VIP Biosynthesis

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Bacterial Biosynthesis of a Multipotent Stilbene**

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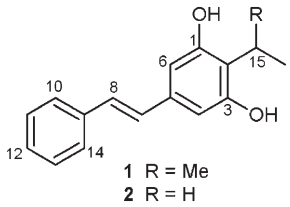
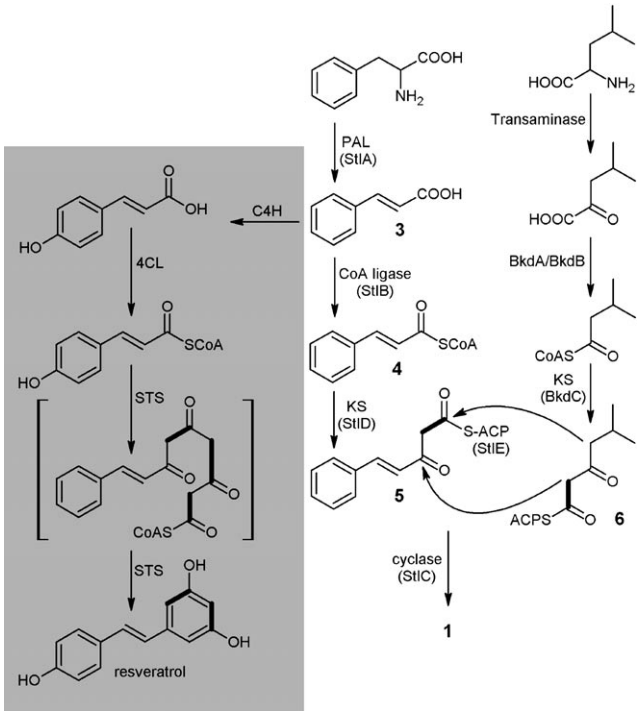
Although the biosynthesis of stilbenes is widespread in plants, the only nonplant organism known to produce this type of compound is the Gram-negative bacterium *Photorhabdus luminescens* (Enterobacteriaceae).[1] *Photorhabdus* is entomopathogenic and the bacteria produce 2-isopropyl-5-[(*E*)-2-phenylvinyl]benzene-1,3-diol (**1**) and small amounts of 2-ethyl-5-[(*E*)-2-phenylvinyl]benzene-1,3-diol (**2**) during the post-exponential phase of growth in both complex media and insect larvae.[2,3]



1 R = Me
2 R = H

In addition to being a pathogen of insects, *Photorhabdus* has a mutualistic interaction with nematodes of the genus *Heterorhabditis*, where the bacteria colonize the gut of the infective juvenile (IJ) stage of the nematode. The IJs live in the soil and, on finding a suitable insect host, enter the insect and migrate to the hemolymph (the combined blood and lymph system) where they regurgitate the *Photorhabdus* cells. The bacteria grow exponentially and kill the insect, probably of septicemia, within 72 h. The nematodes then feed on the bacterial biomass and after several rounds of reproduction a new generation of IJs develops and emerge from the insect cadaver, carrying *Photorhabdus* in their gut.[4] Stilbene **1** has antimicrobial activity, and it has always been assumed that the role of **1** during the *Photorhabdus* life cycle was to protect the insect cadaver from attack from microbial saprophytes living in the soil.[1,5–7] However, it has recently been shown that **1** is also a virulence factor as it inhibits the activity of phenoloxidase, a major component of the insect innate immune response.[8]

In plants, stilbenes are derived from the elongation of cinnamoyl-CoA thioester (**4**) or coumaroyl-CoA thioester with three malonyl-CoA extender units by a stilbene synthase (STS), an enzyme belonging to the type III polyketide synthase (PKS) family (Scheme 1). The resulting tetraketide is then cyclized and decarboxylated to give the stilbene.[9] How is **1** produced in *Photorhabdus*? As in plants, the first step is the production of cinnamic acid (**3**) by the action of StlA (Scheme 1).[10] For further biosynthesis to occur, **3** must



