Supporting Information

Genome Mining in *Streptomyces*. Discovery of an Unprecedented P450-Catalyzed Oxidative Rearrangement That is the Final Step in the Biosynthesis of Pentalenolactone

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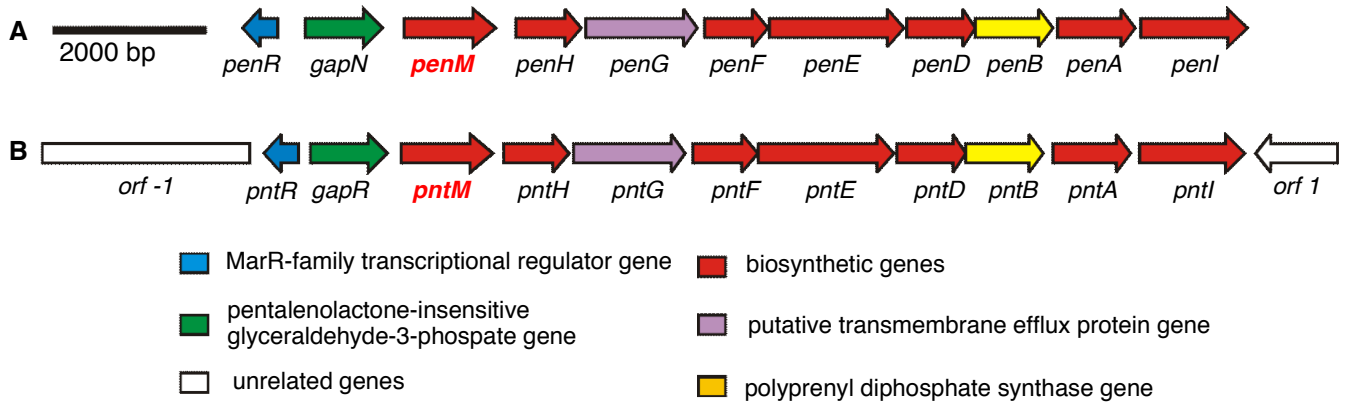
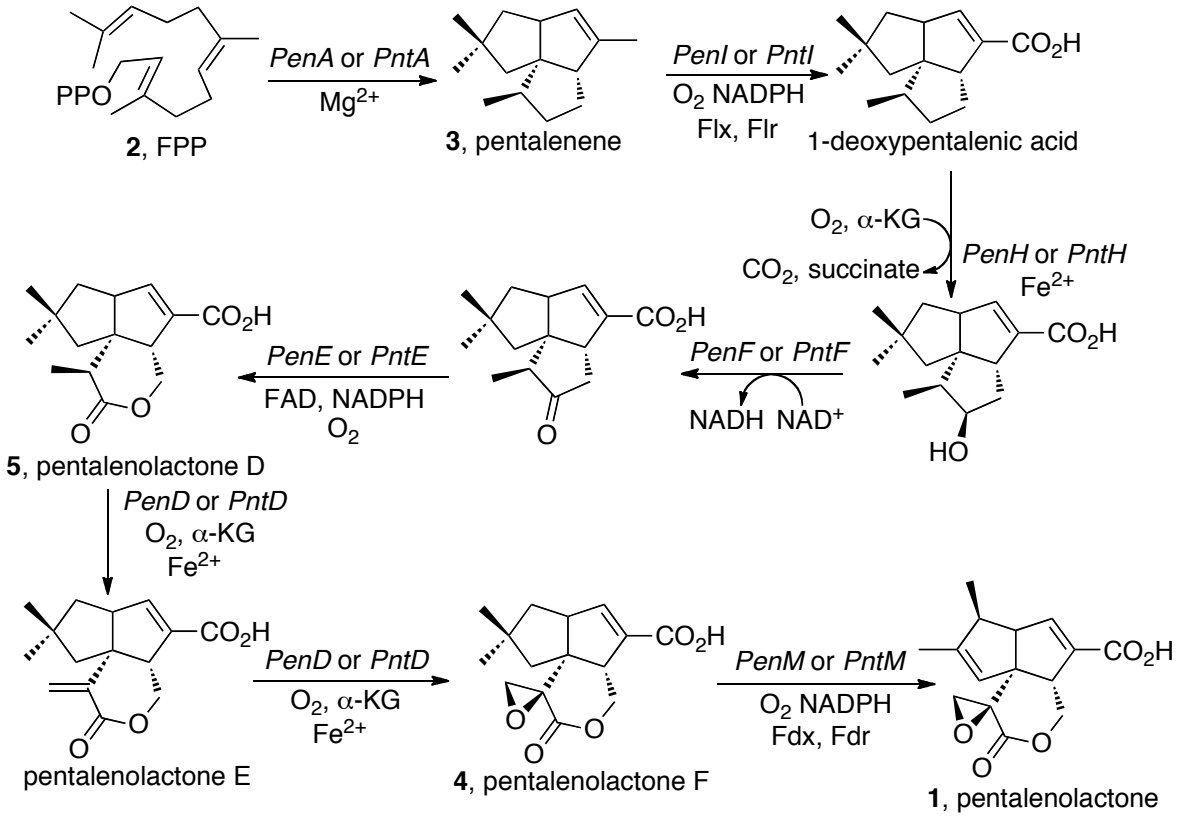


Figure S1. Pentalenolactone Biosynthetic Gene Clusters. A. *S. exfoliatus* UC5319 *pen* cluster. B. *S. arenae* TÜ469 *pnt* cluster.



Scheme 1. Pentalenolactone biosynthetic pathway and role of Pen and Pnt gene products.

EXPERIMENTAL PROCEDURES

Materials. Reagents and solvents purchased from Sigma-Aldrich or Fisher Scientific were of the highest quality available and were used without further purification. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and used according to the manufacturer's specifications. Isopropylthio-β-D-galactopyranoside (IPTG) was purchased from Invitrogen. Ni-NTA affinity resin was purchased from Qiagen. Amicon Ultra Centrifugal Filter Units (Amicon Ultra-15,

30,000 MWCO) were purchased from Millipore. DNA primers were synthesized by Integrated DNA Technologies. Synthetic genes encoding PenM and PntM, optimized for expression in *Escherichia coli*, were prepared by DNA2.0 and supplied in the vector pJ201. The sources of the majority of the strains and plasmids used in this work have been previously described.[1] Plasmid DQ20 harbors a ca. 3.1-kb *Bam*HI fragment from cosmid 1E2 derived from *S. arenae* TÜ469, corresponding to a partial *orf-1*, *pntR*, *gapR*, and partial *pntM* inserted into the *Bam*HI site of pSET152.[1] Plasmid DQ22 harbors a ca. 6.2-kb BamHI fragment from cosmid 1E2 derived from *S. arenae* TÜ469, corresponding to partial *pntM* plus *pntH*, *pntG*, *pntF*, *pntE*, and partial *pntD* inserted into the *Bam*HI site of pSET152.[1] Plasmid pDQ44 is a derivative of the *Streptomyces* gene knockout vector pJTU1278 that has been digested with *Xba*I and *Spe*I and self-ligated.[1,2] Cosmid G21 harbors the *pen* cluster.[1]

