A Gene Cluster for Biosynthesis of the Sesquiterpenoid Antibiotic Pentalenolactone in *Streptomyces avermitilis*†

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ABSTRACT: *Streptomyces avermitilis*, an industrial organism responsible for the production of the anthelminthic avermectins, harbors a 13.4 kb gene cluster containing 13 unidirectionally transcribed open reading frames corresponding to the apparent biosynthetic operon for the sesquiterpene antibiotic pentalenolactone. The advanced intermediate pentalenolactone F, along with the shunt metabolite pentalenic acid, could be isolated from cultures of *S. avermitilis*, thereby establishing that the pentalenolactone biosynthetic pathway is functional in *S. avermitilis*. Deletion of the entire 13.4 kb cluster from *S. avermitilis* abolished formation of pentalenolactone metabolites, while transfer of the intact cluster to the pentalenolactone nonproducer *Streptomyces lividans* 1326 resulted in production of pentalenic acid. Direct evidence for the biochemical function of the individual biosynthetic genes came from expression of the *ptlA* gene (SAV2998) in *Escherichia coli*. Assay of the resultant protein established that PtlA is a pentalenene synthase, catalyzing the cyclization of farnesyl diphosphate to pentalenene, the parent hydrocarbon of the pentalenolactone family of metabolites. The most upstream gene in the cluster, *gap1* (SAV2990), was shown to correspond to the pentalenolactone resistance gene, based on expression in *E. coli* and demonstration that the resulting glyceraldehyde-3-phosphate dehydrogenase, the normal target of pentalenolactone, was insensitive to the antibiotic. Furthermore, a second GAPDH isozyme (*gap2*, SAV6296) has been expressed in *E. coli* and shown to be inactivated by pentalenolactone.

Pentalenolactone (**1**) (Scheme 1) is a sesquiterpenoid antibiotic, first isolated in 1957 from *Streptomyces roseogriseus* (*1*) and subsequently found in the extracts of numerous *Streptomyces* species, including *Streptomyces* UC5319 (*2*), *Streptomyces arenae* (*3*), *Streptomyces chromofuscus*, *Streptomyces griseochromogenes*, *Streptomyces baarnensis*, *Streptomyces omiyaensis*, *Streptomyces albofaciens*, and *Streptomyces viridifaciens* (*4*, *5*). Pentalenolactone has been shown to be active against both Gram-positive and Gram-negative bacteria as well as a variety of fungi and protozoa (*6*) and to inhibit the replication of DNA viruses such as HSV-1 and HSV-2 (*7*). Pentalenolactone also inhibits vascular smooth muscle cell proliferation (*8*). The antibiotic selectively inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] (*9*, *10*). Incubation of rabbit muscle GAPDH with pentalenolactone results in time-dependent, irreversible inactivation of GAPDH as a result of alkylation of the active site Cys-149 residue, apparently by reaction with the electrophilic epoxide moiety of the antibiotic (Scheme 2) (*11*, *12*). Self-resistance in the pentalenolactone producer *S. arenae* is due to the presence of two GAPDH isozymes, an inducible, pentalenolactone-insensitive form and a constitutive, pentalenolactone-sensitive form (*13*−*15*).

Pentalenolactone is derived from the 1-deoxyxylulose 5-phosphate/2-*C*-methylerythritol 4-phosphate pathway of isoprenoid biosynthesis (*16*, *43*). The committed step in pentalenolactone biosynthesis is the cyclization of farnesyl diphosphate (FPP, **2**), the universal precursor of all sesquiterpenes, to pentalenene (**3**), the parent hydrocarbon of the pentalenolactone family of metabolites. Pentalenene synthase from *Streptomyces* UC5319 has been cloned and expressed in *Escherichia coli* (*17*), and its structure has been solved by X-ray crystallography (*18*). The enzyme, a monomer of M_D 38002, requires only Mg^{2+} for activity. Pentalenene synthase contains two Mg^{2+}-binding domains that are universally conserved across all known sesquiterpene synthases: an aspartate-rich DDLFD motif at amino acids (aa) 80−85 and an NSE triad in **N**DIA**S**LEK**E** beginning at Asn-219. Extensive experiments with isotopically labeled FPP have firmly established a cyclization mechanism in which

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[1] Abbreviations: aa, amino acid; DTT, 1,4-dithiothreitol; FPP, farnesyl diphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-3-P, glyceraldehyde-3-phosphate; IPTG, isopropyl β-D-thiogalactopyranoside; NAD^+, β-nicotinamide adenine dinucleotide; NMWL, nominal molecular weight limit; PS, pentalenene synthase; PLBA, pentalenolactone benzylamine salt; ptl, pentalenolactone; TCEP, tris(2-carboxyethyl)phosphine.

