Biosynthetic Pathway

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Reciprocal Cross Talk between Fatty Acid and Antibiotic Biosynthesis in a Nematode Symbiont**

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In contrast to primary metabolites like basic cellular building blocks, secondary metabolites like antibiotics were often regarded as not essential to the organism that produces them,[1] because the genes encoding the biosynthetic pathways involved can be deleted without any change to the fitness of the producing organism. However, such experiments are usually done under artificial lab conditions, where the benefit to the overall fitness of the organism might not be detectable. Additionally, typical secondary metabolites like siderophores are required for growth or play an ecological role.[2] Thus, the concepts of primary and secondary metabolism are currently under debate.[3] Nevertheless, there is evidence that in microorganisms several biosynthetic pathways involved in the production of antibiotics and other secondary metabolites have evolved from basic metabolism through gene duplication and mutation events,[4] ultimately leading to the well-known and often complex biosynthetic gene clusters.[5] This scenario is supported by the fact that typical cellular building blocks like amino acids, fatty acids, or sugars are also universal precursors of secondary metabolites. For example, a ketose-derived hydroxyethyl unit has recently been identified as an extender unit in nonribosomal peptide synthetases.[6] Thus, the boundaries between both types of metabolism are hard to define. However, herein we present—to the best of our knowledge—the first biosynthesis of a true cellular building block (a fatty acid) by a biosynthetic enzyme (an antibiotic-producing polyketide synthase (PKS)) usually perceived as "secondary" and therefore non-essential.

The branched-chain keto acid dehydrogenase (BKD) complex[7] is required for the biosynthesis of branched-chain carboxylic acids playing an important role in metabolism as they serve as starter units for primary metabolites like branched-chain fatty acids[8] but also for several secondary metabolites (e.g. avermectin,[9] myxalamide,[10] myxothiazol[11]). Moreover, it has been shown recently that the extender units for type I polyketide synthases (PKS) can be derived from these branched carboxylic acids.[12] Biochemically, these carboxylic acids are derived from the branched-chain amino acids leucine, valine, and isoleucine by way of a transaminase-catalyzed conversion into the corresponding 2-oxo carboxylic acid, which is then oxidatively decarboxylated by the BKD complex to the corresponding isovaleryl- (IV), isobutyryl- (IB), and 2-methylbutyryl- (2MB) coenzyme A (CoA) thioesters, respectively. The BKD complex shows high similarity to the pyruvate dehydrogenase (PDH) complex, which catalyzes the formation of acetyl-CoA from pyruvate and thus is the entry point in the citric acid cycle.[7,13] The BKD and PDH complexes consist of three enzymes that form a large multienzyme system: E1 is a thiaminepyrophosphate (TPP) dependent dehydrogenase that decarboxylates the respective 2-oxo carboxylic acids to a TPP bound intermediate. This is then transferred as a carboxylic acid to a lipoic acid moiety covalently bound to E2 and from there to free coenzyme A, resulting in the respective CoA thioester (Figure 1 a). The reduced lipoic acid is then regenerated by E3 carrying a covalently bound FAD, which itself is regenerated by free NAD^+. The branched-chain thioesters can then be elongated by way of specific β-ketoacyl-acyl-carrier protein synthase III (FabH) enzymes catalyzing their condensation with malonyl-acyl-carrier-protein (malonyl-ACP) to give the first fatty acid intermediate as found in myxobacteria, streptomycetes, and bacilli.[14] In secondary metabolism these thioesters can be loaded onto ACP domains being part of the PKS loading module, which can then be elongated using the PKS machinery as it has been described in myxobacteria and streptomycetes (Figure 1 a).

