approach was successfully employed to clone the biosynthetic gene clusters of chlorothricin[26,27] and azinomycin B,[35] suggesting a potential application for screening bacterial

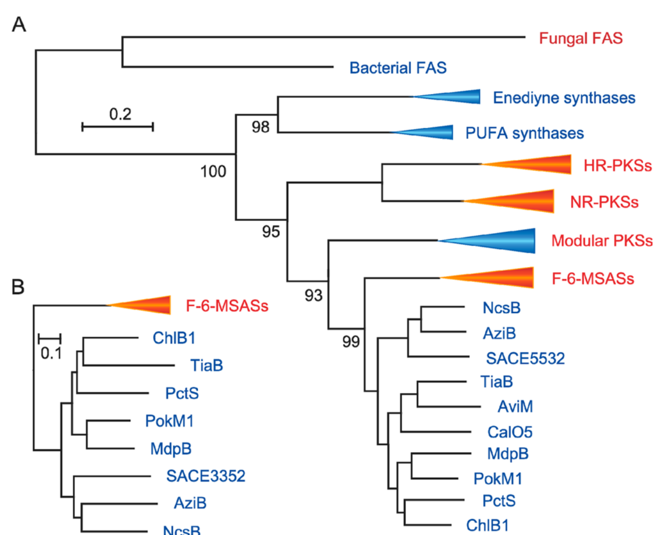

Figure 3. Phylogenetic analysis of different PKSs based on KS domains (A) and full-length proteins (B). Enzymes from bacteria and fungi are shown in blue and orange, respectively. The triangles represent the phylogenetic clades containing multiple sequences of a specific type. Enzymes and their GenBank accession numbers are listed as follows: fungal FAS (FasA from *Aspergillus flavus*, XP_002377327.1), bacterial FAS (Fas from *Mycobacterium leprae*, CAC31572.1), enediyne synthases (3 sequences, including SgcE, ZP_11383500.1; MdpE, AAQ17110.2; and NcsE, AAM78012.1), PUFA synthases (3 sequences, including PfaA from *Photobacterium profundum*, AAL01060.1; Orf8 from *Moritella marina*, BAA89382.2; and OrfA from *Schizochytrium* AAK72879.2), HR-PKSs (5 sequences, including Fum1p, AAD43562.2; FUSS, AAT28740.1; LovF, AAD34559.1; LovB, AAD39830.1; and MkB, ABA02240.1), NR-PKS (5 sequences, including PksA from *A. flavus*, AAS90093.1; PKS1 from *Colletotrichum lagenaria*, BAA18956.1; PKS1 from *Glarea lozoyensis*, AAN59953.1; PKS4 from *Fusarium fujikuroi*, CAB92399.1; and At4 from *Aspergillus terreus*, BAB88689.1); modular PKSs (8 sequences, including PimS1: module 1 and 2, CAC20931.1; MonAI: module 1 and 2, AAO65796.1; EryA1: module 2 and 3, YP_001102988.1; and AveS1: module 2 and 3, NP_822113.1), fungal 6-MSA synthases (4 sequences, including Atx, BAA20102.2; Ppa, CAA39295.1; Glo, AAX35547.1; and Bni, AAK48943.1), and bacterial aromatic iPKS (10 sequences, including ChlB1, AAZ77673.1; PokM1, ACN64831.1; MdpB, ABY66019.1; PctS, BAF92601.1; AviM, AAK83194.1; CalO5, AAM70355.1; TiaB, ADU85988.1; AziB, ABY83164.1; NcsB, AAM77986.1; and SACE_5532, YP_001107644.1). For the analysis shown in B, only iPKSs that share head-to-tail homology are included, and bacterial OSA synthases are omitted because they lack the KR domain. Numbers above branches indicate bootstrap values (1000 replicates) of the major clades. The bootstrap values within each clade are mostly larger than 85. Analyses were performed on the basis of neighbor joining algorithm with the Jones−Taylo−Thornton (JTT) substitution model using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html).

genomes to identify iPKS-programmed, novel aromatic polyketides.