Methods. General methods were as previously described.[1,3,4] Growth media and conditions used for *E. coli* and *Streptomyces* strains and standard methods for handling *E. coli* and *Streptomyces in vivo* and *in vitro* were those described previously, unless otherwise noted.[5,6] All DNA manipulations were performed following standard procedures.[6] DNA sequencing was carried out at the U. C. Davis Sequencing Facility, Davis, CA. All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford, using a Hewlett Packard 8452A Diode Array Uv/Vis spectrophotometer with bovine serum albumin as the standard.[7] Protein purity was estimated using SDS-PAGE gel electrophoresis and visualized using Coomassie Blue stain. GC-MS analyses were carried out using a Hewlett Packard Series 2 GC-MSD, at 70 eV electron impact (EI), operating in positive ion mode, using a HP5MS capillary column (30 m × 0.25 mm) with a solvent delay of 3 min and a temperature program of 60 °C for 2 min, followed by a termperature gradient of 60-280 °C for 11 min at 20 °C/min, and a hold at 280 °C for 2 min. MALDI-TOF measurements were performed on an Applied Biosystems Voyager DE PRO MALDI-TOF bench top mass spectrometer. LC-ESI-MS analysis of recombinant proteins was carried out on a Thermo LXQ LC-ESI-MS equipped with Surveyor HPLC system and Waters Symmetry C18 column (2.1 mm x 50 mm, 3.5 μm).

Isolation of Pentalenolactone F. Authentic pentalenolactone F (**4-Me**) was isolated and purified as the methyl ester from cultures of wild-type *S. exfoliatus* UC5319 by J. K. Sohng, as previously described.[8] The free acid **4** was generated by hydrolysis of **4-Me** in 2 mL of 5% aqueous K_2CO_3 and 5 mL of methanol at reflux for 24 h. After evaporation of the MeOH by rotoevaporation, the aq. solution was acidified to pH 2.0 with 10% HCl and then extracted with $CHCl_3$. The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated to give pentalenolactone F (**4**). In subsequent experiments, pentalenolactone F methyl ester (**4-Me**) was isolated in the same manner from cultures of the *ΔpenM* deletion mutant, *S. exfoliatus* ZD22.

Expression and purification of recombinant PenM and PntM Proteins. The synthetic *penM* and *pntM* genes in the pJ201 vector were prepared by DNA2.0 with codons optimized for expression in *E. coli*. The 5′-end of each coding region was preceded by a synthetic linker 5′-CCATGGGCAGCAGCCATCATCATCACCACCATAGCAGCGGCCTGGTGCCGCGCGGCAGCC-AT-3′ with an *Nco*I site (underlined) containing the ATG start codon and encoding the N-terminal leader peptide MGSSHHHHHHSSGLVPRGSH (thrombin cleavage site in red) upstream of the natural N-terminal Met. Each of the plasmids was digested with *Nco*I and *Xho*I and then cloned into the corresponding sites of pET-28a(+) vector to generate pET28a-*penM* and pET28a-*pntM*, which were individually transformed into *E. coli* BL21(DE3) for expression. Cultures of each *E. coli* transformant were grown at 37 °C in Terrific Broth (TB) media containing 50 μg/ml kanamycin until an OD_{600} of 0.6-0.8, then induced with 0.4 mM IPTG. The cells were further grown at 18 °C overnight, harvested by centrifugation at 5,000*g* for 15 min, and then suspended in lysis buffer (50 mM Tris-HCl, 10% glycerol, 300 mM NaCl, 0.1 mM DTT, 2.7 mM β-mercaptoethanol, 10 mM imidazole, pH 8.0) containing 10 mg/L pepstatin, 10 mg/L phenylmethylsulfonyl fluoride, and 0.2 mg/mL benzamidine. After disrupting the cells by sonication, the cell debris was separated by centrifugation at 20,000*g* for 30 min and the supernatant was applied to a Ni-NTA column equilibrated with lysis buffer. The column was washed with 20 mM imidazole in lysis buffer, and the protein was eluted with 200 mM imidazole in lysis buffer

followed by monitoring by SDS-PAGE. The fractions containing the recombinant His_6-tag-PenM or His_6-tag-PntM proteins were pooled, concentrated, and then dialyzed against storage buffer (50 mM Tris-HCl, 10% glycerol, pH 8.0) using an Amicon Ultra Centrifugal Filter (Ultracel-30K). The purified PenM and PntM concentrations were 21.45 and 26.93 mg/L of culture, respectively. ESI-MS, His_6-tag-PenM, 46428 Da (predicted 46428 for P-Met protein) and His_6-tag-PntM 46196 Da (predicted 46195 for P-Met protein).

Native molecular weight. The native molecular weights of His_6-tag-PenM and His_6-tag-PntM were determined by high resolution size exclusion chromatography on a HiPrep 16/60 Sephacryl S-200 HR column calibrated with standard molecular weight markers ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), and albumin (67.0 kDa). The calculated M_r values for PenM and for PntM were each 50,300 and 50,100, establishing that each protein is a monomer.

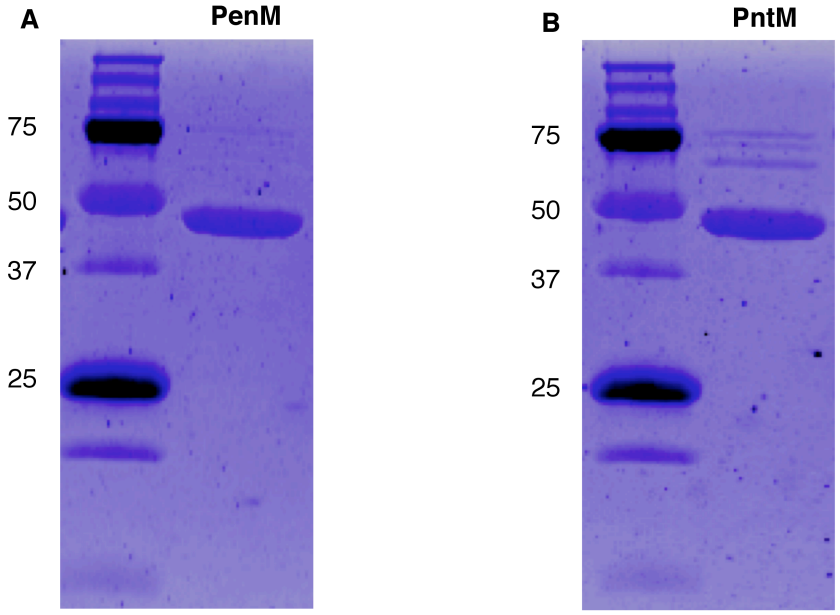


Figure S2. SDS-PAGE analysis of A. His_6-tag-PenM. B. His_6-tag-PntM

UV-visible spectra. The CO-difference spectra of the purified recombinant PenM and PntM were monitored from 400 nm to 500 nm. After directly recording the spectra of the oxidized Fe^{3+} resting forms of PenM and PntM in storage buffer, 1 M sodium dithionite was added to reduce the purified ferric P450 prior to recording the spectra of the reduced Fe^{2+} proteins. The CO-ferrous P450 complex

was generated by gently bubbling of CO gas into the cuvette for 1 min and then comparison with the sodium dithionite-reduced spectra to give the reduced CO-difference spectra. The P450 concentrations of PenM and PntM were calculated using the extinction coefficient ε=91 cm^{-1} mM^{-1}.[9] The P450 content of PenM and PntM was 166 μM and 186 μM, respectively, compared to measured protein concentrations of 156 μM and 168 μM.

