Polyketide Biosynthesis

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Biosynthesis of the Antibiotic Bacillaene, the Product of a Giant Polyketide Synthase Complex of the *trans*-AT Family**

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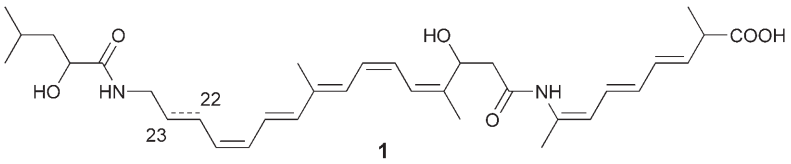
The *pksX* polyketide synthase (PKS) genes were discovered in 1993 during genome sequencing of *Bacillus subtilis* 168.[1] Although they represent one of the earliest examples of type I PKS gene clusters, their function has long remained enigmatic. Remarkably, immunogold labeling studies revealed that the PksX proteins assemble into a giant organelle-like structure with a diameter larger than 0.1 μm, which can be clearly observed by cryoelectron microscopy.[2] A virtually identical gene cluster has recently been identified in the genome of *Bacillus amyloliquefaciens* FZB 42 and was assigned to the biosynthesis of bacillaene,[3] a known antibiotic that had been largely uncharacterized because of its notorious instability.[4] NMR studies of partially purified *B. subtilis* extracts ultimately revealed the structure of bacillaene to be the highly unsaturated enamine acid **1** (Scheme 1).[5]



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Scheme 1. Bacillaene (**1**) and dihydrobacillaene (**2**), products of the *pksX* (*bae*) system in bacilli. **1** carries a double bond between C22 and C23, which is reduced in **2**.

The bacillaene cluster, subsequently termed *bae*, is noteworthy for another unusual feature. The PKS entirely lacks the acyltransferase (AT) domains that are normally present in each biosynthetic module. Instead, the AT activity is complemented by free-standing enzymes.[6] This new type of *trans*-AT architecture is now also known in many other PKS systems.[6–12] The *trans*-AT PKSs have evolved independently[13] and further differ from "regular", *cis*-AT PKSs by the common presence of unusual domains and modules, a peculiarity that represents a significant challenge in studying and engineering such enzymes. Since there is little information available on exact elongation sequences in such PKSs, we aimed to perform genetic studies on the *bae* system. These provided direct insights into almost the entire pathway, including the timing of biosynthetic steps and the function of noncanonical components.

Since bacillaene is highly unstable, we aimed to generate *B. amyloliquefaciens* strains that produced shortened, stabilized model polyketides for biosynthetic studies. This could be achieved by fusing the terminal thioesterase (TE) domain, which normally releases the fully elongated polyketide from the PKS,[14] with various upstream PKS modules (similar experiments have been made on *cis*-AT PKS[15,16]). Our bacillaene producer was the engineered strain *B. amyloliquefaciens* CH12, in which all other polyketide pathways are inactivated.[3] The crude extracts of its cultures contain two bacillaene isomers as predominant compounds and a trace amount of dihydrobacillaene (**2**) in a total yield of 100–200 mg L^{-1} (Figure 1).

To facilitate PKS construction we first deleted the TE region and part of the neighboring gene *baeS*, which encodes a cytochrome P450, by homologous

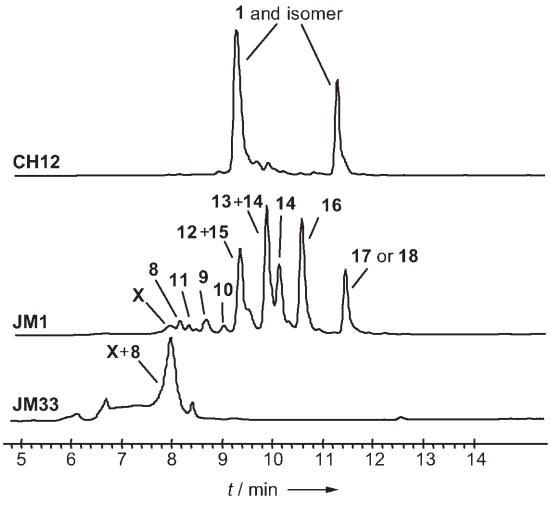

Figure 1. HPLC traces of crude extracts of *B. amyloliquefaciens* CH12, mutant JM1, and mutant JM33 recorded using a diode-array detector. "X" is an unknown compound produced by the mutants.