Selective Ketoreductions in Bacterial Aromatic iPKS-Catalyzed Reactions. Selective keto-reduction is biosynthetically essential for the chemical diversity of polyketides and plays a role in diversifying the small group of bacterial aromatic iPKSs. For example, AziB and NcsB share the same chain length control and cyclization pattern; however, because of different keto-reduction actions, they produce different products varying in the hydroxyl functionality on the NPA rings (5-methyl-1-NPA for AziB and 2-hydroxyl-5-methyl-1-NPA for NcsB). In addition, in bacteria, the catalysis of 6-MSA synthases is highly similar to that of OSA synthases, differing only in the keto-reduction step. The five regiospecific keto-reduction patterns in characterized bacterial aromatic iPKSs are summarized in Figure 2B.

The KR domain of SACE5532 has been studied in vitro using different substrate analogs, with the KR domain of NcsB as a control.[39] Both the KR domains were expressed as the stand-alone proteins that were enzymatically active, as demonstrated by the ability to reduce trans-1-decalone, a nonspecific substrate used for assaying the KR activity.[48] However, only the KR from SACE5532 is active when analogs of the diketide intermediates, S-ethyl acetoacetate and S-acetoacetyl-N-acetylcysteamine (acetoacetyl-S-NAC), were used as the substrates. This result suggests that the programmed keto-reduction in these enzymes is achieved via the discrimination of the polyketide intermediates by the KR domain alone.[39] Previous biochemical studies revealed that fungal 6-MSA synthase only produces a triketide shunt product, i.e., triacetic acid lactone (TAL) in the absence of NADPH, and this shunt product was also produced by the KR mutant enzyme with the mutated NADPH binding site (Figure 4).[17,18,49] Together with the in vitro study on the KR domain of SACE5532, the selective keto-reduction reactions likely occur during the chain elongation process in polyketide biosynthesis. Most of the hydroxyl groups resulting from keto-reduction are eliminated during aromatization; however, (*R*)-mellein does retain the stereochemistry of its precursor (*R*)-hydroxyl group,[39] suggesting that SACE5532 contains a B-type KR that stereospecifically produces (*R*)-hydroxyl (D-hydroxyl) groups.[50,51] Consistent with this observation, all the KR domains of bacterial aromatic iPKSs lack the conserved Trp residue found in A-type KRs but contain a motif similar to the characteristic Leu-Asp-Asp motif of B-type KRs (e.g., the SACE5532 KR contains a Val-Asn-Asp motif).[50,51] Thus, all the KR domains of bacterial aromatic iPKSs are predicted to be specific for generating a D-hydroxyl group; this hypothesis needs further examination.

The diverse keto-reduction pattern in bacterial aromatic iPKSs suggests that alterations of the functionalities on the aromatic polyketides may be accomplished by engineering the KR domains. Several studies have shown that for modular type I PKS, alteration of β-keto in polyketide functionalities can be achieved by modulating the activities of the KR, DH, or ER domains during the chain extension process.[52] Furthermore, OSA and hOSA synthases do not have a functional KR domain but are able to successfully assemble the tetraketide intermediates to generate the mature products (Figure 2B). Consistent with these findings, the bacterial 6-MSA synthase ChlB1, which is involved in the biosynthesis of chlorothricin, was engineered into an OSA synthase (Figure 4) after the replacement of a highly conserved Tyr residue (corresponding to Tyr1540 of ChlB1) with Phe.[53] This Tyr residue participates in forming a catalytic triad along with the Lys and Ser residues and is thus catalytically essential for the KR activity.[54,55] The production of OSA from this mutant enzyme is slightly decreased but comparable to that of 6-MSA produced by the wide type ChlB1.[53] Remarkably, the mutant enzyme is compatible with the downstream tailoring enzymes, leading to the production of two novel OSA-derived chlorothricin analogs that exhibit antibacterial activities comparable to that of the parent compound.[53] In contrast, replacing ChlB1 with