Substrate binding assay. Pentalenolactone F (**4**) dissolved in DMSO was added at increasing concentrations (12.70-170.03 μM) to the already diluted PenM (1.17 μM) and PntM (1.32 μM) in 50 mM Tris-HCl buffer (pH 8.0) with 10% glycerol and the spectra (350-500 nm) were then recorded. A saturation curve was obtained by measuring the absorbance difference (ΔAU=A390 - A420) versus substrate concentration. The calculated K_D values for **4** with PenM and PntM were 153±14 μM and 126±11 μM, respectively.

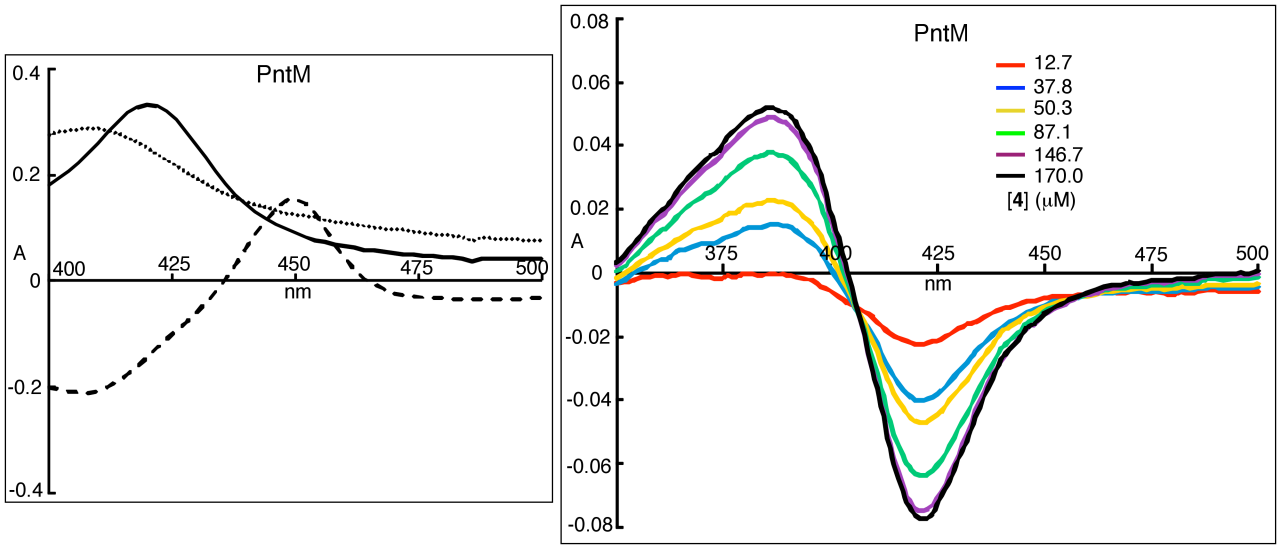


Figure S3. Characterization of PntM - CYP161C2. A. Reduced CO difference spectra. Solid line, free, Fe^{3+}-PntM; dotted line, Fe^{2+}-PntM after $Na_2S_2O_4$ reduction; broken line, reduced-CO complex. B. Titration with pentalenolactone F (**4**), UV difference spectra.

Incubation of PenM and PntM with pentalenolactone F (4). For the *in vitro* enzyme reactions, purified recombinant His_6-tag-PenM (16.6 μM) and His_6-tag-PntM (12.4 μM) were separately added in individual incubations to 20 mM Tris-HCl buffer (pH 8.0) containing pentalenolactone F (**4**, 0.11 mM), 2.5% glycerol, 55 μg/ml spinach ferredoxin, and 0.125 U/ml spinach ferredoxin-$NADP^+$ reductase in total volume of 2 mL. After pre-incubation for 5 min at 37 °C, the reactions were initiated by addition of

2.5 mM NADPH and continued for 4 h at 30 °C. The reactions were terminated by addition of 2 N HCl (20 μL) and the mixtures were then extracted with 3 × 2 mL of dichloromethane. The resulting organic extracts were dried over anhydrous Na_2SO_4, concentrated, and redissolved in 100 μL of methanol. Trimethylsilydiazomethane (TMS-CHN_2, 10 μL of a 2 M solution in hexane) was added to generate pentalenolactone methyl ester (**1-Me**), which was then analyzed by GC-MS. The major reaction product, ret. time 12.90 min (*m/z* 290), was identical by direct comparison with authentic pentalenolactone methyl ester (**1-Me**). Although the substrate **4-Me** and the product **1-Me** are not baseline-resolved in the one-dimensional GC time domain (Δret. time 0.02 min), the individual time-resolved mass spectra represented by vertical slices of each GC-MS chromatogram can cleanly distinguish the two compounds so as to allow the reaction progress to be monitored, as described below for the steady-state kinetics and illustrated in Figure S5. Control incubations with boiled PenM or PntM, without ferredoxin and ferredoxin reductase, or without added NADPH gave only recovered pentalenolactone F.

