Supporting Information
for

A novel and widespread class of ketosynthase is responsible for the head-to-head condensation of two acyl moieties in bacterial pyrone biosynthesis
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Experimental procedures, details of bioinformatic analysis and NMR data of pseudopyronines

Materials and methods

Cultivation of strains. E. coli strains were cultivated in liquid or solid LB-medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) or TB-medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol and 0.17 M KH₂PO₄/0.72 M K₂HPO₄). For preparation of solid media, 1.5% (w/v) Agar was added. For plasmid preparation E. coli strains were cultivated at 37 °C in TB-medium. Kanamycin (50 μg/mL) and chloramphenicol (20 μg/mL) were used as resistant markers. For cultivation of Pseudomonas sp. GM30 and Pseudomonas putida KT2440 the same media was used but strains were grown at 30 °C.

Construction of ppyS mutants. Mutants were either constructed using the TA-cloning strategy [1] or with oligonucleotide-directed mutagenesis. Point mutations were introduced to the pCOLA_ppys vector, which is used to heterologously express
PpyS in *E. coli* BL21(DE3) Star. Mutants C129A, H281A, E105A and E330A were constructed using the TA-cloning strategy, for this method a pair of oligonucleotides (v.p. fw and v.p. rev) were used to amplify the vector, containing the non-mutated gene section using the Phusion polymerase (Fermentas). After separation by agarose gel electrophoresis, the desired fragments were extracted with the GeneJET Gel Extraction Kit (Fermentas) and incubated for 30 minutes at 70 °C with Taq polymerase (Thermo Scientific), resulting in a 3' A-overhang fragment. Another pair of oligonucleotides (ds fw and ds rev), containing the mutated gene section, was used to form the 3' T-overhang fragment carrying the mutation. For ligation both fragments were incubated overnight at room temperature with T4 DNA ligase (Fermentas). The N310A and R121D PpyS mutants were created using oligonucleotide-directed mutagenesis, for which a pair of oligonucleotides was applied to amplify the vector and to introduce the point mutation using the Phusion polymerase (Fermentas) for PCR. For both strategies the ligation mixture was subsequently used to transform *E. coli* DH10B by electroporation (1250 V, 200 Ω and 25 μF). After plasmid extraction the obtained plasmids were verified by sequencing at SeqIT GmbH (Germany, Kaiserslautern). Verified plasmids were used to transform *E. coli* BL21 (DE3) Star along with pACYC_bkdABCngrA [2] by electroporation. For photopyrone biosynthesis both vectors (pCOLA_ppyS and pACYC_bkdABCngrA) are induced with 0.01 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fermentas) for expression in *E. coli* BL21 (DE3) Star.

**Cloning of pseudopyronine synthase (pyrS).** We first constructed pCOLA_pyrS for heterologous expression in *E. coli* BL21 (DE3) Star. Therefore pyrS was cloned from extracted *Pseudomonas* sp. GM30 genomic DNA using the oligonucleotides pyrS_pCOLA_FW and pyrS_pCOLA_Rev. The vector pCOLADuet-1 and PyrS PCR
product were both digested with restriction enzymes BamHI and HindIII and ligated using the T4 DNA Ligase. This mixture was then used to transform *E. coli* DH10B by electroporation (1250 V). After plasmid extraction the obtained plasmid was verified by sequencing at SeqIT GmbH (Germany, Kaiserslautern). The verified plasmid was used to transform *E. coli* BL21 (DE3) Star by electroporation. The construction of pCom10_pyrS was performed using the Gibson assembly method [3]. Therefore the vector was amplified via PCR using the oligonucleotides pCom10_Fw and pCom10_Rev. PyrS was amplified using the oligonucleotides pyrS_pCom_Fw and pyrS_pCom_Rev and pCOLA_pyrS as template, the oligonucleotides were previously modified with a 30 bp 3’ overhang which are homologues to the amplified pCom10 product. Both products were then incubated with the Gibson assembly mix for 1 h at 50 °C. This mixture was then used to transform *E. coli* DH10B by electroporation as described earlier. The plasmid was obtained by using the extraction protocol described previously.

**Electrotransformation of *Pseudomonas* strains.** *Pseudomonas putida* KT2440 and *Pseudomonas* sp. GM30 were grown over night at 30 °C in liquid LB media. To prepare cells for electro transformation 2 mL of fresh liquid LB media were inoculated with an overnight culture (1:100) and were then grown for 3 hours at 30 °C. The cells were then centrifuged and washed twice with cold water. Centrifuged cells were then resuspended in 50 μL cold water and cells were kept on ice. 1 μL of plasmid was used to transform *Pseudomonas* strains by electroporation (2500 V).

**Analytical scale culture extraction.** In order to detect photopyrone production in the wildtype and mutant strains by means of HPLC/MS, 20 mL of liquid LB-medium, containing the appropriate resistant markers, were inoculated with an overnight culture to an optical density of OD$_{600}$ = 0.05 and cultivated for 3.5 h at 37 °C. Then
0.01 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fermentas) and 2% Amberlite™ XAD16 (Sigma-Aldrich) were added to the culture, which was cultivated for 48 h at 16 °C. For detection of pseudopyronines 20 mL of liquid LB-medium, containing the appropriate resistant marker and 2% Amberlite™ XAD16, were inoculated with an overnight culture (1:100). Expression was induced with addition of 0.05% (v/v) dicyclopropyl ketone. The *Pseudomonas* containing cultures were then incubated for 72 h at 30 °C. After 48 h again 0.05% (v/v) of dicyclopropyl ketone was added. Cultures were harvested by centrifugation (4000 rpm, 10 min, 18 °C) followed by removal of the supernatant. Amberlite™ XAD16 resins were extracted with 30 mL of methanol and incubated for 1 h under constant rotation followed by a filtration step (Folded Filters (Quality), grade: 3 m/N, Munktell) to remove cells and resins. The elution step was repeated once with 10 mL of methanol. The methanol extract was then concentrated to dryness using a rotary evaporator. The solid residue was redissolved in 2 mL of methanol and a 1:10 dilution was analyzed by means of HPLC/MS. Extracts were analyzed using a Dionex UltiMate 3000 system coupled to a Bruker Daltonik AmaZon X mass spectrometer, a RP18-column (50 mm × 2.1 mm × 1.7 μm; Waters GmbH) and an acetonitrile/0.1% formic acid in H₂O gradient, ranging from 5 to 95% in 22 min at a flow rate of 0.6 mL/min. The production of *ppyS* mutants of 4 was calculated against standard concentrations of the main compound photopyrone D (4) produced by wildtype *ppyS*. The retention time of 4 under these conditions was 10.5 min.