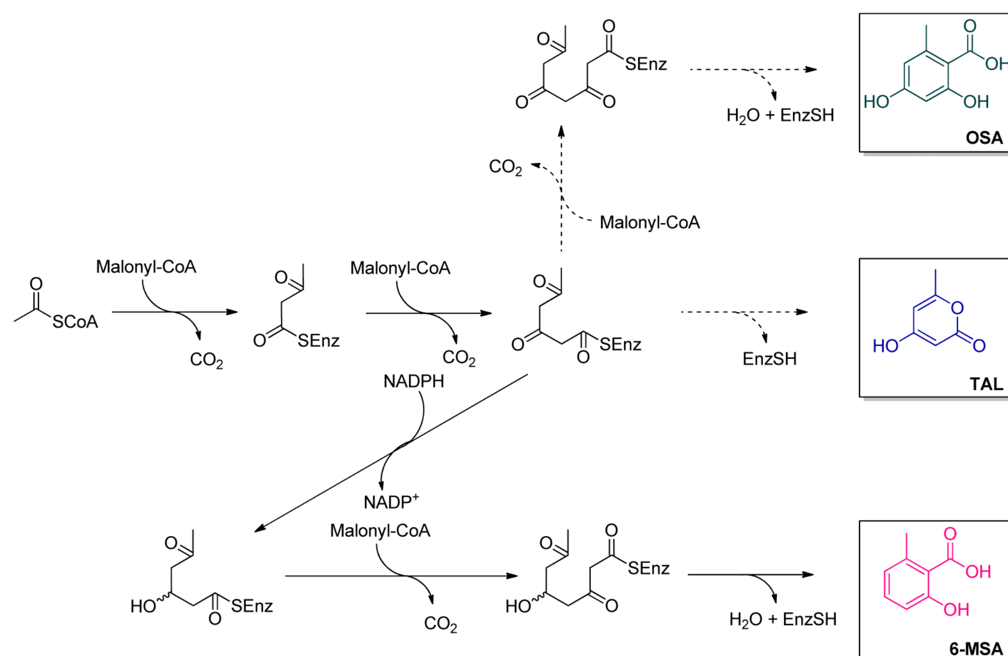

Figure 4. Different reactions catalyzed by fungal and bacterial 6-MSA synthases and their mutants. The products include 6-MSA, TAL synthesized by KR-mutated fungal 6-MSA synthases (mutation in the NADPH binding site A*x*P*xx*A) or by native fungal 6-MSA synthase in the absence of NADPH, and OSA synthesized by KR-mutated ChlB1 (Y1450F at the active site). Solid arrows indicate the native enzyme-catalyzed reactions, whereas dashed arrows show the shunt or engineered reactions.