In the entomopathogenic bacterium *Photorhabdus luminescens*, we have recently identified the biosynthetic gene cluster for isopropylstilbene (IPS).[15] The formation of this compound is different from the well-known stilbene biosynthesis in plants in that the resorcinol ring is generated by way

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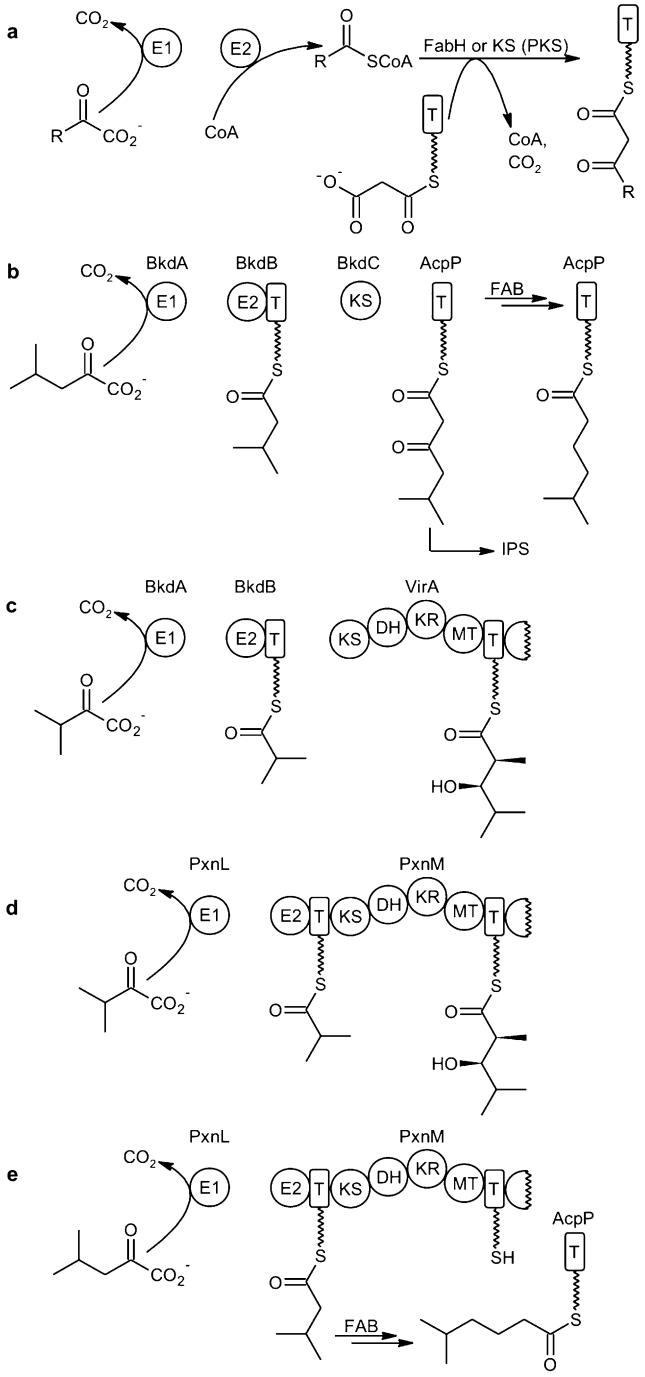

Figure 1. Examples of BKD arrangements. Standard BKD complex resulting in soluble CoA thioesters, which can be used as starting units for fatty acids and polyketides (a), BkdABC involved in isopropylstilbene (IPS) and fatty acid biosynthesis (FAB) in *Photorhabdus* (b), and BKD as part of virginiamycin biosynthesis in *Streptomyces virginiae* (c) and *Xenorhabdus nematophila* (d). The formation of iso-FAs by PxnL and PxnM is shown in (e). Thiolation (T), ketosynthase (KS), dehydratase (DH), ketoreductase (KR), methyltransferase (MT), fatty acid biosynthesis (FAB). For other details see text.

of condensation of two different thioesters, one of which results from elongation of a leucine-derived isovaleryl moiety with a malonyl unit.[15] A detailed reinvestigation of the *bkdABC* operon responsible for the biosynthesis of this thioester revealed that 1) BkdA corresponds to the E1 enzyme, 2) BkdB, the E2 enzyme, has an additional C-terminal acyl carrier protein (ACP) domain, and 3) BkdC, a ketosynthase (KS), shows greatest homology to KS of the *trans*-acyltransferase (*trans*-AT) PKS[16] (Supporting Information, Figure S1). 4) The E3 enzyme is not encoded in this operon but might be used from primary metabolism. This indicates that the product of the E1 and E2 subunits is not soluble isovaleryl-CoA as found in standard BKD complexes (Figure 1a) but is directly loaded to the ACP domain of BkdB, which would then interact with BkdC to generate 5-methyl-3-oxohexanoyl (5MOH) ACP (Figure 1b).