Scheme 1. Stilbene biosynthesis in plants (gray box) and *P. luminescens*. Intact acetate units are shown in bold. PAL: phenylalanine ammonium lyase (StlA), CoA ligase: StlB, KS: cinnamoyl-CoA condensing ketosynthase (StlD) and isovaleryl-CoA condensing ketosynthase (BkdC), ACP: StlE and FA ACP, cyclase: StlC, Bkd: branched-chain keto acid dehydrogenase (BkdA, BkdB), C4H: cinnamate-4-hydroxylase, 4CL: 4-coumaroyl-CoA ligase.

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be activated to its thioester **4** by a coenzyme A ligase. The corresponding gene was readily identified in this study as there are only two genes predicted to encode CoA ligases in the genome of *Photorhabdus*[11] (*plu2134* and *plu0760*) that are not associated with the biosynthesis of siderophores or fatty acids. Both genes were disrupted by plasmid insertion and only the disruption of *plu2134* (renamed *stlB*) resulted in the loss of the production of **1**, with the expected concomitant accumulation of **3** in the media (Figure 1 b). The structure of **1** is unusual in that there is an isopropyl group at the 2-position of the molecule. Feeding experiments with ^{13}C-labeled compounds[12] (or ^{12}C labeled in a ^{13}C background[13]) confirmed that two acetate units were incorporated (most likely as malonyl-CoA) into **1**, with one of the actetate groups being decarboxylated to form the second aromatic ring structure (see Tables S1 and S2 in the Supporting Information). Moreover, the isopropyl moiety appears to be generated from leucine-derived isovaleryl-CoA (IV-CoA), as both leucine and isovalerate groups are incorporated (see Table S2 in the Supporting Information). Based on a hypothetical plantlike biosynthesis, **1** could be produced by the sequential elongation of **4** with malonyl-CoA, isopropyl-malonyl-CoA (derived from IV-CoA), and finally another malonyl-CoA by a stilbene synthase. As STSs usually only accept malonyl-CoA as an extender unit,[9] the predicted use of two different elongation units for the production of **1** would be unusual and no gene encoding a putative STS could be identified in the genome of TT01 (data not shown). STS (and type III PKSs in general) use the free CoA-esters of the starter and elongation units, whilst both type I and type II PKSs work on precursors bound to the acyl-carrier protein (ACP). ACPs are activated from their *apo* form to a cofactor-bearing *holo* form through the action of phosphopantetheinyl transferases (PPTs). It was previously shown that a PPT (encoded by *ngrA*) was required for the production of **1** in *Photorhabdus*, thus suggesting that type I and/or type II PKS activity is required for its production.[14] However, we could not identify a typical type I or type II PKS in the genome of TT01 that might be involved in the biosynthesis of **1**.