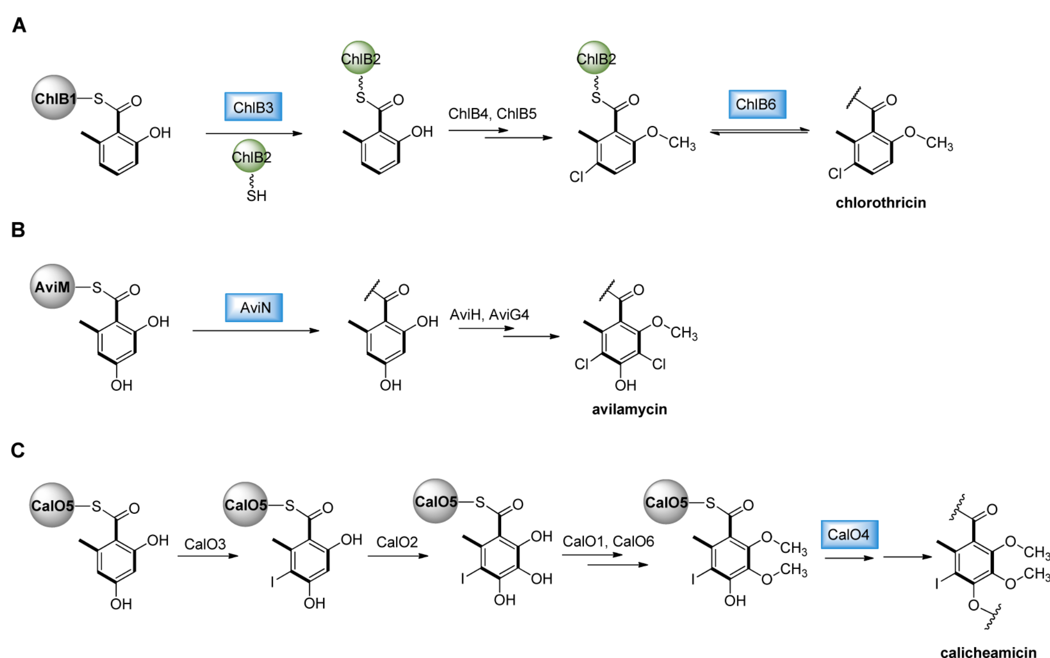

Figure 5. Hypothesis regarding the AT-mediated polyketide off-loading and associated post-iPKS modifications in the biosynthesis of chlorothricin (A), avilamycin (B), and calicheamicin (C). The off-loading mechanism in the biosynthesis of pactamycin and tiacumicin B could be similar to that of avilamycin. ATs are represented by blue rectangles, and the iPKSs and ACP are depicted as gray and green circles, respectively. It should be noted that the mechanisms shown in B and C are only speculative and require testing.

exogenous bacterial OSA synthase AviM in avilamycin biosynthesis yields only trace amounts of OSA-derived analogs,[53] which may be due to inefficient crosstalk between the exogenous PKS and the downstream enzymes.

Uncharacterized Catalytic Issues for Bacterial Aromatic iPKSs. One of the remaining questions regarding the catalysis of bacterial aromatic iPKSs is their priming mechanism. Unlike fungal NR-PKSs, which utilize a specialized SAT domain for loading the starter unit (Figure 2A), bacterial iPKSs do not have a SAT domain for priming polyketide biosynthesis. The fungal 6-MAS synthase Axt and some HR-PKSs, such as LovB, can use malonyl-CoA for chain priming by loading of malonate and subsequent decarboxylation to make the acetyl starter unit.[44,56] Distinctly, the bacterial aromatic iPKS SACE5532 produced (*R*)-mellein in vitro only in the presence of acetyl-CoA, malonyl-CoA, and NADPH.[39] How