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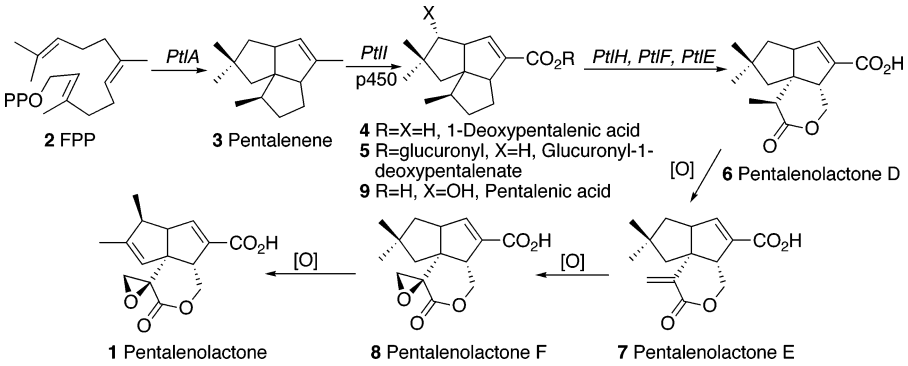
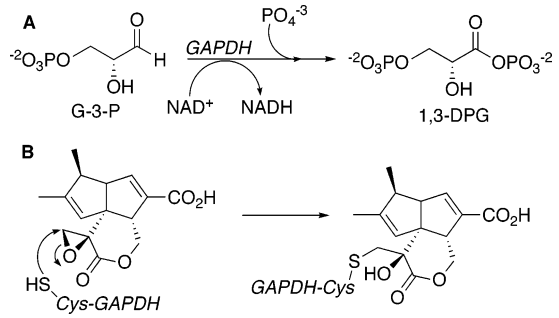
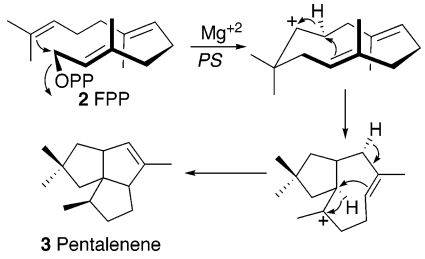
Scheme 1: Biosynthesis of Pentalenolactone (**1**) from Farnesyl Diphosphate (**2**, FPP) Showing Known Metabolites and the Proposed Role of Enzymes Encoded by the *S. avermitilis* Pentalenolactone Gene Cluster



Scheme 2: (A) GAPDH-Catalyzed Conversion of D-Glyceraldehyde 3-Phosphate (G-3-P) to 1,3-Diphosphoglycerate (1,3-DPG) and (B) Inactivation of GAPDH by Pentalenolactone



Scheme 3: Mechanism of Cyclization of FPP (**2**) to Pentalenene (**3**) Catalyzed by Pentalenene Synthase (PS)



FPP (**2**), folded as shown, undergoes ionization and cyclization via an intermediate humulyl cation to pentalenene (**3**) (*19*, 20) (Scheme 3). Refeeding of labeled pentalenene (**3**) to cultures of *Streptomyces* UC5319 and analysis of the derived labeled pentalenolactone (**1**) have confirmed the intermediacy of pentalenene in the pentalenolactone biosynthetic pathway (*20*). A variety of plausible intermediates in the conversion of pentalenene to pentalenolactone have been isolated, including 1-deoxypentalenic acid (**4**) (as the derived glucuronyl ester **5**) (*4*), pentalenolactone D (**6**) (*21*), pentalenolactone E (**7**) (*22*), and pentalenolactone F (**8**) (*21*, *23*, *24*), as well as pentalenic acid (**9**) (*25*), a demonstrated shunt metabolite of the main pentalenolactone biosynthetic pathway (*20*) (Scheme 1).