To test this hypothesis, we expressed the *bkd* genes from *Photorhabdus luminescens* TT01 in *E. coli* and analyzed the fatty acid (FA) profile of the resulting strains. Iso-FAs were only produced when the complete *bkdABC* operon was expressed together with a phosphopantetheinyl transferase (PPTase) required for the attachment of the phosphopantetheinyl arm to the ACP domain (Figure 2a). Without PPTase activation only trace amounts of iso-FAs were detected. These results also show that neither BkdAB nor the KS BkdC alone are sufficient for iso-FA production, which is in accordance with similar data in *Photorhabdus*.[15] Thus these results show the interaction of BkdC, a secondary metabolism-type KS of the *trans*-AT PKS type[16] with the *E. coli* fatty acid biosynthesis machinery most likely using the fatty acid biosynthesis ACP, AcpP (Figure 1b).

A BLAST-P database search with BkdB led to the identification of the corresponding homologue from *P. asymbiotica* carrying the complete *bkdABC* operon but also to the *bkdAB* operon from *Streptomyces virginiae*.[17] In *S. virginiae*,

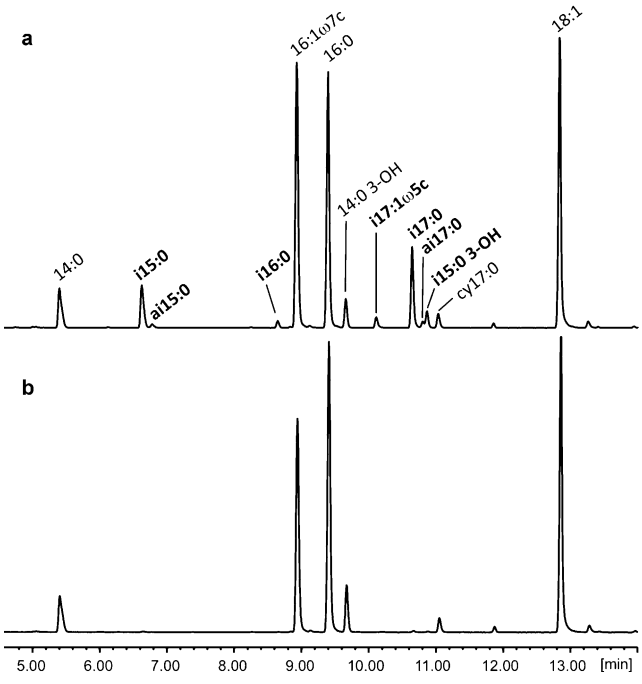

Figure 2. GC/MS chromatograms of *E. coli* strains. Expression of *bkdABC*+*mtaA* (a) and a control plasmid without any *bkd* genes (b). Expression of *bkdABC* without *mtaA*, *bkdAB* with and without *mtaA*, and *bkdC* with and without *mtaA* resulted in chromatograms identical to the control (b).

BkdAB have been shown to be required for virginiamycin M production as they provide the required isobutyryl-CoA starting unit. Here, BkdB also contains an additional C-terminal ACP domain, which was not mentioned in its previous analysis (Figure 1c).[17] Additionally, another E2 candidate from the entomopathogenic bacterium *Xenorhabdus nematophila* was identified. Here the E2 domain is part of a much larger PKS, as it was also recently shown in pristinamycin II_a biosynthesis (Figure 1d).[18] Pristinamycin II_a is a precursor for one part of the clinically used two-component antibiotic synercid that belongs to the streptogramin-type class of antibiotics.[19] A detailed analysis of the biosynthesis gene cluster (Supporting Information, Table S3) in *X. nematophila* allowed the prediction of the natural product resulting from the activity of this hybrid of *trans*-AT PKS and non-ribosomal peptide synthetase (NRPS), which to our surprise, was proposed to produce pristinamycin II_a or a close derivative thereof. Thus the biosynthetic gene cluster was named *pxn* (for pristinamycin in *Xenorhabdus nematophila*; Supporting Information, Figure S2).