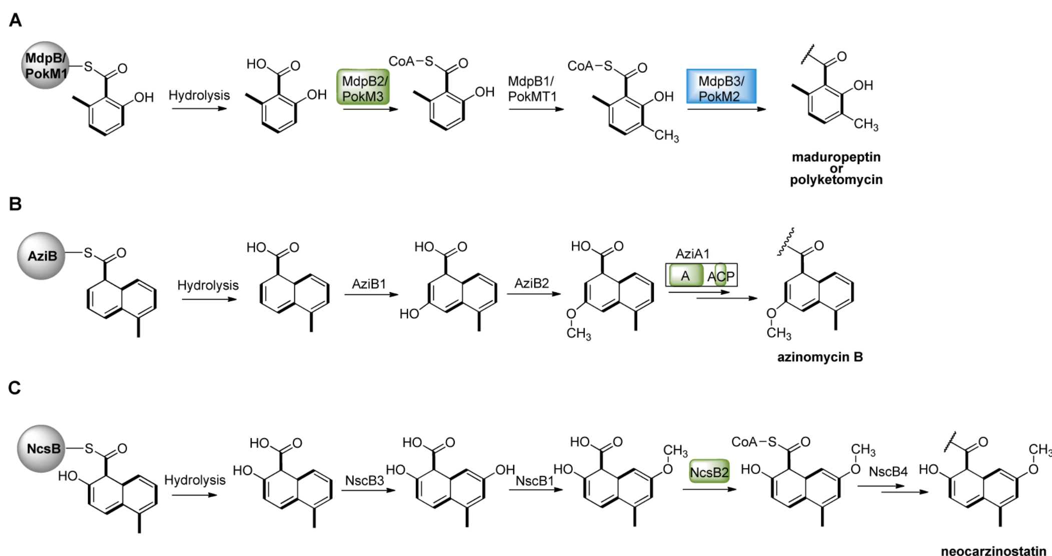

Figure 6. Hypothesis regarding the hydrolytic polyketide off-loading and corresponding post-iPKS modifications in the biosynthesis of maduropeptide/polyketomycin (A), azinomycin B (B), and neocarzinostatin (C). The promiscuous adenylation enzymes and the A domain responsible for activating the free carboxyl group of aromatic polyketides are represented by light blue rectangles. The iPKSs are depicted as gray circles.

the starter units (acetyl in most cases and propionyl in hOSA biosynthesis) are loaded onto the bacterial aromatic iPKSs is unclear. It could be hypothesized that this process may be achieved via a self-acylation mechanism; however, this hypothesis needs to be tested in future biochemical studies.

Another interesting aspect of the catalytic mechanism is the function of the DH domain, which is present in all bacterial aromatic iPKSs (Figure 2A). Mutagenesis studies have indicated that this domain is of catalytic importance because replacing the His residue within the conserved H*xxx*G*xxxx*P motif of the DH domain with a Phe residue abolished 5-methyl-1-NPA production in azinomycin biosynthesis[35] and significantly reduced 6-MSA production in chlorothricin biosynthesis.[53] Bacterial OSA and hOSA synthases do not appear to produce the β-hydroxypolyketide intermediates required for DH activity, raising the question of whether the so-called DH domain really functions as a dehydratase. Notably, psymberin, a sponge-symbiotic bacterial natural product produced by a NRPS-PKS hybrid system, also contains an OSA-like moiety, and the PKS module involved in the biosynthesis of this moiety (PsyD module 10) contains an inactive DH domain lacking conserved catalytic residues.[57] The DH domain of the fungal 6-MSA synthase was recently shown to be essential for the hydrolytic release of 6-MSA from the enzyme and was accordingly renamed as a thioester hydrolase (TH) domain.[56] Whether thioester hydrolysis is the sole function of this domain needs further investigation. Notably, unlike fungal NR-PKSs that contain a PT domain for the regiospecific cyclization of the nascent polyketide chain (Figure 2A), the factor that controls the cyclization pattern in the catalysis of bacterial aromatic iPKSs is unclear. Despite having minimal sequence similarity to known enzymes, the crystal structure of the PT domain displays a distinct "double hot dog" fold that is a variant of those observed in the DH domains of animal FASs and bacterial modular PKSs.[42] This structural similarity suggests that the DH domain of bacterial aromatic iPKSs could possibly play a role in the cyclization reaction to maturate the polyketide products.

Two Different Mechanisms for Polyketide Off-Loading. The aromatic polyketides produced by bacterial iPKSs are released from the enzymes by distinct ways and are subject to a diverse array of post-iPKS modifications. The mechanism for polyketide off-loading could be roughly grouped into two types. The first type is found in the biosynthesis of chlorothricin, pactamycin, avilamycin, tiacumicin B, and calicheamicin, during which a dedicated AT is utilized to release the polyketides products from PKSs (Figure 5). In contrast, the second type is found in the biosynthesis of maduropeptin, polyketomycin, azinomycin B, neocarzinostatin, and (*R*)-mellein, during which iPKSs release the polyketide products by direct hydrolysis (Figure 6). For the second type, in all cases except (*R*)-mellein, the released polyketides are then activated by an adenylating enzyme before or after modification(s) on the aromatic rings and are subsequently incorporated into the scaffolds of certain natural products.