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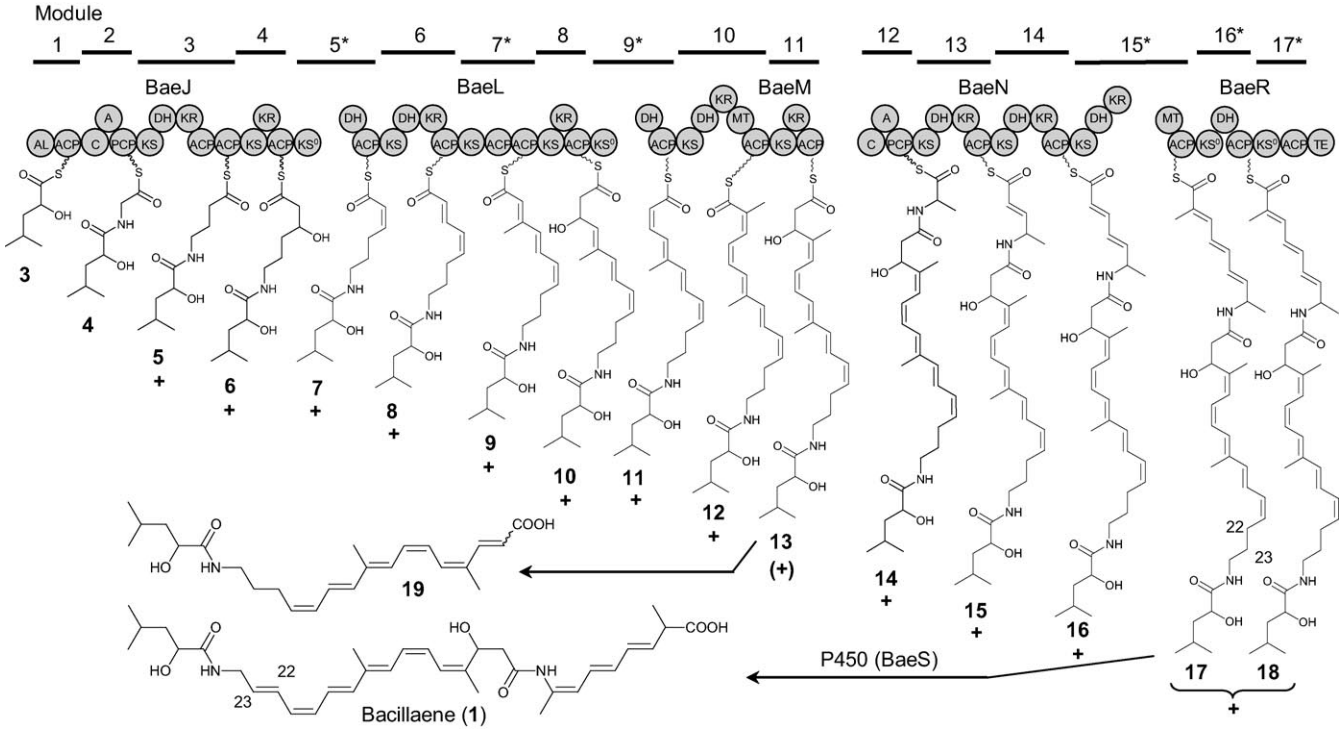
recombination to yield mutant JM1. To our surprise, when the culture extracts were checked by LC-HRMS for the expected disappearance of **1**, we detected small but, consistently present, amounts of novel compounds that were not found in extracts of the original producer (Figure 1). In total, 13 metabolites were detected with masses in the range of 217 to 582 Da. The high-resolution masses suggested the presence of 10 to 34 carbon and 1 or 2 nitrogen atoms (Table 1), and the UV spectra of the largest compounds indicated the presence of conjugated pentaene chromophores. To test whether these compounds were stalled intermediates that were spontaneously released from the PKS, we engineered two strains containing the TE relocated to modules 3 and 6, respectively. While the module 3 fusion did not yield any compounds, the module 6 mutant JM33 produced a subset of the JM1 series that lacked the larger polyketides (Figure 1). This finding suggested that virtually the entire *trans*-AT pathway is directly observable by mass spectrometry. A similar phenomenon of precursor hydrolysis is known for the rifamycin *cis*-AT PKS after deletion of the off-loading amide synthase RifF.[17] The release of all PKS intermediates has to our knowledge not been reported for TE domains.