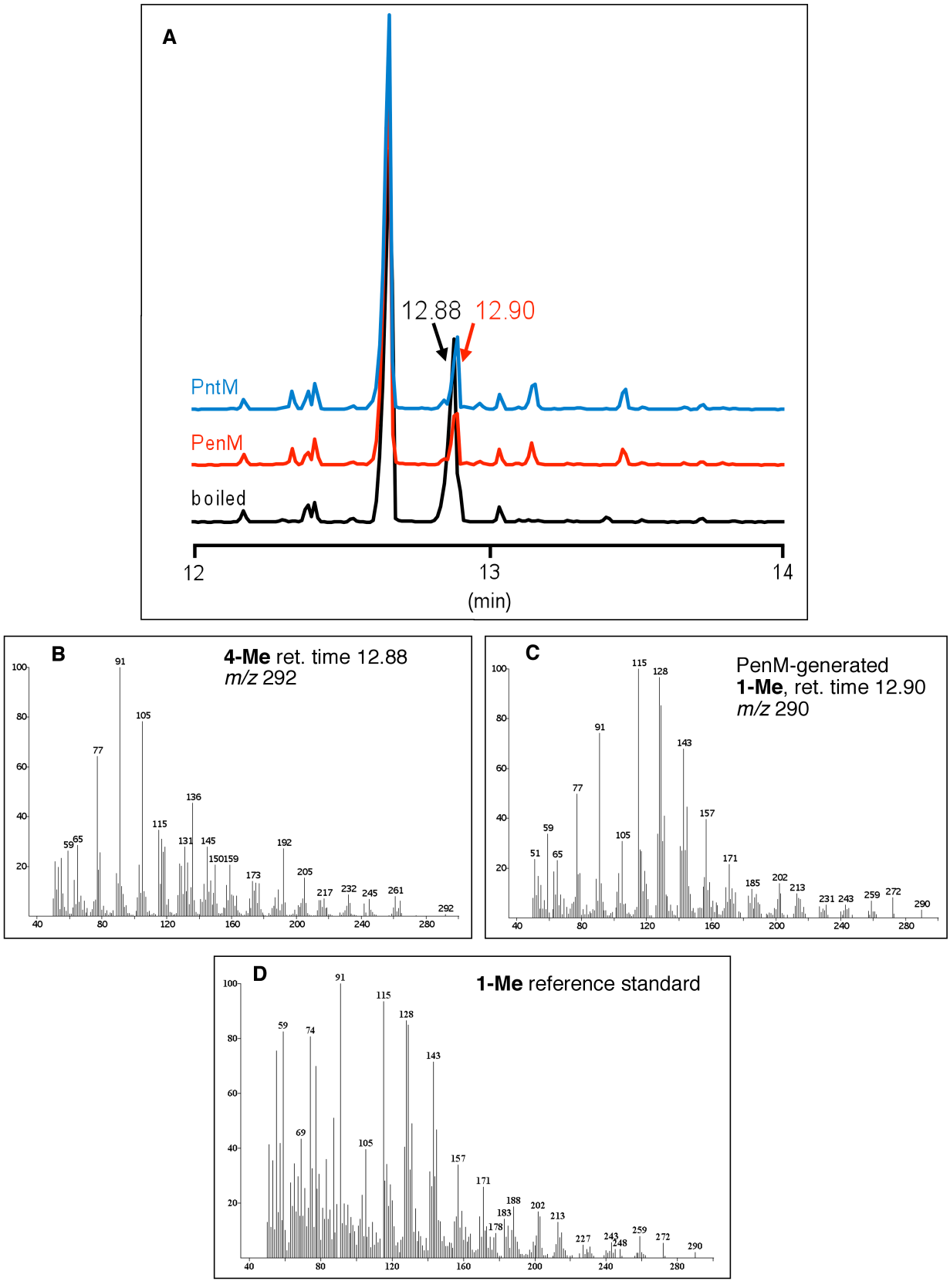


Figure S4. GC-MS analysis of incubation of PenM and of PntM with pentalenolactone F (**4**). A. GC-MS, incubation with PenM, PntM, and boiled PenM. B. Mass spectrum of pentalenolactone F methyl ester (**4-Me**), ret. time 12.88 min. C. Mass spectrum of pentalenolactone methyl ester (**1-Me**), ret. time 12.90 min. D. Mass spectrum of **1-Me** reference standard.

Steady-state kinetic analysis of PenM- and PntM-catalyzed oxidations. Kinetic assays were carried out at 30 °C with PenM (1.16 μM) and PntM (1.86 μM) in 20 mM Tris-HCl buffer (pH 8.0) containing 2.5% glycerol, 44 μg/mL spinach ferredoxin, 0.1 U/mL spinach ferredoxin-$NADP^+$ reductase, and pentalenolactone F (**4**) (71.9 – 863.3 μM) in a total volume of 1 mL. After pre-incubation for 5 min, the reactions were initiated by addition 0.6 mM NADPH and then continued for 10 min. The reactions were quenched with 2 N HCl to give a final pH 2, extracted with dichloromethane, and then analyzed by GC/MS, using a predetermined calibration for quantitation of pentalenolactone methyl ester (**1-Me**), which gave a linear response of peak area vs. [**1-Me**] with an R^2 = 0.9987. The steady-state kinetic parameters were calculated from the observed initial velocities and substrate concentrations by direct fitting to the Michaelis-Menten equation (Kaleidagraph 4.0) to give K_m (**4**) of 343±101 μM and a V_{max} 12.2±2.1 μM/min, corresponding to a k_{cat} of 10.5±1.8 min^{-1} for PenM and K_m(**4**) 428±100 μM and a V_{max} 16.4±1.8 μM/min, corresponding to a k_{cat} of 8.8±0.9 min^{-1} for PntM. Reported standard deviations in the steady-state kinetic parameters represent the calculated statistical errors in the non-linear, least squares regression analysis.

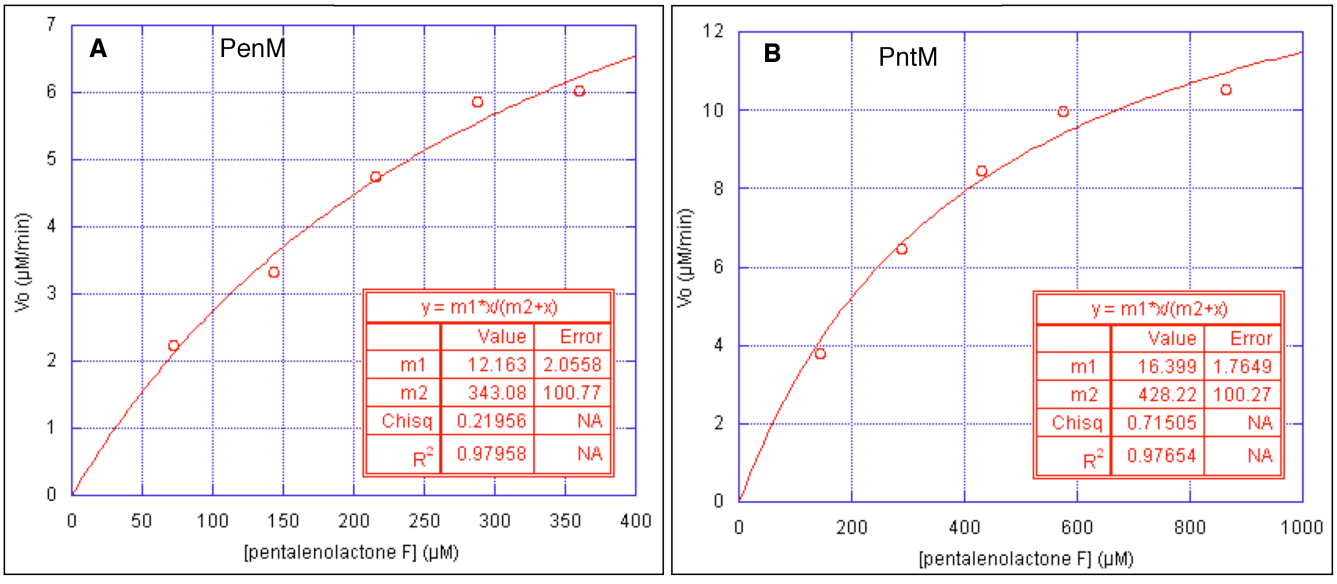


Figure S5. Steady-State kinetic analysis of conversion of pentalenolactone F (**4**) to pentalenolactone (**1**). A. PenM. B. PntM