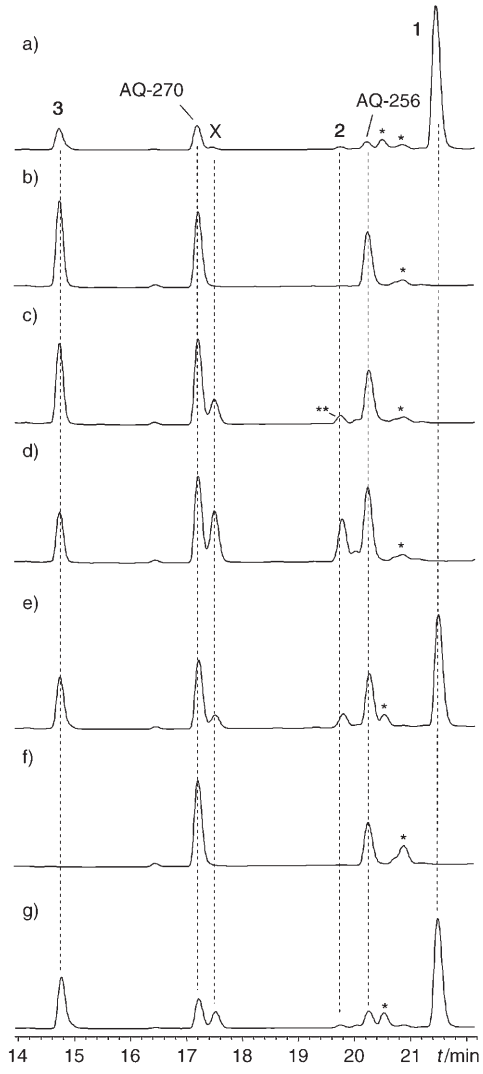

Figure 1. HPLC/UV chromatograms of selected *P. luminescens* strains. Detection wavelength 200–350 nm. a) TT01 (wild-type), b) *stlB::Cat*, c) *stlC::Cat*, d) Δ*bkdA*, e) Δ*bkdA* + iso15:0, f) *stlA::Kn*, and g) *stlA::Kn* + **3**. Peaks corresponding to **1**, **2**, and **3** are assigned. AQ-270 and AQ-256 are the major anthraquinones from TT01,[12] X is an unknown derivative of **3**, and additional unknown compounds are indicated by an asterisk (*). The peak marked by two asterisks (**) in (c) is an unknown compound that appears to have a similar retention time as **2** (see (d) and (e)), but differs in both the MS and UV data. All chromatograms are drawn to the same scale.

During a genetic screen to identify genes involved in the production of **1**, we identified a Tn5 insertion mutant that was unable to synthesize **1**, even in the presence of added **3**. The site of insertion in this mutant was mapped to *plu1884*, a gene predicted to be part of the three-gene operon *plu1883–plu1885* (we have renamed these genes *bkdA*, *bkdB*, and *bkdC*, respectively; Figure 2). Nonpolar deletion mutations in each of these three genes confirmed their involvement in the biosynthesis of **1** (Figure 1 d and unpublished results). Interestingly, **2** which only differs from **1** in that the isopropyl group has been replaced by an ethyl group accumulated in these mutants.

Therefore, assuming that we were looking for an atypical PKS, we searched the TT01 genome for genes encoding ACPs that were not part of loci-containing genes encoding nonribosomal peptide synthetases (NRPS) or NRPS/PKS hybrids. In this way, three ACP-encoding genes were identified: *plu2834* as part of the normal fatty acid (FA) biosynthesis, *plu0765* as part of an unusual biosynthesis gene cluster, and *plu2165* which is part of a three-gene operon, *plu2163–plu2165* (renamed *stlC*, *stlD*, and *stlE*, respectively). The other genes of this operon encode a ketosynthase (encoded by *plu2164* (*stlD*)) and a protein (encoded by *plu2163* (*stlC*)) with homology to a putative cyclase from *Pseudomonas aurantiaca* (Figure 2).[15] This cyclase is involved in the biosynthesis of 2,5-dialkylresorcinol, which is similar to the phenolic ring of **1**. Moreover, it was shown recently that structurally related dihydrocoumarins can be produced by engineered fungal iterative type I PKS enzymes.[16] Disruption of the *stlCDE* operon, by plasmid insertion into either *stlC* or *stlD*, resulted in a stilbene-negative mutant that accumulated high amounts of **3**, thus confirming the role of these genes in the production of **1** (Figure 1 c).