**Preparative extraction and purification.** For the isolation of compounds 9–11 from *Pseudomonas* sp. GM30, the strain was cultivated in 6 L of LB-medium, with an addition of 2% Amberlite™ XAD16 for 3 days at 30 °C. Cultures were harvested by centrifugation (4000 rpm, 10 min, 18 °C) followed by removal of the supernatant.
Amberlite™ XAD16 resins were extracted with methanol and incubated for 1 h under constant rotation followed by a filtration step to remove cells and resins. The methanol extract was then concentrated to dryness using a rotary evaporator. The solid residue (4.6 g) was redissolved in 10 mL of water, then 20 mL of ethyl acetate was added and the mixture was shacked in a separating funnel. This step was repeated two times with the addition of 20 mL of ethyl acetate and separation. The ethyl acetate phase was then concentrated to dryness using a rotary evaporator. The solid residue (0.7 g) was redissolved in a 70% dimethyl sulfoxide (DMSO), 20% methanol and 10% isopropanol mixture. Compounds were then isolated with a Waters Bridge XBridge™ Prep C18 5 µm OBD™ 19 × 150 mm Column (S/N) and a Waters HPLC-MS system as described in the following: Waters 3100 Mass Detector, Waters 2998 Photodiode Array Detector, Waters SFO System Fluidics Organizer, Waters 515 HPLC Pump, Waters 2545 Binary Gradient Module, Waters Selector Value, Waters 2767 Sample Manager. The purification was performed at a flow rate of 24 mL/min with an acetonitrile–water (0.1% formic acid) gradient: 0–26 min 50–75%, 26.1–30 min 76–95%. The combined fractions were then concentrated to dryness using a rotary evaporator to give 4.8 mg, 35.6 mg and 1.6 mg of 9, 10 and 11, respectively.

**NMR.** 1D and 2D nuclear magnetic resonance (NMR) spectra for purified compounds were recorded on a Bruker DRX 500 spectrometer using deuterated dimethyl sulfoxide as solvent and internal standard.

**HR-ESI-MS.** Determination of exact masses of 9–11 were carried out using a Dionex Ultimate 3000 RSLC coupled to a Bruker microTOF-Q II equipped with an ESI source. The following masses were detected: for 9, \( m/z \) 267.1956 (calcd for \( [C_{16}H_{26}O_3 + H]^+ \), 267.1955, \( \Delta = 0.38 \) ppm); 10, \( m/z \) 295.2270 (calcd for \( [C_{18}H_{30}O_3 + \)
H]⁺, 295.2268, Δ = 0.68 ppm); 11, m/z 323.2583 (calc’d for [C₂₀H₃₄O₃ + H]⁺, 323.2581, Δ = 0.62 ppm).

**Homology modelling.** The protein sequences of PpyS and PyrS were used as queries for BLASTP [4] searches in the PDB [5], to identify the most similar available structure in the PDB. This resulted in the identification of OleA (sequence identity 27%, E-value 1e-14, PDB: 3S21) from *Xanthomonas campestris* for PpyS and OleA (sequence identity 37%, E-value 4e-10, PDB: 3S21) for PyrS-. These template structures were used to create a sequence alignment applying the ClustalW algorithm [6]. The homology models were generated using the Homology Modelling Tool integrated in MOE 2012.10 (Molecular Operating Environment; Chemical Computing Group Inc., Montreal, Canada) and the ClustalW sequence alignment was imported. A series of ten models was created, for further processing the one with the highest packing quality score was chosen and energy minimized applying the AMBER12EHT (integrated in MOE) force field. All figures showing protein structures in this work, were created using MOE.

**Docking.** Protein–ligand docking calculations were carried out using the program GOLD (version 5.2) [7] using the empirical scoring function for advanced protein–ligand docking CHEMPLP [8]. For each docking study the result with the highest docking score is shown in this work.

**Phylogenetic analysis.** A PHYML [9] tree (50 bootstraps) was calculated using a ClustalW alignment (gap opening: 10; gap extension: 0.1), which was generated using the collected ketosynthases. For visualization and calculation of the alignment as well as the PHYML tree the Geneious software (Biomatters Ltd., New Zealand) was used.
**Supplementary Tables and Figures**

**Table S1**: Strains used in this work.

| Strain       | Genotype                                                                 | Reference |
|--------------|--------------------------------------------------------------------------|-----------|
| *E. coli* DH10B | $\text{F}_mcrA \ (\text{mrr-hsdRMS-mcrBC}), \ 80\text{lacZ}_{\Delta, \ M15, \ \Delta \ \text{lacX74} \ \text{recA1} \ \text{endA1} \ \text{araD} \ 139}$ \ $\	ext{\Delta (ara, leu)7697 galIJ galK \ \lambda. \ \text{rpsL} (\text{Str}) \ \text{nupG}}$ | [10]      |
| *E. coli* BL21 (DE3) Star | $\text{F- ompT hsdSB(rB-, mB-) gal dcm}$ | Invitrogen |
| *Pseudomonas* sp. Wildtype | | [11]      |
| *Pseudomonas putida* KT2440 | | [12]      |
| **WT** | BL21 (DE3) Star:pCOLA\_ppyS, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | [2]       |
| **C129A** | BL21 (DE3) Star:pCOLA\_ppyS\_C129A, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | this work |
| **H281A** | BL21 (DE3) Star:pCOLA\_ppyS\_H281A, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | this work |
| **N310A** | BL21 (DE3) Star:pCOLA\_ppyS\_N310A, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | this work |
| **E105A** | BL21 (DE3) Star:pCOLA\_ppyS\_E105A, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | this work |
| **R121D** | BL21 (DE3) Star:pCOLA\_ppyS\_R121D, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | this work |
| **E330A** | BL21 (DE3) Star:pCOLA\_ppyS\_E330A, pACYC\_bkdABC\_ngrA, Km$^R$, Cm$^R$ | this work |
| *E. coli* DH10B | *E. coli* DH10B:pCOLA\_pyrS | this work |
| *E. coli* BL21 (DE3) Star | *E. coli* BL21 (DE3) Star:pCOLA\_pyrS | this work |
| *E. coli* DH10B | *E. coli* DH10B:pCom10\_pyrS | this work |
P. putida KT2440  Pseudomonas putida  this work
pCom10_pyrS  KT2440:pCom10_pyrS