Construction of *ΔpenM* mutant *S. exfoliatus* ZD22. A pair of primers DQ84F/R (DQ84F: 5′-GGTCGGATCCAGTTCGGTCAGCC-3′, *Bam*HI underlined; DQ84R:

5′-AACGGTAAGCTTGGGAGTTCGTTCA-3′, *Hin*dIII underlined) was designed to amplify an 1860-bp DNA fragment as the left arm of *penM* deletion. A pair of primers DQ85F/R (DQ85F: 5′-CGACGAAAGCTTCGTCACCTTCTGA-3′, *Hin*dIII underlined; DQ85R: 5′-GCGGGTACCAGCGAGACCAGCAG-3′, *Kpn*I underlined) was designed to amplify a 1752-bp DNA fragment as the right arm of *penM* deletion. Cosmid G21 harboring the *pen* gene cluster[1] was used as the PCR template. The 1860-bp DNA fragment digested with *Bam*HI and *Hin*dIII and the 1752-bp DNA fragment digested with *Hin*dIII and *Kpn*I were inserted into the corresponding *Bam*HI and *Kpn*I sites of vector pDQ44 to generate plasmid pDQ60. Plasmid pDQ60 was sequenced to prove that there had been no errors introduced during PCR amplification. The 1170-bp DNA fragment of *penM* from 15 nt to 1184 nt was replaced by a 6-bp DNA fragment corresponding to the *Hin*dIII site from pDQ60. The remaining portions of *penM* retained in pDQ60 were 14 bp from the 5′-end and 13 bp from the 3′-end. The two arms from the *pen* cluster used in homologous recombination were the flanking 1838-bp and 1731-bp segments (Figure 2). The plasmid pDQ60 was conjugated into *S. exfoliatus* UC5319 and grown on plates overlaid with thiostrepton. The exconjugants were inoculated to onto SFM agar plates (20 g soy flour, 20 g mannitol, 20 g agar in 1 L water[5]) with thiostrepton to confirm the resistance of single crossover strains and then they were inoculated to SFM medium plate without any antibiotic in order to obtain double crossover strains under relaxed conditions. The primer pair DQ86F/R (DQ86F: 5′-GACGAATCGGGCGAGGAA-3′; DQ86R: 5′-GAGGTGACGAGGTCAGAGGGAC-3′) was designed to select the *penM* mutant. The PCR product of wild type strain was 1645 bp and the PCR product of the *ΔpenM* mutant was 481 bp (Figure S6). An internal 1170 bp of *penM* from 15 nt to 1184 nt on the chromosome of *S. exfoliatus* UC5319 was replaced by a 6 bp *Hin*dIII scar to generate *S. exfoliatus* ZD22, a *ΔpenM* in-frame deletion mutant.

Construction of *ΔpntM* mutant *S. arenae* ZD23. A *Pvu*II + *Bam*HI DNA fragment of ca. 2222 bp from plasmid pDQ20 carrying a portion of the *S arenae pnt* cluster and a 5′-fragment of *pntM*[1] and a *Bam*HI + *Kpn*I DNA fragment of ca. 1883 bp from plasmid pDQ22 carrying a downstream portion of

the *pnt* cluster from *S. arenae* including a 3′-fragment of *pntM*[1]were inserted into the *Eco*RV + *Kpn*I sites of pBluescriptII SK(+) to generate pDQ52 harboring *pntR*, *gapR*, *pntM*, *pntH* and *pntG*. The plasmid pDQ52 was digested with *Eco*RI and *Kpn*I and the 4105 bp DNA fragment was recovered and inserted into *Eco*RI- and *Kpn*I-digested pDQ44 to generate plasmid pDQ53 (Figure S6). pDQ53 was transformed into *E. coli* BW25113/pIJ790.[10,11] A pair of primers DQ82F/R (DQ82F: 5′-ATGACGGATCTCCCGAGGTTACCGTTCGACAACCCGGACATTCCGGGGATCCGTCGACC-3′, DQ82R: 5′-TCAGAAGGTGACGGGAATTTCGTCGAACCCGCCGGTGATTGTAGGCTGGAGCTGCTTC-3′) was used for PCR-amplification of an *Eco*RI + *Hin*dIII DNA fragment carrying *oriT* and *aac(3)IV* of ca. 1382 bp that was recycled from plasmid pIJ773 to give a 1447-bp DNA fragment that was transformed into *E. coli* BW25113/pIJ790 containing pDQ53 to generate plasmid pDQ54.[10] The 1119-bp DNA fragment of *pntM* from 40 nt to 1158 nt was replaced by *oriT* and *aac(3)IV* (ca. 1369 bp) on pDQ54. The plasmid pDQ54 from *E. coli* BW25113 was transformed into *E. coli* ET12567/pUZ8002. The resultant unmethylated pDQ54 was isolated from *E. coli* ET12567/pUZ8002, digested with *Xba*I, and ligated to generate pDQ57, from which the 1369-bp *oriT* and *aac(3)IV* DNA fragment had been deleted and replaced by an 81-bp scar. Plasmid pDQ57 was conjugated into *S. arenae* TÜ469 and thiostrepton was used to select exconjugants. The single crossover strains were inoculated onto an SFM plate without any antibiotic in order to select double crossover strains under relaxed conditions that had lost thiostrepton resistance. The primer pair DQ83F/R (DQ83F: 5′-GCTCATCGGGTCGTCCCTGTA-3′); DQ83R: 5′-GGCGTAGTCGCTGAAAGTGTCC-3′)) was designed to select the *ΔpntM* mutant. The PCR product of the wild type strain was 1563 bp and the PCR product of the *ΔpntM* mutant was 525 bp (Figure S6). An internal 1119 bp of *pntM* from 40 nt to 1158 nt on the chromosome of *S. arenae* TÜ469 was replaced by a 81-bp scar to generate *S. arenae* ZD23, a *ΔpntM* in-frame deletion mutant.