AT-Mediated Polyketide Off-Loading and Associated Post-iPKS Modifications. Chlorothricin biosynthesis serves as a unique example of post-iPKS modifications that center on a discrete ACP protein (Figure 5A).[58] A closely linked gene cassette consisting of *chlB*1−*B6* resides within the chlorothricin biosynthetic locus, and the genes of this cassette are proposed to encode the enzymes involved in the biosynthesis and modification of the 6-MSA moiety.[26] ChlB2 is a discrete ACP, whereas ChlB3 and ChlB6 are two ATs with high sequence similarity to each other and are homologous to the ketoacyl-ACP synthase III family of enzymes that initiate elongation in type II fatty acid synthase systems.[59] Despite that both ChlB3 and ChlB6 transferred the salicyl moiety from salicyl-S-NAC to the holo-form of ChlB2, only ChlB3 was able to load the 6-methylsalicyl moiety from the ChlB1 ACP domain onto the holo-ChlB2 (Figure 5A).[58] Conversely, only ChlB6 was able to transfer the 5-chloro-6-methyl-*O*-methylsalicyl group from ChlB2 onto the demethylsalicyl chlorothricin intermediate to produce the mature product.[58] This reaction can also proceed in the reverse direction by deacylating chlorothricin in the presence of holo-ChlB2 to produce demethylsalicyl chlorothricin and salicylated ChlB2 (Figure 5A).[58] Intriguingly, mutation of a conserved Cys residue in ChlB3 to Ser significantly decreased the activity of ClhB3, allowing for the

detection of a ChlB3-associated salicyl intermediate. These results supported that the acyl transfer reaction is mediated by the conserved Cys residue of ChlB3,[58] as opposed to most ATs that possess a Ser residue as the nucleophile to channel the acyl group. These studies clearly established an ACP-centered strategy that involves two distinct acyl transfer steps for the modifications and attachment of the 6-MSA moiety in chlorothricin biosynthesis. Although other modifications, including ChlB4-catalyzed halogenation and ChlB5-catalyzed O-methylation, have yet to be characterized in vitro, these two reactions can be assumed to occur on the ACP-tethered substrates.

The biosynthesis of pactamycin, avilamycin, tiacumicin B, or calicheamicin involves only one ChlB3/ChlB6 homologue and, in most cases, does not contain a discrete ChlB2-like ACP. There is an ACP protein, PacK, in the pactamycin biosynthetic pathway;[30,31] however, its homologue was also found in the biosynthesis of mitomycin C,[60] a compound that does not have an aromatic polyketide moiety, therefore excluding the involvement of PacK in polyketide off-loading. Because the 6-MSA moiety is not further modified in pactamycin biosynthesis, it is likely that, after the assembly of the 6-methylsalicyl moiety by iPKS PctS, the ChlB3/ChlB6 homologue PctT directly transfers the 6-methylsalicyl moiety from PctS onto the demethylsalicyl pactamycin intermediate to yield pactamycin. A similar off-loading mechanism might also be utilized in the biosynthesis of tiacumicin B and avilamycin, and modifications on the OSA rings may occur after their attachment onto certain biosynthetic intermediates (Figure 5B). Genetic knockout of the halogenase gene *tiaM* in the tiacumicin B producing strain resulted in a tiacumicin B analog that lacks the two chlorine atoms on the OSA ring.[34] This dechlorinated analog can be chlorinated in vitro by TiaM to yield tiacumicin B and an analog containing only one chlorine atom on the OSA moiety,[34] supporting that the sequential chlorination reactions occur as the last step for tiacumicin B maturation. AviH, which shares high sequence similarity with TiaB, likely chlorinates the OSA moiety of avilamycin in a similar manner.[32] This chlorination could be followed by the O-methylation catalyzed by AviG4, which was supported by systematic knockout studies in avilamycin biosynthesis (Figure 5B).[61]