Pseudomonas sp.  Pseudomonas sp. GM30:pCom10_pyrS  this work
Gm30  pCom10_pyrS

Table S2: Plasmids used in this work.

| Plasmid                  | Genotype                                               | Reference     |
|--------------------------|--------------------------------------------------------|---------------|
| pCOLADuet-1              | ColA ori, Km\(^\text{R}\), T7lac promoter             | Merck Millipore |
| pACYCDuet-1              | CloDF13 ori, Cm\(^\text{R}\), T7lac promoter          | Merck Millipore |
| pCOLA_ppyS               | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | [2]           |
| pACYC\_bkdABC\_ngrA      | CloDF13 ori, Cm\(^\text{R}\), T7lac promoter, bkdABC, ngrA | [2]           |
| pCOLA_ppyS\_C129A        | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | this work     |
| pCOLA_ppyS\_H281A        | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | this work     |
| pCOLA_ppyS\_N310A        | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | this work     |
| pCOLA_ppyS\_E105A        | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | this work     |
| pCOLA_ppyS\_R121D        | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | this work     |
| pCOLA_ppyS\_E330A        | ColA ori, Km\(^\text{R}\), T7lac promoter, ppyS        | this work     |
| pCom10                   | pBR322 ori, Km\(^\text{R}\), alkB promoter             | [13]          |
| pCom10_pyrS              | pBR322 ori, Km\(^\text{R}\), alkB promoter, pyrS        | this work     |
| pCOLA_pyrS               | ColA ori, Km\(^\text{R}\), T7lac promoter, pyrS        | this work     |

Table S3: Oligonucleotides used in this work.

| Oligonucleotide | Sequence                                                        |
|-----------------|----------------------------------------------------------------|
| Cys129_Fw.ds    | CGCCGCGAACGCT                                                |
| Cys129_Rev.ds   | CCGTTCGCGGCAT                                                |
| Cys129_Fw.V.P   | TGGGTGCGAGCTGGATAGGATTAATCATAGT                              |
| Cys129_Rev.V.P  | CTACAACATCGTAGTTCCTACAAAGTTAGCC                              |
| Glu105_Fw.ds    | TAGCGCCAGCTAACAGTACTTT                                       |
| Glu105_Rev.ds   | AGTACTGTTAGCAGCTGCCATAG                                      |
| Glu105_Fw.V.P   | TTGCTCTAAAGGCACTAGGGCTACTGTT                                  |
| Glu105_Rev.V.P  | GAAGCGCGCGGCAACTGGGCTACTGG                                    |
| His281_Fw.ds    | TTTTTTATGCGACAAGGAGTACTGCAAACGACGAC                          |
| His281_Rev.ds   | CGTTTTGGTGAACCTGGCTGCAGAAAAT                                  |
| His281_Fw.V.P   | GGGCACGCGCTGGTGAACCCATGAGAAGATG                              |
| Protein            | Organism                      | Accession number |
|--------------------|-------------------------------|------------------|
| **OleA homologues**|                               |                  |
| 1 3-Oxoacyl-ACP synthase | S. sp. NRRL F-5555          | WP_030402327     |
| 2 3-Oxoacyl-ACP synthase | Streptomyces                 | WP_031086294     |
| 3 3-Oxoacyl-ACP synthase | S. sp. NRRL F-5650          | WP_031039494     |
| 4 3-Oxoacyl-ACP synthase | A. rifamycini               | WP_026404743     |
| 5 3-Oxoacyl-ACP synthase | M. rosea                    | WP_036407026     |
| 6 3-Oxoacyl-ACP synthase | N. candida                  | WP_043622914     |
| 7 3-Oxoacyl-ACP synthase | S. pristinaespiralis        | WP_005309093     |
| 8 3-Oxoacyl-ACP synthase | T. sp. 28                   | WP_045191732     |
| 9 3-Oxoacyl-ACP synthase | S. hofmanni                 | WP_017748751     |
| 10 3-Oxoacyl-ACP synthase | L. araneosa                 | WP_007281261     |
| 11 OleA              | X. campestris pv. campestris| 3S21_A           |
| 12 3-Oxoacyl-ACP synthase | S. amylolyticus              | AKF07269         |
| 13 3-Oxoacyl-ACP synthase | B. muris                    | WP_017822397     |
| 14 3-Oxoacyl-ACP synthase | A. phenanthrenivorans       | WP_004353199     |
| 15 3-Oxoacyl-ACP synthase | Arthrobacter                 | WP_018779660     |
| 16 3-Oxoacyl-ACP synthase | A. sp. MWB30                | KIA73109         |
| 17 3-Oxoacyl-ACP synthase | L. rubra                    | WP_021808728     |
| 18 3-Oxoacyl-ACP synthase | M. yannicii                 | WP_040569064     |
| 19 3-Oxoacyl-ACP synthase | M. sp. B19                  | WP_026096098     |
| 20 3-Oxoacyl-ACP synthase | M. testaceum               | WP_04360932      |
| 21 3-Oxoacyl-ACP synthase | M. testaceum StLB037        | BAJ73499         |
| 22 3-Oxoacyl-ACP synthase | M. sp. SUBG005              | KEP74827         |
| **PpyS homologues**|                               |                  |
| 23 3-Oxoacyl-ACP synthase | X. nematophilia             | WP_010847197     |
| 24 3-Oxoacyl-ACP synthase | X. nematophilia ATCC 19061  | WP_003713506     |