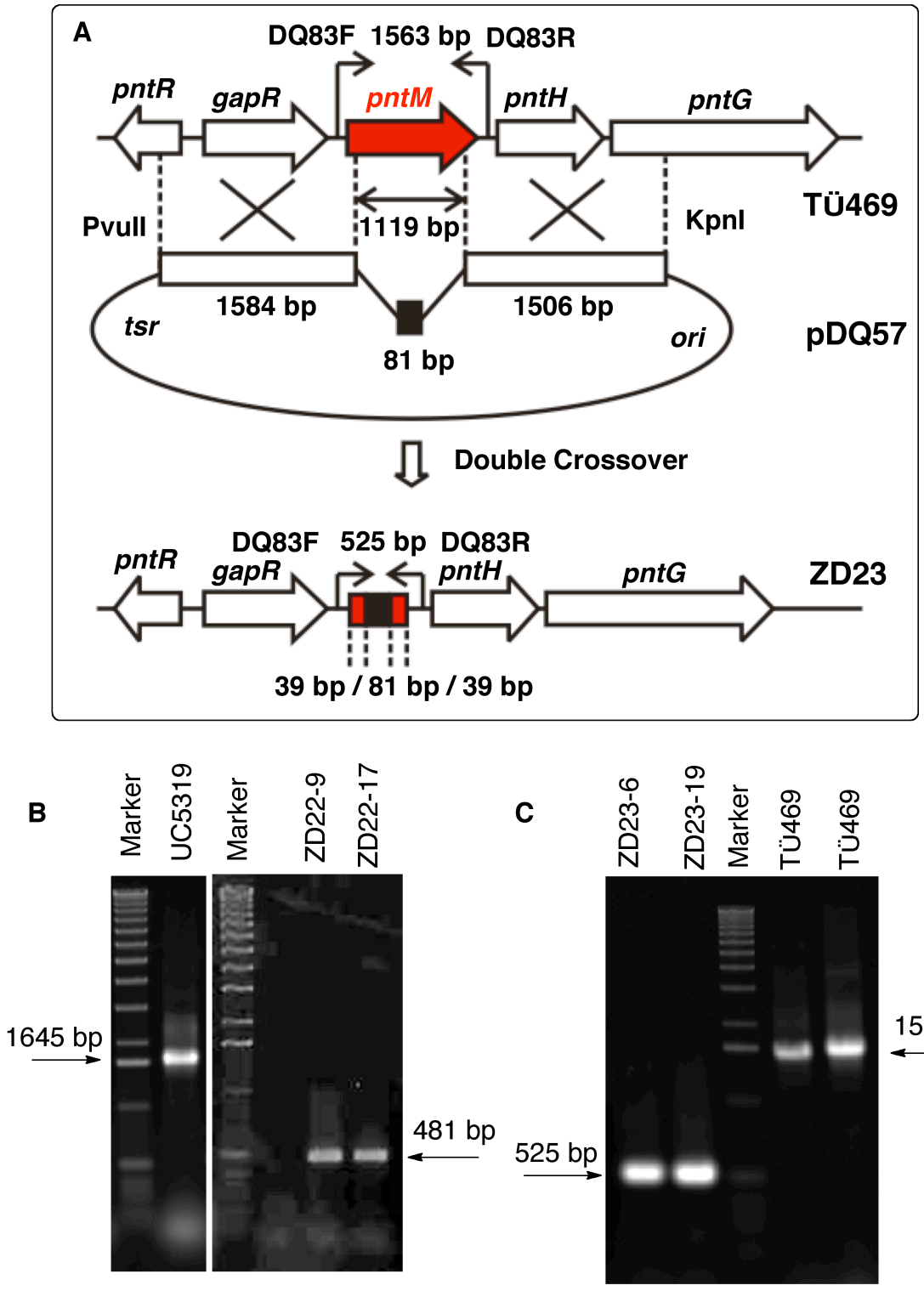


Figure S6. A. Construction of *ΔpntM* deletion mutant *S. arenae* ZD23 B. PCR verification of the *ΔpenM* deletion mutant. C. PCR verification of the *ΔpntM* deletion mutants.

Analysis of pentalenolactone production in wild-type *S. exfoliatus* UC5319 and *S. arenae* TÜ469 and deletion mutants *S. exfoliatus* ZD22/*ΔpenM* and *S. arenae* ZD23/*ΔpntM*. The liquid medium used in fermentation of *S. exfoliatus* UC5319 and ZD22 contained 0.2% NaCl, 0.5% $CaCO_3$, 1% corn

gluten meal, 0.1125% Bactodextrose, 0.2% blackstrap molasses, and 2% corn starch, pH 7.2.[8] After incubation at 30 °C for 6 days, the culture was acidified to pH 2.4 with H_2SO_4 and extracted with chloroform. The organic layer was dried over anhydrous Na_2SO_4, concentrated, methylated with TMS-CHN_2 and analyzed by GC-MS. The liquid synthetic medium used for fermentation of *S arenae* TÜ469 and ZD23 contained 4% mannitol, 0.25% asparagines, 0.2% $(NH_4)_2SO_4$, 0.1% NaCl, 0.3% K_2HPO_4•$3H_2O$, 0.1% $MgSO_4$•$7H_2O$, 0.04% $CaCl_2$•$2H_2O$, 0.002% $FeSO_4$•$7H_2O$, 0.001% $ZnSO_4$•$7H_2O$, pH 6.2.[12] The strains were fermented at 30 °C for 6 days. The fermentation broth was adjusted to pH 2.5 with HCl and extracted with chloroform. The organic layers were dried over anhydrous Na_2SO_4, concentrated, methylated with TMS-CHN_2 and analyzed by GC-MS.