Subsequent analysis of *X. nematophila* extracts obtained under different cultivation conditions revealed a peak with 526.4 *m/z* $[M+H]^+$ in extracts of *X. nematophila* infected *Galleria mellonella* larvae. HR-MALDI MS of this peak (526.2539 *m/z* $[M+H]^+$, Δppm 1.65) allowed the determination of its molecular formula as $C_{28}H_{35}O_7N_3$, which is identical to pristinamycin II_a (**1**). So far, all derivatives of this class of compounds have only been found in streptomycetes.[19] Interestingly, the pristinamycin II_a biosynthetic gene clusters in *X. nematophila* and *S. pristinaspiralis* are very similar (Supporting Information, Table S3). Although the G/C content for the *pxn* operon (46%) is very similar to other genes in *X. nematophila* (43–49%) but significantly different to the *sna* operon in *S. pristinaspiralis* (76%), the *pxn* cluster is enclosed by transposases (Supporting Information, Table S3) indicating that it might result from horizontal gene transfer. Further analysis of the *X. nematophila* genome revealed that a gene cluster for the biosynthesis of the peptide component of the streptogramin antibiotics (e.g. pristinamycin I_a)[18] is missing, which is unexpected for these two-component antibiotics. To confirm the involvement of the *pxn* biosynthetic gene cluster in the production of **1**, the gene *pxnL* was disrupted using plasmid insertion, resulting in the loss of the signal for **1**, as expected (Figure 3a). Additionally, labeling experiments were performed to confirm the structure of **1**. Briefly, L-[2,3,4,4,4,5,5,5-D_8]valine and L-[methyl-D_3]-methionine (5 mM) were injected into *G. mellonella* larvae 48 hours after injection of *X. nematophila*. At this time the insects were already dead, but the bacteria were still growing and producing **1** inside the insect cadaver (Supporting Information, Figure S3) and therefore the expected incorporation of the [D_7]isobutyryl and [D_3]methyl units were detected (Figure 3b–d).

Fatty acid (FA) analysis of *X. nematophila* showed a small amount of odd-numbered iso-FAs (iso-odd FAs) and such FAs are also part of the secondary metabolites prexenocoumacins A and C in this strain.[20] However, no *bkd* genes other than *pxnLM* could be detected in the genome. To test whether PxnLM are also responsible for the biosynthesis of iso-FAs in

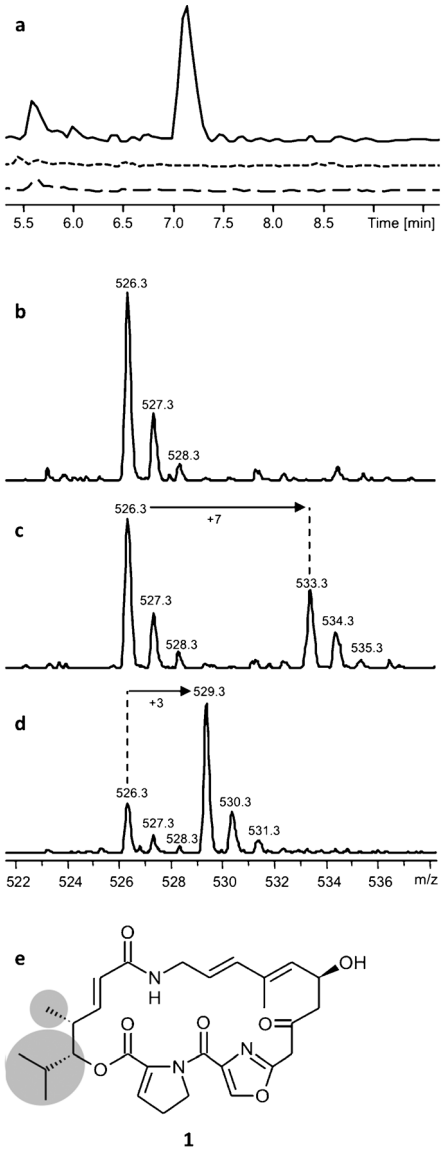

Figure 3. a) HPLC/MS chromatograms of extracts obtained from *Galleria mellonella* infected with different *Xenorhabdus nematophila* strains. Wildtype (continous line), *pxnL*:*cat* (broken line), and Δ*pxnF* (dotted line). All chromatograms are drawn to the same scale. b–d) ESI-MS data for **1** without labeled compounds (b), and after addition of L-[D_8]valine (c), and L-[methyl-D_3]methionine (d). Structure of pristinamycin II_a (**1**) highlighting the methionine- and valine-derived moieties (e).