**Table S4:** Ketosynthases used for the phylogenetic tree. The sequences are ordered clockwise according to their location in the respective branches. All KS showing the conserved glutamic acid residue identified as catalytically important are shown in red.
| No. | Description                              | Accession          |
|-----|------------------------------------------|--------------------|
| 25  | 3-Oxoacyl-ACP synthase                   | X. nematophila     |
| 26  | 3-Oxoacyl-ACP synthase                   | X. bovienii        |
| 27  | 3-Oxoacyl-ACP synthase                   | X. bovienii        |
| 28  | PpyS                                     | P. luminescens TT01|
| 29  | 3-Oxoacyl-ACP synthase                   | P. luminescens TT01|
| 30  | 3-Oxoacyl-ACP synthase                   | P. luminescens TT01|
| 31  | 3-Oxoacyl-ACP synthase                   | P. sp. PH1b        |
| 32  | 3-Oxoacyl-ACP synthase                   | P. sp. St29        |
| 33  | 3-Oxoacyl-ACP synthase                   | P. sp. Os17        |
| 34  | 3-Oxoacyl-ACP synthase                   | P. mosselii        |
| 35  | 3-Oxoacyl-ACP synthase                   | P. mosselii        |
| 36  | PyrS                                     | P. sp. GM30        |
| 37  | 3-Oxoacyl-ACP synthase                   | P. sp. URL14HWK12:16|
| 38  | 3-Oxoacyl-ACP synthase                   | P. sp. W15Feb9B    |
| 39  | 3-Oxoacyl-ACP synthase                   | B. sp. UYPR1.413   |
| 40  | 3-Oxoacyl-ACP synthase                   | B. bannensis       |
| 41  | 3-Oxoacyl-ACP synthase                   | B. mimosarum       |
| 42  | 3-Oxoacyl-ACP synthase                   | B. nodosa          |
| 43  | 3-Oxoacyl-ACP synthase                   | B. heileia         |
| 44  | 3-Oxoacyl-ACP synthase                   | B. phytofirmans PsJN|
| 45  | 3-Oxoacyl-ACP synthase                   | B. phytofirmans    |
| 46  | 3-Oxoacyl-ACP synthase                   | B. sp. WSM2230     |
| 47  | 3-Oxoacyl-ACP synthase                   | B. sp. WSM2232     |
| 48  | 3-Oxoacyl-ACP synthase                   | B. sp. CCGE1003    |
| 49  | 3-Oxoacyl-ACP synthase                   | B. sp. CCGE1003    |
| 50  | 3-Oxoacyl-ACP synthase                   | B. sp. WSM3556     |
| 51  | 3-Oxoacyl-ACP synthase                   | B. graminis        |
| 52  | 3-Oxoacyl-ACP synthase                   | B. sp. URHA0054    |
| 53  | 3-Oxoacyl-ACP synthase                   | B. sp. CCGE1001    |
| 54  | 3-Oxoacyl-ACP synthase                   | B. sp. CCGE1001    |
| 55  | 3-Oxoacyl-ACP synthase                   | B. phenoliruptrix BR3459a|
| 56  | 3-Oxoacyl-ACP synthase                   | Burkholderia       |
| 57  | 3-Oxoacyl-ACP synthase                   | L. anisa           |
| 58  | 3-Oxoacyl-ACP synthase                   | L. pneumophila     |
| 59  | 3-Oxoacyl-ACP synthase                   | S. sp. CNB091      |
| 60  | 3-Oxoacyl-ACP synthase                   | A. mirum          |
| 61  | 3-Oxoacyl-ACP synthase                   | A. azurea          |
| 62  | 3-Oxoacyl-ACP synthase                   | S. sp. MspMP-M5    |
| 63  | 3-Oxoacyl-ACP synthase                   | N. abscessus       |
| 64  | 3-Oxoacyl-ACP synthase                   | N. sp. CNY236      |
| 65  | 3-Oxoacyl-ACP synthase                   | N. farcinica       |
| 66  | 3-Oxoacyl-ACP synthase                   | N. higoensis       |
| 67  | 3-Oxoacyl-ACP synthase                   | G. maliensis       |
| 68  | 3-Oxoacyl-ACP synthase                   | T. campyonemoides  |
| 69  | 3-Oxoacyl-ACP synthase                   | A. sp. PCC 7108    |
| 70  | 3-Oxoacyl-ACP synthase                   | M. sp. SC2         |
| 71  | 3-Oxoacyl-ACP synthase                   | M. rosea           |
| 72  | 3-Oxoacyl-ACP synthase                   | M. sp. SB2         |
| 73  | 3-Oxoacyl-ACP synthase                   | M. sp. T1-4       |
| 74  | 3-Oxoacyl-ACP synthase                   | C. fritschii       |
| 75  | 3-Oxoacyl-ACP synthase                   | C. acetobutylicum  |
| 76  | 3-Oxoacyl-ACP synthase                   | P. lactis          |
| 77  | 3-Oxoacyl-ACP synthase                   | B. thuringiensis   |
| 78  | 3-Oxoacyl-ACP synthase                   | B. sp. 1NLA3E      |
| 79  | 3-Oxoacyl-ACP synthase                   | O. scapharcae      |
| 80  | 3-Oxoacyl-ACP synthase                   | P. polymerma      |
| 81  | 3-Oxoacyl-ACP synthase                   | P. polymerma      |
| 82  | 3-Oxoacyl-ACP synthase                   | P. sp. Aloe-11     |
| 83  | 3-Oxoacyl-ACP synthase                   | P. terrae          |