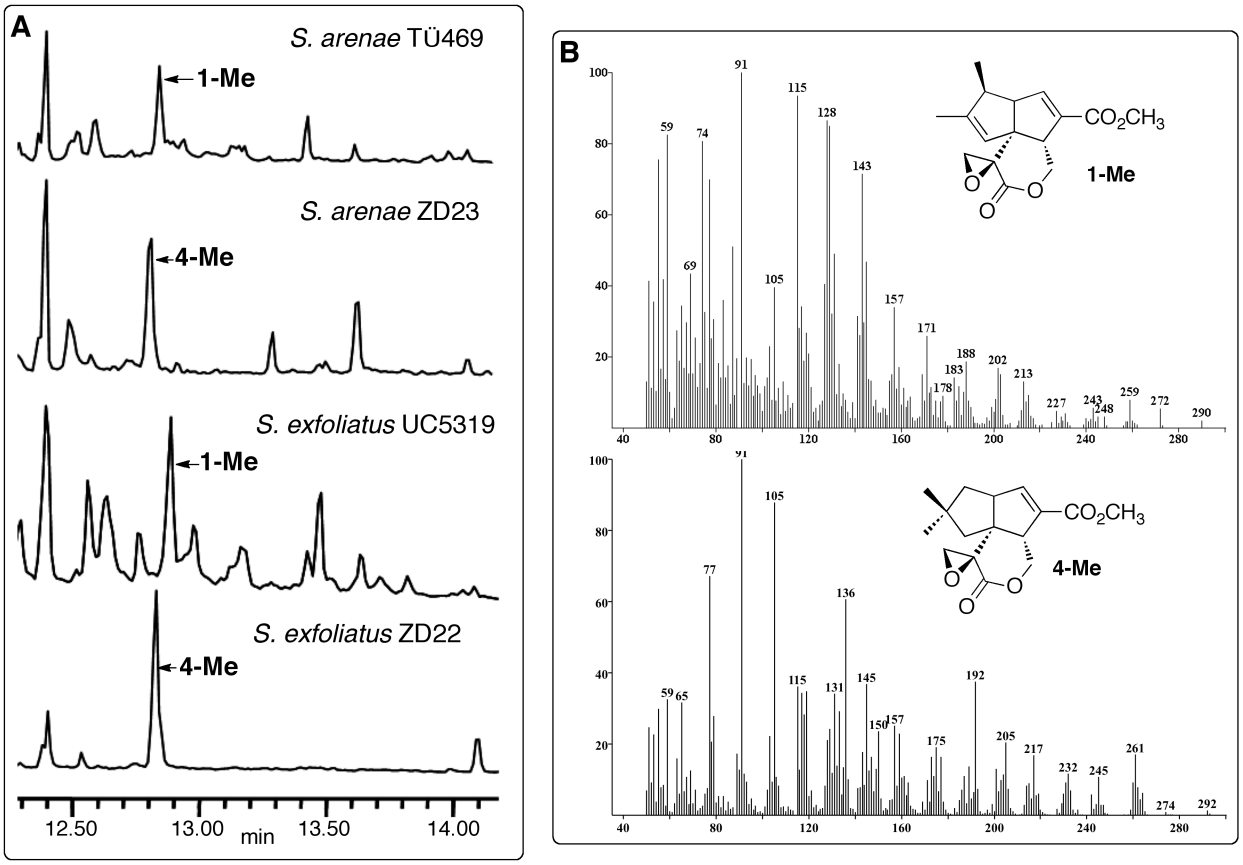


Figure S7. GC-MS analysis of *S. exfoliatus* ZD22/*ΔpenM* and *S. arenae* ZD23/*ΔpntM* deletion mutants. A. GC analysis of wild-type and mutant strains. B. Mass spectra of **1-Me** and **4-Me**.

Complementation of *S. exfoliatus* ZD22/*ΔpenM* and *S. arenae* ZD23/*ΔpntM* deletion mutants with *penM* and *pntM*. The primer pair DQ94F/R (DQ94F: 5′-GACGGACCATATGACGGATCTC-3′, *Nde*I site underlined; DQ94R: 5′-GCGATCTAGATCAGAAGGTGACG-3′, *Xba*I site underlined) was

designed to amplify the *pntM* gene. The 1217-bp PCR product was digested with *Xba*I and inserted into the *Eco*RV + *Xba*I sites of pBluescriptII SK(+) to generate plasmid pDQ67. The primer pair DQ95F/R (DQ95F: 5′-ACCTGACCATATGAACGAACTC-3′, *Nde*I site underlined; DQ95R: 5′-GCGATCTAGATCAGAAGGTGAC-3′, *Xba*I site underlined) was designed to amplify the *penM* gene. The 1217-bp PCR product 1217 bp was digested with *Xba*I and inserted into the *Eco*RV + *Xba*I sites of pBluescriptII SK(+) to generate plasmid pDQ68. pDQ67 and pDQ68 were each digested with *Nde*I and *Not*I. The ca. 1.2-kb DNA segments corresponding to *pntM* and *penM* were recovered and inserted into the *Nde*I + *Not*I sites of the integrative plasmid vector[13] pIB139 to generate plasmids pDQ73 and pDQ74, respectively, which were then conjugated into the *pntM* mutant ZD23 and *penM* mutant ZD22 to give *S. exfoliatus* ZD22::pDQ73, *S. exfoliatus* ZD22::pDQ74, *S. arenae* ZD23::pDQ73 and *S. arenae* ZD23::pDQ74. A pair of universal primers, M13F/R (M13F, 5′-CCCAGTCACGACGTTGTAAAACG-3′; M13R, 5′-AGCGGATAATTTCACACAGG-3′) were synthesized to check those strains, according to the sequence of the vector. The predicted 1.5 kb PCR products (1.2 kb *pntM* or *penM* and ca. 300 bp DNA from pIB139) were amplified from ZD22::pDQ73, ZD22::pDQ74, ZD23::pDQ73 and ZD23::pDQ74. Each of the complemented mutants was cultivated in liquid medium as described above and the methylated organic extracts were analyzed by GC-MS.

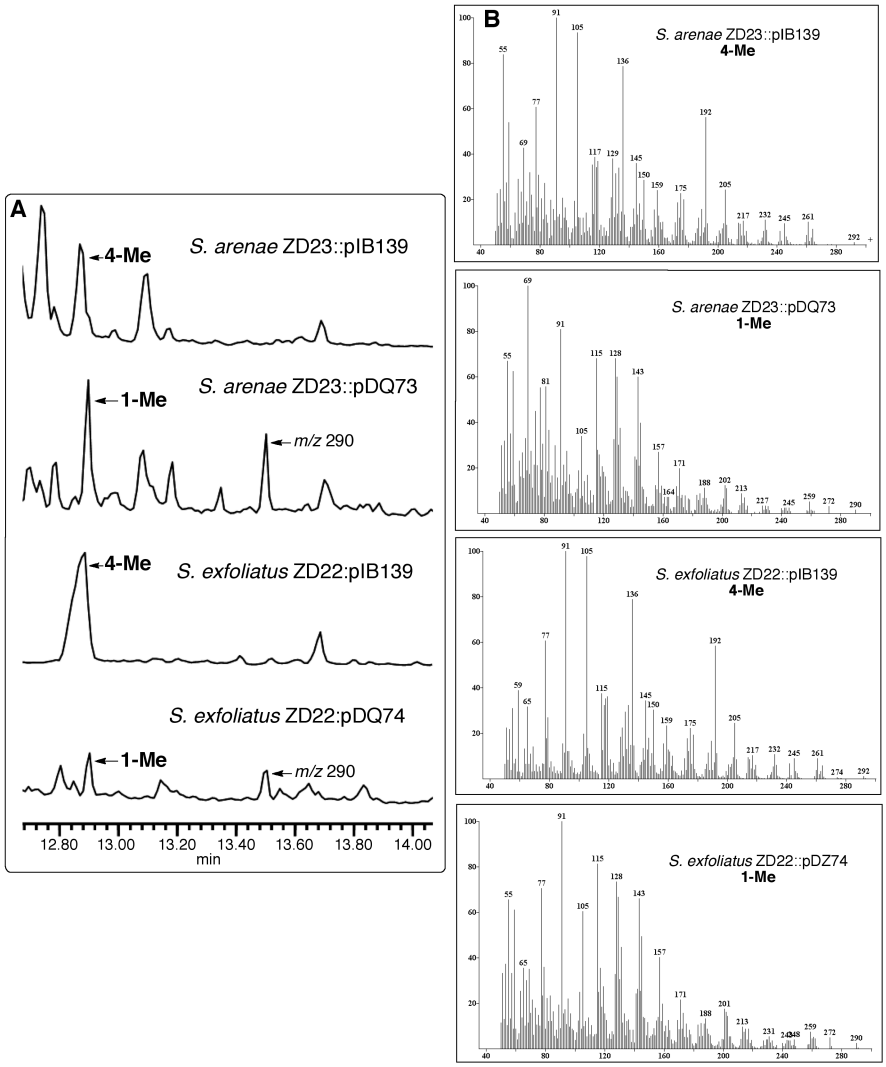


Figure S8. GC-MS analysis of *S. exfoliatus* ZD22/*ΔpenM* and *S. arenae* ZD23/*ΔpntM* deletion mutants complemented with *penM* and *pntM*. A. Mutants complemented with *pntM* (pDQ73) and *penM* (pDQ74). Controls with plasmid pIB139 without insert. B. Mass spectra of **1-Me** and **4-Me**.