The OSA moiety of calicheamicin is subject to more extensive modifications, including the putative CalO3-catalyzed iodination, CalO2-catalyzed hydroxylation, CalO6-catalyzed C2 O-methylation and CalO1-catalyzed C3 O-methylation. The proposed reaction sequence shown in Figure 5C is based on a combination of biochemical studies, ligand-binding analysis, crystal structure studies, and docking analysis.[62,63] Intriguingly, these studies have suggested that the modifications occur on substrates that bind to a pantetheinyl arm, which comes from either a CoA or an ACP.[62,63] Because neither CoA-ligase nor discrete ACP is known to be involved in calicheamicin biosynthesis, it is unclear how these modifications are achieved. Docking studies with the crystal structure of cytochrome P450 hydroxylase CalO2 and the homologous model of the ACP domain of OSA synthase CalO5 have revealed a putative docking site within CalO2 for accommodating the CalO5 ACP domain, which involves a well-ordered helix along the CalO2 active site cavity that is different from other P450s.[62] Although appearing less likely, this result suggests a possibility that CalO5 may hold the nascent OSA moiety on its ACP and then recruits other enzymes to modify OSA in calicheamicin biosynthesis (Figure 5C). Further studies are needed to explore these intriguing issues.

Hydrolytic Polyketide Off-Loading and Associated Post-iPKS Modifications. The biosyntheses of maduropeptin and polyketomycin employ a strategy distinct from that of chlorothricin and pactamycin for modifying the resulting 6-MSA moiety. The maduropeptin biosynthetic gene cluster does not encode a ChlB3/ChlB6 homologue but instead contains a gene *mdpB2*, which encodes an adenylation enzyme and is closely linked to the 6-MSA synthase gene *mdpB*.[28] In vitro studies showed that MdpB2 efficiently converted 6-MSA to its CoA thioester in the presence of ATP and CoA,[64] suggesting that the resulting polyketide is hydrolytically released as a free carboxylic acid from the PKS. Interestingly, the C-methylation catalyzed by MdpB1 occurs on the CoA-tethered 6-MSA substrate instead of 6-MSA.[64] The attachment of the aromatic ring to the amine group of a sugar moiety may be catalyzed by the putative AT MdpB3 (Figure 6A).[64] Polyketomycin biosynthesis is likely similar to maduropeptin biosynthesis in MSA modification because its gene cluster encodes a set of enzymes PokM1, PokMT1, and PokM3 that are highly homologous to MdpB, MdpB1, and MdpB2, respectively.[29] This gene cluster also encodes an AT PokM2 that may be used to attach the C-methylated 6-MSA moiety to produce the final product (Figure 6A).[29] Thus, the building of the aryl group in maduropeptin and polyketomycin likely depends on the CoA-based modification and subsequent acyl transfer.

The production of 5-methyl-1-NPA and 2-hydroxyl-5-methyl-1-NPA in the biosynthesis of azinomycin B and neocarzinostatin, respectively, may share a similar post-iPKS modification strategy: that is, the NPA moieties are both released from the iPKSs by hydrolysis, and the modifications occur on the free carboxylic NPA substrates. In azinomycin biosynthesis, the P450 hydroxylase AziB1 catalyzes the regiospecific C-3 hydroxylation on the 5-methyl-1-NPA, and the resulting hydroxyl group is methylated by the *O*-methyltransferase AziB2 to yield the fully modified 3-methoxy-5-methyl-NPA. The modified NPA moiety is then incorporated into a nonribosomal peptide synthase (NRPS) system by AziA, which consists of an adenylation domain and an ACP domain (Figure 6B).[36] In neocarzinostatin biosynthesis, the C-7 regiospecific hydroxylation on 2-hydroxyl-5-methyl-1-NPA and its subsequent methylation on the nascent C-7 hydroxyl group are catalyzed by the P450 hydroxylase NcsB3 and *O*-methyltransferase NcsB1, respectively.[65−67] The resulting 2-hydroxyl-7-methoxy-5-methyl-NPA is then activated by NscB2 as a CoA thioester and transferred to the enediyne core by the putative AT NcsB4 (Figure 6C).[38]