**Closest BLAST-P hits for XcIC**

[14]
| No. | Gene Name | Organism | Accession Number |
|-----|-----------|----------|-----------------|
| 84  | 3-Oxoacyl-ACP synthase | P. peoriae | WP_010345468.1 |
| 85  | FabH     |          |                 |
| 86  | CorB     | C. coraloides | ADI59524 |
| 87  | Myxopyron ketosynthase | M. fulvus | AGS77282 |
| 88  | FabHB    | B. subtilis | NP_388898 |
| 89  | FabH     | N. punctiforme | YP_001865657 |
| 90  | FabH     | B. subtilis | NP_389015.1 |
| 91  | FabH     | P. luminescens | NP_930069 |
| 92  | FabH     | E. coli | NP_287225 |
| 93  | FabH     | S. griseus | YP_001826619 |
| 94  | FabH     | S. echinatus | AAV84077 |
| 95  | NP_626634 | S. coelicolor A3(2) | NP_626634 |
| 96  | FabH     | S. avermitilis | BAC73499 |
| 97  | Q54206   | S. glaucescens | Q54206 |
| 98  | FdmS     | S. roseofulvus | AAC18104 |
| 99  | CAM58805_S._sp._BenQ | S. sp. CM020 | ACI88883 |
| 100 | ZhuH 1MZJ | S. sp. A2991200 | CAM58805 |
| 101 | FrnI     | S. roseofulvus | AAC18104 |
| 102 | AlnI     | S. coelicolor A3(2) | NP_626634 |
| 103 | Plu1885  | P. luminescens | NP_929153 |
| 104 | NanA8    | S. nanchangensis | AAP42874 |
| 105 | EryAll   | S. erythraea | YP_001102990 |
| 106 | TylGi KSQ | S. fradiae | AAB66504 |
| 107 | MerA     | S. violaceusniger | AJB97437 |
| 108 | TamAI    | S. sp. 3079 | ADC79637 |
| 109 | OleAI KSQ | S. antibioticus | AAF82408 |
| 110 | HedT     | S. griseoruber | AAP85336 |
| 111 | 3-Oxoacyl-ACP synthase | R. blandensis | WP_008043745.1 |
| 112 | 3-Oxoacyl-ACP synthase | X. nematophila | YP_003714026.1 |
| 113 | 3-Oxoacyl-ACP synthase | X. nematophila | WP_010848687.1 |
| 114 | 3-Oxoacyl-ACP synthase | M. sp. PE36 | WP_006034384.1 |
| 115 | 3-Oxoacyl-ACP synthase | P. profundum | WP_132684.1 |
| 116 | 3-Oxoacyl-ACP synthase | P. damselae | WP_005305524.1 |
| 117 | 3-Oxoacyl-ACP synthase | P. sp. AK15 | WP_007465048.1 |
| 118 | 3-Oxoacyl-ACP synthase | P. leiognathi | WP_008989540.1 |
| 119 | 3-Oxoacyl-ACP synthase | P. sp. SKA34 | WP_006644045.1 |
| 120 | 3-Oxoacyl-ACP synthase | P. angustum | WP_005364526.1 |
| 121 | FabF     | M. sp. 4-46 | YP_001771620 |
| 122 | FabF     | C. pinensis | ACU62401 |
| 123 | cpin1855 | C. pinensis | YP_003121552 |
| 124 | Dfer_1997 | D. fermentans | YP_003086385 |
| 125 | FabB     | A. pleuro pneumoniae | ZP_00134992 |
| 126 | FabB     | C. sp. 30_2 | ZP_04562837 |
| 127 | NP_416826 | E. coli | NP_416826 |
| 128 | FabB     | S. boydii | YP_001881145 |
| 129 | NP_344945 | S. pneumoniae | NP_344945 |
| 130 | FabF     | T. thermophilus | YP_143679 |
| 131 | FabF     | N. punctiforme | YP_001867862 |
| 132 | FabF     | B. subtilis | NP_389016 |
| 133 | NP_645683 | S. aureus | NP_645683 |
| 134 | FabF     | P. luminescens | NP_930065 |
| 135 | FabF     | E. albertii | ZP_02902779.1 |
| 136 | FabF     | E. coli | NP_287229 |
| 137 | NP_415613 | E. coli | NP_415613 |
| 138 | FabF     | S. avermitilis | BAC70003 |

**Type II PKS KS a**
139 SimA2  
S. antibioticus  
AF324838.4
140 TcmL  
S. glaucescens  
AA67516
141 EncB  
S. maritimus  
AAF81729
142 ActIA  
S. coelicolor A3(2)  
SCO5087
143 NcnB  
S. arenae  
AAD20268

**FabB**

144 AntD (Plu4191)  
P. luminescens  
NP_931374
145 EncA  
S. maritimus  
AAF81729
146 ActIB  
S. coelicolor A3(2)  
SCO5087
147 NcnA  
S. arenae  
AAD20267
148 TcmK  
S. davawensis  
CCK26894
149 SimA1  
S. antibioticus  
AAK06784

**ChlB6; CerJ; KSIII DpsC-like**

150 ChlB6  
S. antibioticus  
AAZ77679
151 CerJ  
S. tendae  
AE91069
152 CosE  
S. olindensis  
ABC00733
153 DpsC  
S. peucetius  
AAA65208
154 AknE2  
S. sp. SPB74  
ZP_04991255.1
155 BAB72048  
S. galilaeus  
BAB72048
156 PokM2  
S. diastatocchromogenes  
ACN64832
157 CalO4  
S. aurantiaca  
ZP_01462124
158 NcnA  
S. arenae  
AAD20267

Closest BLAST-P hits for XclB

167 3-Oxoacyl-ACP synthase III  
B. sp. EniD312  
WP_00911263.1
168 3-Oxoacyl-ACP synthase III  
A. nasoniae  
CBA73264.1
169 3-Oxoacyl-ACP synthase III  
P. carotovorum  
WP_010301235.1
170 3-Oxoacyl-ACP synthase III  
P. pacifica  
WP_006975318.1
171 3-Oxoacyl-ACP synthase III  
C. stagnale  
YP_007317906.1
172 3-Oxoacyl-ACP synthase III  
N. punctiforme  
YP_001865628.1
173 3-Oxoacyl-ACP synthase III  
R. sp. PCC 7116  
YP_007056099
174 3-Oxoacyl-ACP synthase III  
S. cyanobacteria  
YP_007130807.1
175 3-Oxoacyl-ACP synthase III  
Calothrix sp. PCC 6303  
YP_007138278
176 3-Oxoacyl-ACP synthase III  
N. punctiforme  
YP_001868566.1
177 3-Oxoacyl-ACP synthase III  
R. sp. PCC 7116  
YP_007057764.1