Table 1: Measured and theoretical high-resolution masses of bacillaene intermediates detected in JM1.[a]

Deduced intermediate[b]	$[M+H]^+$ calculated	$[M+H]^+$ measured
5 ($C_{10}H_{19}NO_4$)	218.1387	218.1386
6 ($C_{12}H_{23}NO_5$)	262.1649	262.1642
7 ($C_{12}H_{21}NO_4$)	244.1543	244.1535
8 ($C_{14}H_{23}NO_4$)	270.1700	270.1694
9 ($C_{17}H_{27}NO_4$)	310.2013	310.2008
10 ($C_{19}H_{31}NO_5$)	354.2275	354.2276
11 ($C_{19}H_{29}NO_4$)	336.2169	336.2158
12 ($C_{22}H_{33}NO_4$)	376.2482	376.2474
13 ($C_{24}H_{37}NO_5$)	420.2750	402.2639[c]
14 ($C_{27}H_{42}N_2O_6$)	491.3116	491.3109
15 ($C_{29}H_{44}N_2O_6$)	517.3272	517.3249
16 ($C_{31}H_{46}N_2O_6$)	543.3429	543.3404
17 or **18** ($C_{34}H_{50}N_2O_6$)	583.3742	583.3726

[a] As assessed by HPLC coupled with ESI-MS. [b] Molecular formula of the free acid. [c] Putative spontaneous dehydration product.

Interestingly, all the recorded masses were larger by two units than those calculated from a previously proposed biosynthetic model.[5] The MS and UV data would be in full agreement if one terminal hexaene double bond in bacillaene (**1**) is generated in a post-PKS step (Scheme 2). In the smallest compound detected (the free acid of **5**), the only possible position of this bond corresponds to the C22–C23 double bond in **1**. This is also the position that is reduced in dihydrobacillaene (**2**), which suggests that this compound is desaturated by a post-PKS enzyme. It has recently been shown[18] that incubation of a total *B. subtilis* extract containing **1** and **2** with the purified cytochrome P450 PksS (BaeS) and a crude protein extract used as the reductase led to the disappearance of **2**, thus indicating a P450-mediated reaction. In our mutant strain JM1, BaeS had been inactivated together with the TE, and in accordance only **2**, and not the dehydrogenation product **1**, was detectable. Thus, these results establish that BaeS is directly involved in the desaturation of **2**. The formation of a single bond was unexpected, since the corresponding module lacks an enoylreductase domain that normally performs the reduction. Such



Scheme 2. Updated model of bacillaene biosynthesis. Intermediates marked with "+" have been detected as free acids by MS. Intermediate **13** was detected only as the dehydrated form **19**.

missing domains are another common peculiarity of *trans*-AT PKSs.

The accumulation of shunt products also provided valuable insights into the timing of other unusual steps. A common feature of *trans*-AT PKSs is the conversion of a keto function into a carbon branch, termed the β-branch.[8,9,19–22] Previous studies of enzymatically loaded single ACPs of the bacillaene[19] and curacin[20] PKS showed that such moieties are introduced by aldol addition of acetyl-ACP and subsequent Grob fragmentation. Evidence that this reaction takes place during and not after chain elongation was obtained by deleting β-branch genes of the myxovirescin[21,22] and mupirocin[23,24] pathway, which resulted in abolished or derailed production. Since the exact masses of the bacillaene intermediates were available, we could now observe directly the precise timing of this reaction. Comparison of the masses revealed that a C_3H_4 unit is introduced during the elongation step that corresponds to the site of the branched position at C17 (conversion of **8** into **9**). Consequently, formation of the β-branch must take place at the β-ketothioester stage.

The third notable feature is the presence of the noncanonical bimodular sequence KS KR ACP KS^0 DH ACP (KS^0 being a non-elongating KS) with a split downstream module on two proteins. This unusual pattern occurs twice and is also present in several other *trans*-AT systems, such as the difficidin PKS.[3] According to the structure of **1**, the bimodule introduces a single extension unit bearing a double bond. Three scenarios could be imagined for the formation of the double bond: 1) the downstream DH dehydrates in a *trans* manner an intermediate tethered to the upstream module, 2) ketoreduction and dehydration occur on two different modules, or 3) the DH is inactive, and the double bond is generated by an external enzyme. Interestingly, for each *bae* bimodule we detected a pair of intermediates with a mass difference of 18 Da, which corresponds to the loss of water (**6**/**7** and **10**/**11**). This finding suggests that stalled hydroxylated intermediates are being attached to the upstream modules and dehydrated downstream. Scenarios (1) and (3) with a *trans*-acting DH are therefore very unlikely, as in these cases no β-hydroxythioesters could accumulate. The most plausible mechanism is therefore a sequential dehydration of two modules.

In summary, this work highlights the utility of combined genetic and chemical strategies to gain detailed insights into the biosynthesis of even highly unstable natural products. The giant bacillaene multienzyme complex, the archetype member of the steadily growing class of *trans*-AT PKSs, contains a number of noteworthy features: a P450 moiety with PKS-like chemistry, on-line β-branch introduction, and the sequential introduction of double bonds into noncanonical bimodules. These insights into aspects shared by many other *trans*-AT PKSs will contribute towards analyzing and tailoring these enzymes more efficiently.

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