A common feature in the hydrolytic off-loading pathway is that all of the adenylation enzymes (MdpB3, PokM3, NcsB2, and AziA1 in the biosyntheses of maduropeptin, polyketomycin, neocarzinostatin, and azinomycin B, respectively) show some extent of catalytic promiscuity. NcsB2 was shown to activate a set of 1-NPA analogues with different substitutions at the 2-, 5-, or 7-position.[38] Similarly, AziA1 was shown to tolerate a diverse array of substitutions on the naphthalenyl ring and was even able to activate 2-NPA analogues.[36] MdpB3 appeared less promiscuous in comparison with NcsB2 and AziA1, but it was still able to activate three of the five different substrate analogues tested with variable functionalities on the phenyl ring.[64] The catalytic promiscuity of these adenylation enzymes may be useful in biochemical engineering studies such as mutasynthesis and chemoenzymatic synthesis.

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CONCLUSION AND OUTLOOK

In terms of the chemical process, iPKSs are impressive catalysts: they load the starter and extender units and sequentially assemble them via multiple rounds of catalytic turnover to produce a polyketide chain of defined length; they selectively perform modifications at specific positions to produce varied functionalities in the growing polyketide chain; they channel and accommodate the nascent polyketide chain and maintain favorable kinetics to cyclize it regioselectively to yield a specific structural outcome; and all of these processes are controlled and catalyzed in an organized and efficient manner by a single enzyme. Compared with fungal PKSs, bacterial aromatic iPKSs constitute a relatively simple system for understanding the rationale behind the catalysis of these impressive enzymes, as well as various off-loading and post-PKS modifications. As discussed herein, although a combination of genetic and biochemical studies have begun to shed light on the mechanisms of these enzymes, many issues await further investigation. A detailed understanding of these enzymes will not only enrich our knowledge of mechanistic enzymology but could also benefit future bioengineering and synthetic biology studies to produce novel natural products with improved biological activities.

Another important part of future studies on bacterial aromatic iPKSs may be mining and characterizing novel iPKS-derived natural products. Because most of the aromatic polyketides resulting from bacterial iPKSs are building blocks of larger natural products that exhibit various pharmaceutical properties, including the antibacterial, antitumor, and antiplasmodial activities, studying bacterial iPKSs may help identify novel bioactive natural products. The distinct phylogeny of these enzymes could facilitate these efforts with the use of either PCR-based approaches or in silico analysis. With increasing information about bacterial genomes, such studies could be very fruitful, particularly at a time when new chemotherapeutic drugs are urgently needed.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

6-MSA, 6-methylsalicyclic acid; ACP, acyl carrier protein; AT, acyltransferase; CMeT, *C*-methyltransferase; CoA, coenzyme A; CON, condensation-like; DEBS, 6-deoxyerythronolide B synthase; DH, dehydratase; ER, enoyl reductase; FAS, fatty acid synthase; hOSA, homoorsellinic acid; HR, highly reducing; iPKS, iterative type I PKS; KR, ketoreductase; KS, ketosynthase; NPA, naphthoic acid; NR, nonreducing; NRPS, nonribosomal peptide synthase; OSA, orsellinic acid; PKS, polyketide synthase; PT, product template; PUFA, polyunsaturated fatty acid; SAT, starter unit-ACP transacylase; S-NAC, *N*-acetylcysteamine; TAL, triacetic acid lactone; TH, thioester hydrolase

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