KS adjacent to XclA homologues

178 3-Oxoacyl-ACP synthase  
C. sp. PCC 7822  
YP_003899222.1
179 3-Oxoacyl-ACP synthase  
N. punctiforme  
YP_001865657.1
180 3-Oxoacyl-ACP synthase  
A. cylindrica  
YP_007155727.1

**KS type III PKS**

181 Chs-like  
R. baltica  
NP_868579
182 BPS (PLN03172)  
H. androaemum  
Q8SAS8
183 CHS H. (PLN03173)  
H. androaemum  
Q9FUB7
184 CHS9  
M. sativa  
AAA02827
185 STS  
P. quinquefolia  
AAM21773
186 BAS  
R. palmatum  
AAK82824
187 bpsA  
B. subtilis str. 168  
NP_390087
188 MXAN_6639  
M. xanthus  
YP_634756
189 PKS10  
M. tuberculosis  
NP_216176
190 PKS11  
M. tuberculosis  
NP_216181
191 Cpz6 Capramyzin ketosynthase  
Streptomyces sp. MK730–62F2
192 Germicidin synthase  
Streptomyces coelicolor  
3V71_A
193 RppA S  
S. antibioticus  
BAB91443
| 194 | RppA | S. avermitilis | NP_828307 |
| 195 | RppB | S. antibioticus | BAB91444 |
| 196 | O3I_37171 | N. brasiliensis | ZP_09843377 |
| 197 | M446_0174 | M. sp. 4-46 | YP_001767187 |
| 198 | cpin6850 | C. pinensis | YP_003126452 |
| 199 | BFO_3187 | T. forsythia | YP_005015826 |
| 200 | NiAsoDRAFT_0547 | N. soli | ZP_09632794 |
| 201 | Mucpa_6793 | M. paludis | ZP_09618305 |
| 202 | Oweho_0889 | O. hongkongensis | YP_004988545 |
| 203 | CHU_0390 | C. hutchinsonii | YP_677020 |
| 204 | Fluta_1447 | F. taffensis | YP_004344279 |
| 205 | Dfer_5797 | D. fermentans | YP_003090150 |
| 206 | BZARG_2045 | B. argentinensis | ZP_08820341 |
| 207 | Lacal_2074 | L. sp. 5H-3-7-4 | YP_004580348 |
| 208 | Aeqsu_0932 | A. sublithincola | YP_006417450 |
| 209 | Zobellia_2074 | Z. galactanivorans | YP_004736513 |
| 210 | Lbys_1508 | L. byssophila | YP_003997574 |
| 211 | HMPREF0204_10987 | C. gleum | ZP_07085127 |
| 212 | PM13_02465 | C. sp. CF314 | ZP_10726507 |
| 213 | HMPREF0156_01383 | B. taxon 274 str. F0058 | YP_06803320 |
| 214 | HMPREF9071_0527 | C. taxon 338 str. F0234 | YP_08201061 |
| 215 | CAPGl0001_0843 | C. gingivalis | YP_04056582 |
| 216 | HMPREF1154_2288 | C. sp. CM59 | ZP_10800679 |
| 217 | HMPREF1320_1701 | C. taxon 335 str. F0486 | EJF37460 |
| 218 | HMPREF1321_1154 | C. taxon 412 str. F0487 | ZP_10366882 |
| 219 | CAPSP0001_1216 | C. sputigena | ZP_03390203 |
| 220 | Coch_0547 | C. ochracea | YP_003140666 |
| 221 | HMPREF1319_0374 | C. ochracea | EJF43732 |
| 222 | HMPREF1977_1456 | C. ochracea | ZP_07866642 |
| 223 | Weevi_1554 | W. virosa | YP_004238932.1 |
| 224 | HMPREF9716_01579 | M. odoratimimus | EKB07937 |
| 225 | Myrod_1723 | M. odoratus | ZP_09672239 |
| 226 | HMPREF9711_01694 | M. odoratimimus | EKB04829 |
| 227 | HMPREF9712_01161 | M. odoratimimus | ZP_09523568 |
| 228 | Fco1_11845 | F. columnare | YP_004942963 |
| 229 | FP2279 | F. psychrophilum | YP_001297136 |
| 230 | PM10_02641 | F. sp. CF136 | ZP_10730768 |
| 231 | FF52_12311 | F. sp. F52 | ZP_10481912 |
| 232 | Fjoh_1102 | F. johnsoniae | YP_001193454 |
| 233 | FJSC11DRAFT_3961 | F. sp. JSC-11 | ZP_08987753 |
| 234 | Micag_1820011 | M. aeruginosa | CCI22605 |
| 235 | Ds_105116 | M. psychrophilum | YP_065553 |
| 236 | DaAHT2_1139 | D. psychrophilus | YP_003890456 |
| 237 | MidDRAFT_4065 | delta proteobacterium MLMS-1 | ZP_01289639 |
| 238 | CBGD1_514 | S. gotlandica | ZP_05070248 |
| 239 | SMGD1_1386 | S. gotlandica | EHP29910 |
| 240 | Sdel_2118 | S. deleyianum | YP_003305165 |
| 241 | Sulba_2257 | S. barnesii | YP_006405107 |
| 242 | Armi_2310 | A. nitrofigilis | YP_003656468 |
| 243 | HMPREF9401_0244 | A. butzleri | ZP_07890833 |
| 244 | Hbal_2902 | H. baltica | YP_003061270 |
| 245 | Parca3_01010003428 | P. arctica | ZP_10280196 |
| 246 | PspsU_010100018642 | P. spongiae | ZP_10300425 |
| 247 | PSJM300_17945 | P. stutzeri | AFN79642 |
| 248 | MDS_0597 | P. mendocina | YP_004378380 |
| 249 | Psefu_0435 | P. fulva | YP_004472512 |
| 250 | Plu2164 | P. luminescens | NP_929424 |
| 251 | PAU-RAVE6-3077 | P. asymbiotica | CAR66906 |
| 252 | PAU_02401 | P. asymbiotica | YP_003041237 |
| 253 | PchloO6_4243 | P. chlororaphis | ZP_10172862 |
|   | Genomic Location | Species | Accession Number |
|---|-----------------|---------|-----------------|
| 254 | DarB | *P. chlororaphis* | AAN18032 |
| 255 | Pch3084_3967 | *P. chlororaphis* | EJL05977 |
| 256 | PMI20_00702 | *P. sp. GM17* | ZP_10707840 |
| 257 | Daro_2368 | *D. aromatic* | YP_285574 |
| 258 | azo0292 DarB | *A. sp. BH72* | YP_931796 |
| 259 | Rter_3974 | *R. ferrirucedens* | YP_525203 |
| 260 | Sli1_0359 | *S. lithotrophicus* | YP_003522988 |
| 261 | PMI12_02025 | *V. sp. CF313* | ZP_10567997 |
| 262 | Varp_3389 | *V. paradoxxus* | YP_002945272 |
| 263 | Varpa_2231 | *V. paradoxxus* | YP_004154548 |
| 264 | Col_2002 | *M. haemolytica* | ZP_05992665 |
| 265 | COK_0379 | *M. haemolytica* | ZP_05988513 |
| 266 | HMPREF9417_0595 | *H. parainfluenzae* | ZP_08147854 |
| 267 | HMPREF9952_1824 | *H. pittmaniae* | ZP_08755481 |
| 268 | HMPREF9064_0174 | *A. segnis* | ZP_07888807 |
| 269 | ATCC33389_0196 | *A. aphrophilus* | EGY32238 |
| 270 | NT05HA_1737 | *A. aphrophilus* | YP_003008155 |
| 271 | HMPREF9335_01583 | *A. aphrophilus* | EHB98432 |
| 272 | GOWU000324_02596 | *K. oralis* | ZP_04603113 |
| 273 | EIKCOROL_00456 | *E. corrodens* | ZP_03712789 |
| 274 | HMPREF9371_1043 | *N. shayegani* | ZP_08866538 |
| 275 | HMPREF9370_1914 | *N. wadsworthii* | ZP_08940206 |
| 276 | NEIFLAIOT_02523 | *N. flavescens* | ZP_03720660 |
| 277 | HMPREF0604_01363 | *N. mucosa* | ZP_07993739 |
| 278 | NEIFL0001_0036 | *N. flavescens* | ZP_04757628 |
| 279 | NEISUBOT_03200 | *N. subflava* | ZP_05983976 |
| 280 | NEISICOT_02133 | *N. sicca* | ZP_05318975 |
| 281 | HMPREF9418_1128 | *N. macacae* | ZP_08684521 |
| 282 | HMPREF1051_1749 | *N. sicca* | EIG27057 |
| 283 | HMPREF1028_00835 | *N. sp. GT4A_CT1* | ZP_08888860 |
| 284 | HMPREF9016_01947 | *N. taxon 014 str. F0314* | ZP_06980826 |
Table S5: NMR spectroscopic data (400 MHz, J in Hz) of pseudopyronine A, B and C in DMSO-d$_6$.