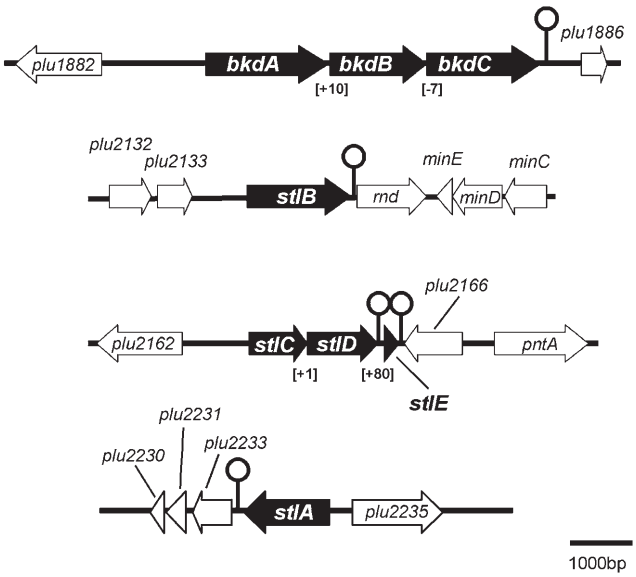

Figure 2. Organization of the genetic loci involved in stilbene production in *Photorhabdus*. The genes involved in stilbene biosynthesis are shown in black and flanking genes in white. Distances (in base pairs) between the different genes are given in brackets and the terminator positions are shown. For a detailed analysis see the Supporting Information.

The genes *bkdA* and *bkdB* are predicted to encode the E1 and E2 components of a branched-chain ketoacid dehydrogenase (Bkd), and *bkdC* is predicted to encode an unusual ketosynthase (KS). Bkd activity is required for the degradation of the branched-chain amino acids leucine, isoleucine, and valine to isovaleryl- (IV-), 2-methylbutyryl-, and isobutyryl-CoA, respectively. We have shown by feeding experiments that leucine and isovalerate are incorporated into **1** (see Table S2 in the Supporting Information). IV-CoA is an important precursor for the biosynthesis of odd-numbered iso-FAs, the dominant family of fatty acids in *Photorhabdus*. Analysis of the fatty acid profile of Δ*bkdA*, Δ*bkdB*, and Δ*bkdC* revealed that these mutants failed to produce iso-FAs, thereby confirming that all these genes are involved in the synthesis of iso-FAs (see Table S3 in the Supporting Information). Interestingly, we were able to fully or partially restore the production of **1** to the wild-type level in these three mutants by the addition of either isovalerate (which also partially restored the wild-type FA profile (see Table S3 in the Supporting Information)) or iso15:0, the dominant FA in *Photorhabdus* (see Figure 1e). This finding indicates that β-oxidation of FAs can also generate stilbene precursors.

From this data we predict that the biosynthesis of **1** in *P. luminescens* occurs by the StlC-catalyzed condensation of (4*E*)-3-oxo-5-phenylpent-4-enoyl (3OPPE) thioester (**5**) with a 5-methyl-3-oxohexanoyl (5MOH) thioester (**6**). Therefore, the major difference to the biosyntheses of dialkylresorcinol[15] and dihydrocoumarin[16] is that **5** substitutes for an aliphatic thioester, thereby resulting in an unprecedented stilbene biosynthesis. Although both compounds might be ACP-bound, they might also be present as CoA esters (see Scheme 1). Thioester **5** is ultimately derived from phenylalanine, which is converted into **3** and **4** by StlA and StlB, respectively. The ACP (StlE) is loaded with malonyl-CoA by an acyltransferase and condensed with the cinnamoyl-CoA by StlD. Similarly, **6** is derived from a BkdC-catalyzed elongation of leucine-derived IV-CoA (from BkdA and BkdB) with malonyl-ACP. In a control study where **6** is not being produced (namely, by using a strain containing a mutation in *bkdA*, *bkdB*, or *bkdC*), *Photorhabdus* produce **2** rather than **1**—probably through the ligation of **5** with 3-oxohexanoyl (3-OH) thioester. 5MOH-ACP/CoA is an intermediate in branched-chain FA biosynthesis/degradation whilst 3-OH-ACP/CoA is an intermediate in the metabolism of straight-chain FAs. Therefore, there is a clear interaction between the production of **1** and metabolism of FAs. We suggest that the ACP involved in the production of **6** is likely to be the ACP involved in FA biosynthesis. This proposal is supported by our observation that there is no other gene encoding an appropriate ACP in the TT01 genome. Likewise, given the close relationship between the production of **1** and FA metabolism, we suggest that FabD is the acyltransferase predicted to be involved in the loading of the malonyl-CoA units onto both ACP and StlE.