Complementation of *S. avermitilis* SUKA16 *ΔptlEΔptlD*/*ermEp::pntE-pntD* with *penM* and *pntM*.

A segment containing *penM* or *pntM* was prepared from plasmid pDQ67 or pDQ68, respectively, by digestion with *Nde*I and *Xba*I. The 1200-bp *Nde*I/*Xba*I fragments of *penM* and *pntM* were subcloned into a large *Nde*I/*Xba*I segment of pKU460*hph::ermEp* to generate pKU460*hph::ermEp-penM* and pKU460*hph::ermEp-pntM*, respectively. For the enhancement of activity of the electron transport proteins, the *S. avermitilis* ferredoxin (*fdxD*, *sav3129*) and ferredoxin reductase (*fprD*, *sav5675*) genes were co-expressed with *penM* or *pntM*. A segment containing *fdxD* and *fprD* genes was prepared from pKU460::*xylAp-sav3129-sav5765*[14] by digestion with *Xba*I/*Hin*dIII. The resultant 1644-bp *Xba*I/*Hin*dIII segment was introduced into large fragments of pKU460*hph::ermEp-penM* and pKU460*hph::ermEp-*

pntM to generate pKU460*hph::ermEp-penM-fdxD-fprD* and pKU460*hph::ermEp-pntM-fdxD-fprD*, respectively. To introduce each plasmid into the previously described host strain *S. avermitilis* SUKA16 *ΔptlEΔptlD*/*ermEp::pntE-pntD*,[1] each recombinant plasmid was first introduced into *E. coli* GM2929 *hsdS::*Tn*10* to obtain preparations of unmethylated DNA. Protoplasts of *S. avermitilis* SUKA16 *ΔptlE ΔptlD* carrying pKU464*aac(3)IV::ermEp-pntE-pntD* were transformed using polyethylene glycol by each unmethylated recombinant plasmid preparation as described previously.[15] Each transformant was selected by overlaying hygromycin B. Spores of each transformant were prepared from growth on YMS medium[16] and each spore suspension in 20% glycerol (v/v) was stored at -30 °C. The liquid synthetic medium used for transformants of *S. avermitilis* SUKA16 double mutants and GC-MS analysis of the methylated extracts have been described previously.[3]

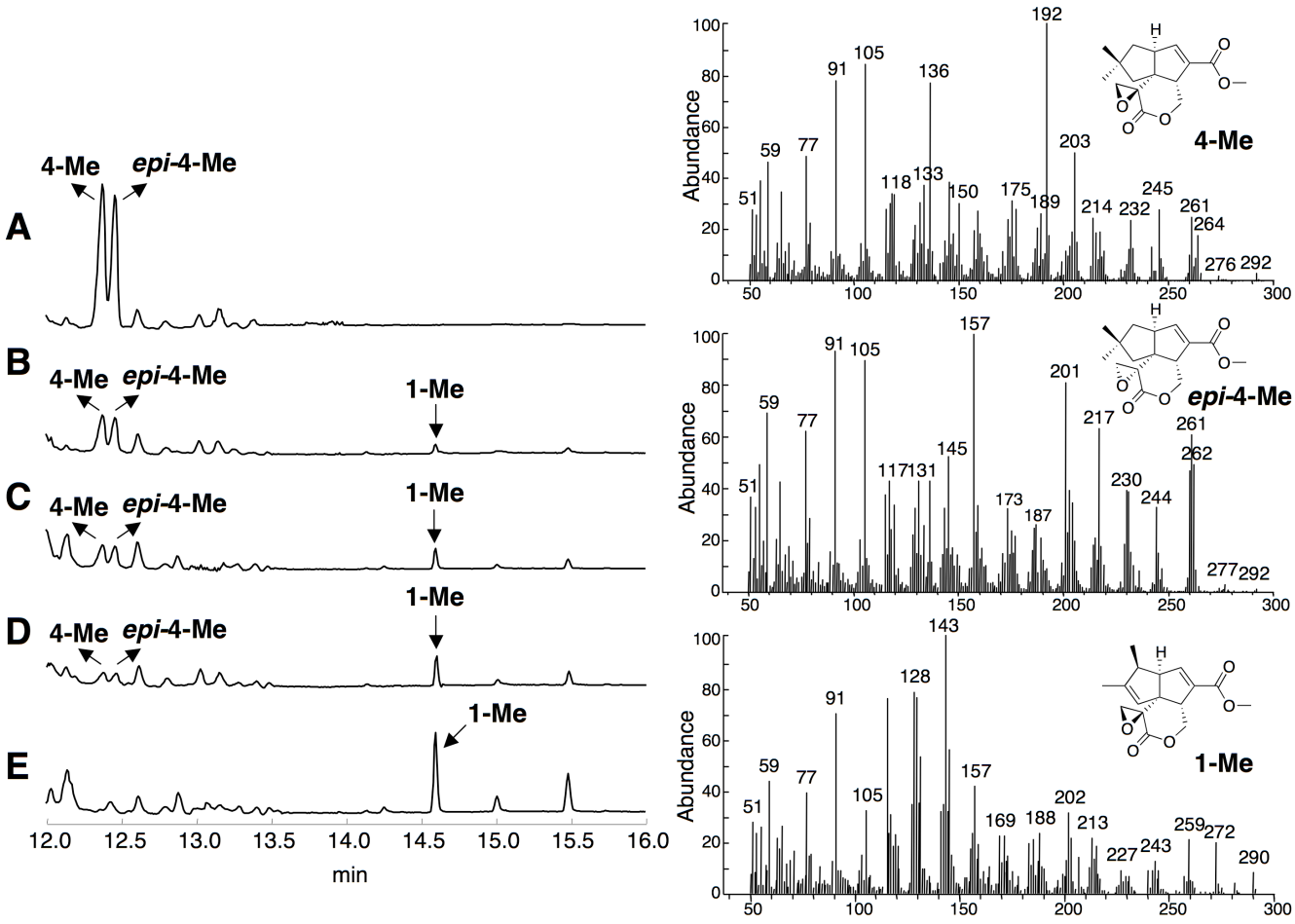


Figure S9. GC-MS analysis of *S. avermitilis* SUKA16 *ΔptlE ΔptlD* double mutants carrying *ermEp::pntE-pntD* complemented with *penM* and *pntM*. A. *S. avermitilis* SUKA16 *ΔptlE ΔptlD* double mutant carrying pKU464*aac(3)IV::ermEp-pntE-pntD*. B. Complementation of *ΔptlE ΔptlD* mutant carrying *pntE-pntD* with *penM*. C. Complementation of *ΔptlE ΔptlD* mutant carrying *pntE-pntD* with *pntM*. D. Complementation of *ΔptlE ΔptlD* mutant carrying *pntE-pntD* with *penM-fdxD-fprD*. E. Complementation of *ΔptlE ΔptlD* mutant carrying *pntE-pntD* with *pntM-fdxD-fprD*.

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