| Position | Pseudopyronine A | Pseudopyronine B | Pseudopyronine C |
|----------|------------------|------------------|------------------|
|          | δ$_C$ (δ$_H$ (J in Hz)) | δ$_C$ (δ$_H$ (J in Hz)) | δ$_C$ (δ$_H$ (J in Hz)) |
| 2        | 164.9 -             | 165.0 -             | 166.9 -             |
| 3        | 100.7 -             | 101.3 -             | 101.5 -             |
| 3a       | 22.7 2.23 (t, 8.0, 2H) | 22.7 2.24 (t, 8.0, 2H) | 22.8 2.22 (t, 8.0, 2H) |
| 3b       | 27.5 1.34 (m, 2H)   | 27.5 1.35 (t br, 8.0, 2H) | 27.7 1.34 (t br, 8.0, 2H) |
| 3c       | 28.5 1.24 (m, 2H)   | 28.6 1.26 (m, 2H)   | 28.8 1.23 (m, 2H)   |
| 3d       | 30.4 1.28 (m, 2H)   | 31.2 1.25 (m, 2H)   | 31.2 1.24 (m, 2H)   |
| 3e       | 22.0 1.24 (m, 2H)   | 22.0 1.26 (m, 2H)   | 22.1 1.25 (m, 2H)   |
| 3f       | 13.8 0.86 (m, 3H)   | 14.0 0.84 (m, 3H)   | 13.9 0.85 (m, 3H)   |
| 4        | 162.2 -             | 164.8 -             | 165.1 -             |
| 5        | 99.9 5.91 (s, 1H)   | 99.2 5.94 (s, 1H)   | 100.4 5.86 (s, 1H)  |
| 6        | 162.2 -             | 162.6 -             | 162.0 -             |
| 6a       | 32.5 2.36 (t, 8.0, 2H) | 32.6 2.37 (t, 8.0, 2H) | 32.6 2.34 (t, 8.0, 2H) |
| 6b       | 25.8 1.52 (m, 2H)   | 26.2 1.51 (t br, 8.0, 2H) | 26.2 1.50 (t br, 8.0, 2H) |
| 6c       | 31.1 1.26 (m, 2H)   | 28.6 1.26 (m, 2H)   | 28.6 1.26 (m, 2H)   |
| 6d       | 21.7 1.26 (m, 2H)   | 28.2 1.28 (m, 2H)   | 28.2 1.26 (m, 2H)   |
| 6e       | 13.7 0.86 (m, 3H)   | 31.1 1.25 (m, 2H)   | 28.6 1.28 (m, 2H)   |
| 6f       | 22.0 1.26 (m, 2H)   | 28.6 1.28 (m, 2H)   | 31.2 1.26 (m, 2H)   |
| 6g       | 13.8 0.84 (m, 3H)   | 22.1 1.26 (m, 2H)   | 13.9 0.85 (m, 3H)   |
| 6h       |                   |                   |                   |
| 6i       |                   |                   |                   |