As **1** is a major secondary metabolite produced by all strains of *Photorhabdus* investigated so far (see Figure S1 in the Supporting Information), we were interested in determining if this molecule had any role in the interaction with the nematode. We tested IJ recovery into self-fertile hermaphrodites, as this is the first step in the post-infection development that requires either signals present in the insect hemolymph or "food signals" produced by the bacteria.[17] The rate of recovery of IJs on BMM901 cells (stilbene-negative; *stlA*::*Kn*) is only 5–15% that observed with wild-type TT01 bacteria, which suggests that **1** is a major component of the bacterial food signal (Figure 3). Moreover, almost complete recovery could be obtained by feeding either **3** or purified **1** to BMM901, or by supplying a copy of the *stlA* gene (encoded on pBMM901) to BMM901. However, nematodes did not recover when inoculated onto agar plates supplemented with **1** but with no bacteria, a finding that suggests that,

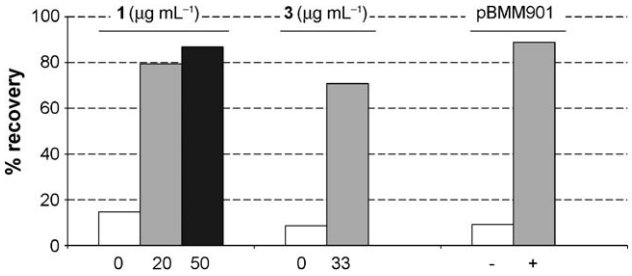

Figure 3. Nematode recovery (in%) with and without stilbenes. Forty infective juvenile (IJ) nematodes of *H. bacteriophora* were added to BMM901 (*stlA*::*Kn*) grown on lipid agar plates, and the total number of IJs that recovered after four days was counted in several plates (*n*) and compared to the recovery on wild-type *P. luminescens* (100%). The lipid agar was supplemented with the indicated concentrations of either **1** ($n=12$) or **3** ($n=7$). Alternatively, BMM901 was transformed with either pBMM901 (*stlA*$^+$, +) or the plasmid vector alone (−) ($n=6$).[18]

although **1** is required, it is not sufficient for nematode recovery (data not shown).

In summary, we have shown that the stilbene in *Photorhabdus* is synthesized by a novel biosynthetic pathway that clearly evolved independently from the well-established pathway in plants. Moreover, **1** is a multipotent molecule that not only acts as an antibiotic and inhibitor of the insect immune system but may also be a signal between kingdoms which is required for normal growth and development of the nematode partner. Therefore, it might play an essential role in both mutualism and pathogenicity and is therefore a central buiding block in the interplay between bacteria, nematodes, and insect larvae. In fact, such an essential function might explain the presence of **1** in all *Photorhabdus* strains investigated so far.

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[18] The data is nonparametric. To represent the data graphically, the level of IJ recovery (in %) is represented relative to the recovery observed when IJs were added to wild-type TT01 cultured under the same conditions. Therefore, taking the addition of **1** as an example, 480 IJs (12 plates, each inoculated with 40 IJs) were inoculated onto either TT01 or BMM901 bacteria growing on lipid agar, and the percentage of IJ recovery was calculated. Nonparametric statistical analysis of this data (Mann-Whitney) shows that the number of IJs recovering on BMM901 growing on lipid agar not supplemented with **1** or **3** is significantly different to the number that recover on either wild-type bacteria or bacteria growing on lipid agar plates supplemented with **1** or **3** ($P \leq 0.01$). Similarly, the number of IJs recovering on BMM901 carrying the plasmid vector is significantly different from both wild-type and BMM901/pBMM901 ($P \leq 0.01$).