X. nematophila, the FA profile of the *pxnL* mutant was compared to the wildtype. Here, a complete loss of all iso-odd FAs (iso-13:0, iso-15:0, and iso-15:0 3-OH) was detected in the *pxnL*:*cat* mutant (Supporting Information, Table S4). FA analysis of the Δ*pxnF* mutant showed no difference from the wildtype (Table S4), because *pxnF* is part of a different transcriptional unit in the *pxn* biosynthetic gene cluster (*pxnABCDEFGHIJK* and *pxnLMNO*) and its deletion does not affect the start of the biosynthesis (*pxnLMNO*; Supporting Information, Figure S2). As iso-odd FAs are produced in *X. nematophila* under normal growth conditions in rich medium, *pxnLMNO* must be expressed during vegetative

growth. The fact that **1** cannot be detected under these conditions might result from missing expression of the *pxnABCDEFGHIJK* operon.

The described BKD arrangements (Figure 1) show different levels of complexity. Standard BKD complexes produce soluble CoA thioesters that can serve as starter units for iso-FA and secondary metabolite biosynthesis (Figure 1a).[8] In stilbene biosynthesis in *Photorhabdus* (Figure 1b), the E2 and ACP are part of BkdB and therefore can bind the isovaleryl moiety at its ACP domain.[15] Interaction with the stand-alone *trans*-AT PKS-like ketosynthase BkdC allows for the formation of 5MOH bound to AcpP from fatty acid biosynthesis, which is then used for the biosynthesis of FAs and secondary metabolites like IPS. In *S. virginiae* a similar E1/E2 setup most likely interacts with the first KS domain of VirA,[21] the first PKS enzyme in virginiamycin biosynthesis (Figure 1c).[17] In the biosynthesis of pristinamycin II_a in *S. pristinaspiralis*[18] and *X. nematophila*, the E2 enzyme is part of the first PKS module, indicating a full transition from basic metabolism to antibiotic biosynthesis (Figure 1d). In *X. nematophila*, this BKD activity is then also responsible for the formation of iso-FAs by way of oxidative decarboxylation of 2-oxoisocaproate from leucine, as can be found in the FA profile but also in acyl moieties of other secondary metabolites like prexenocoumacins A and C.[20]

FAs can be derived from more advanced biosynthetic pathways, as in the formation of polyunsaturated fatty acids or mycolic acids.[22,23] However, to our knowledge, this is the first example of a PKS intermediate being used as substrate for the biosynthesis of basic cellular building blocks (here FA). We postulate that PxnLM can convert 2-ketoisocaproate (transaminated leucine) to isovaleryl-ACP, which can then interact with the KS from the FA biosynthesis machinery of *X. nematophila* instead of continuing the biosynthesis of **1** (Figure 1e). Proof-reading functions of PxnM domains downstream of the E2 domain might ensure that only isobutyryl-ACP is elongated to **1** as no pristinamycin or virginiamycin with an isovalerate moiety is known. Thus, longer intermediates, having a methyl branch derived from module 2 of PxnM (Supporting Information, Figure S2) might be cleaved off by the type II TE PxnK. Although the evolutionary order of the four BKD arrangements cannot be determined from the current data, the fact that four different arrangements can be found in nature gives a nice example of the interplay of primary and secondary metabolism in general. Future research will focus on the stoichiometry of the different complexes. E1, E2, and E3 of the PDH complex (and probably also of the standard BKD complex), which might resemble the ancestor complex owing to its importance to energy metabolism, follows a 24:24:12 stoichiometry leading to a 4.6 MDa protein complex with a diameter of 300 Å.[24]

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