Pseudopyronine A

Pseudopyronine B

Pseudopyronine C
**Figure S1:** Analysis of amino acid substitutions for PpyS activity regarding the biosynthesis of photopyrone D (PPYD, 4). The data is calculated in dependence to the production of the PpyS wildtype enzyme. Cys129, His281 and Asn310 form the catalytic triade, Glu105 is proposed to act as a catalytic base, Arg121 is located at the dimerization interface and Glu330 was used as a neutral control. In PpyS-C129A, -N310A, -E105A and -R121D the production of 4 was not detectable (n. d.) anymore.
Figure S2: Extracted-ion chromatograms (EICs) of the most abundant photopyrones, which are produced heterologously in *E. coli* wildtype and mutant strains. 2 (m/z 267.2 [M + H]+), 3 (m/z 281.2 [M + H]+), 4 (m/z 295.2 [M + H]+), 5 (m/z 309.2 [M + H]+), 6 m/z 323.3 [M + H]+. As the data for PpyS N310A, E105A and R121D look identical to C129A showing a complete loss of photopyrone production, they are not shown here.
Figure S3: Structure of OleA-dimer (PDB: 3ROW) from Xanthomonas campestris (A). Modeled structure of PpyS-dimer from P. luminescens TT01 (B), which was generated using the OleA structure as a template: Red (α-helices), yellow (β-sheets), blue (turns), white (random coils). The superposition of OleA (blue) and PpyS (red) structures (C) revealed a root-mean-square deviation (RMSD) of 2.0 Å. To picture the dimer interface of PpyS the surface of chain α was calculated (green, magenta, and white represent a lipophilic, hydrophilic and neutral surface area, respectively) and the ribbon of chain β is shown (D). At the interface the atoms of Arg121β are represented as spheres, this residue is predicted to be involved in the dimerization of PpyS by interacting with Asp137α. The mutation of Arg121 led to a complete loss of photopyrone production. Furthermore Glu330 is shown in cyan spheres, this residue was mutated as a control, which should not influence the photopyrone production as indeed shown in Figure S1.
Figure S4: Structure of the FabH-dimer (PDB: 1HN9) from *E. coli* (A). Structure of FabH-monomer (PDB: 1HNJ) from *E. coli* along with co-crystallized malonyl-CoA (B). The superposition of both these structures (C) reveals the location of the substrate malonyl-CoA within the dimeric structure and the distance to Phe87\(\beta\), which is the analogue to Glu105 of PpyS. With a shortest distance of ca. 5.3 Å to the ligand this residue seems not to be directly involved in the catalysis.
Figure S5: Last step of myxopyronin A [15] and corallopyronin A [16] biosynthesis. The western (red) and the east (blue) chains are thought to be condensed by the catalytic activity of the KS MxnB or CorB, respectively. The intermediate shown here follows from the nucleophilic attack of the deprotonated $\alpha$-carbon of the western chain with the carbonyl carbon of the eastern chain. Both intermediates were used for docking studies.
Figure S6: A multiple sequence alignment (ClustalW, standard parameters) of PpyS from *P. luminescens* TT01, YP_004230959 (YP42) from *Burkholderia* sp. CCGE1001, WP_016949109 (WP16) from *Anabaena* sp. PCC 7108, WP_007967127 (PyrS) from *Pseudomonas* sp. GM30, OleA from *X. campestris*, FabH from *E. coli*, CorB from *C. coralloides*, MxnB from *M. fulvus*, Cpz6 [17] (caprazamycin biosynthesis) from *Streptomyces* sp. MK730-62F2 and Gcs [18] (germicidin synthase) from *S. coelicolor*. Highly conserved residues are shown in grey, conserved catalytic triad and position of E105 from PpyS are highlighted in black.
Figure S7: Extracted-ion chromatograms (EICs) of pseudopyronines. 9 (m/z 267.2 \([M + H]^+\)), 10 (m/z 295.2 \([M + H]^+\)), 11 (m/z 323.2 \([M + H]^+\)). In *Pseudomonas putida* KT2440 no pseudopyronines could be detected (top chromatogram). All chromatograms are drawn to the same scale.
Figure S8: Structure of OleA-dimer (PDB: 3ROW) from *Xanthomonas campestris* (A). Modeled structure of pseudopyronine synthase (PyrS)-dimer from *Pseudomonas* sp. GM30 (B), which was generated using the OleA structure as a template. The superposition of OleA (blue) and PyrS (red) structures (C) revealed a root-mean-square deviation (RMSD) of 3.3 Å. The modeled PyrS-dimer structure with covalently to Cys124 docked pseudopyronine B intermediate (19, D). In a green sphere representation Glu100 is shown, which is the analogue to Glu105 of PpyS.
Figure S9: A detailed view of the proposed PyrS-binding pocket with covalently to Cys124 docked substrate (17, A) and intermediat (19, B) of pseudopyronine B. The catalytic triade consists of Cys124, His277 and Asn306. The cavity of the binding pocket is shown in a line representation, where green represents a lipophilic surface area, magenta a hydrophilic and white a neutral. Possible formed hydrogen bonds are shown as dashed blue lines.
Scheme S1: Proposed biosynthesis of 10 by PyrS from *Pseudomonas* sp. GM30. In the first step octanoic acid (17) is covalently bound to the active site Cys124. The deprotonated $\alpha$-carbon nucleophilically attacks a 3-oxodecanoyl thioester (18, $R = \text{ACP}$ or CoA) to form the covalently bound intermediate (19). Due to a spontaneous or a catalyzed deprotonation (by E100) the pyrone ring is formed and 10 is released from PyrS.
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$^1$H-NMR spectrum of pseudopyronine C, DMSO-\textit{d6}
$^{13}\text{C}$ -NMR spectrum of pseudopyronine C, DMSO-$d_6$
COSY spectrum of pseudopyronine C, DMSO-$d_6$
HSQC spectrum of pseudopyronine C, DMSO-d6
HMBC spectrum of pseudopyronine C, DMSO-$d_6$