Little is known about the organization of genes for entire terpenoid biosynthetic pathways. The intermediates of the biosynthesis of the fungal diterpene gibberellic acid have been intensively studied, and many of the relevant enzymes and genes have been characterized from both plant and fungal sources (*26*). Similarly, many of the intermediates of trichothecane mycotoxin biosynthesis are known, and a short biosynthetic gene cluster has been identified, although few of the open reading frames (ORFs) have been characterized biochemically (*27*). Croteau and collaborators have systematically identified and characterized many of the genes responsible for the biosynthesis of the antitumor diterpene taxol, a formidable task that has been considerably complicated by the fact that the relevant structural genes are not clustered in Pacific yew (*Taxus brevifolia*) (*28*).

Streptomyces avermitilis [*S. avermectinius* is a junior homotypic synonym of *S. avermitilis* (*29*)] is the producer of the potent anthelminthic macrolide avermectin which is widely used in human and animal medicine. The complete genome sequence of *S. avermitilis* was determined in 2003, revealing the presence of 7575 ORFs in the 9.03 Mb linear chromosome, with an average GC content of 70.7% (*30*, *31*). Thirty gene clusters related to secondary metabolites were recognized, corresponding to nearly 7% of the genome. Among these gene clusters were at least six encoding putative terpenoid biosynthetic pathways. Centered at 3.75 Mb in the *S. avermitilis* chromosome is a ~13.4 kb cluster containing 13 unidirectionally transcribed ORFs, among which is the 1011 bp *ptlA* (SAV2998), encoding a protein of 336 aa with 76% identity and 85% similarity to the pentalenene synthase of *Streptomyces* UC5319 (Figure 1 and Table 1; see Figure S1 in Supporting Information for sequence alignment). Just upstream of *ptlA* is *ptlB* (SAV2997), which encodes a typical farnesyl diphosphate synthase. Of the remaining 11 ORFs, 8 encode putative redox enzymes, including mono- or dioxygenases and a dehydrogenase, while *ptlR* (SAV3000) corresponds to a typical transcriptional regulator. Significantly, the most upstream gene in the cluster, *gap1* (SAV2990), encodes a 335 aa protein with 88% identity and 92% similarity to the previously identified pentalenolactone-resistant GAPDH of *S. arenae* (see Figure S2, Supporting Information). Since many bacterial antibiotics are coex-

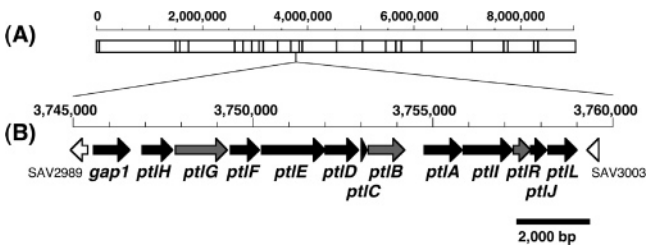


FIGURE 1: Gene cluster for pentalenolactone biosynthesis in *S. avermitilis*. *Ase*I physical map of a linear chromosome of *S. avermitilis* (A) and a region involving pentalenolactone biosynthesis (B). Gene names and definitions are given in Table 1.

Table 1: Pentalenolactone (*ptl*) Gene Cluster of *S. avermitilis*[a]

gene	ID	start nt	end nt	definition	best match[b]
gap1	SAV2990	3745497	3746501	glyceraldehyde-3-phosphate dehydrogenase	*ptlK*, pentalenolactone resistance gene
ptlH	SAV2991	3746862	3747719	putative hydroxylase	PF05721, phytanoyl-CoA dioxygenase (PhyH)
ptlG	SAV2992	3747777	3749231	putative transmembrane efflux protein	PF07690, major facilitator superfamily
ptlF	SAV2993	3749307	3750119	putative oxidoreductase	PF00106, short-chain dehydrogenase
ptlE	SAV2994	3750177	3751961	putative monooxygenase	PF00743, flavin-binding monooxygenase-like
ptlD	SAV2995	3751962	3752882	putative dioxygenase	PF02668, taurine catabolism dioxygenase TauD, TfdA family
ptlC	SAV2996	3752934	3753110	hypothetical protein	
ptlB	SAV2997	3753107	3754120	polyprenyl diphosphate synthase	probable farnesyl diphosphate synthase (idsA2)
ptlA	SAV2998	3754725	3755735	pentalenene synthase	PF03936, terpene synthase family, metal-binding domain
ptlI	SAV2999	3755792	3757141	cytochrome P450	CYP183A1
ptlR	SAV3000	3757201	3757662	putative AraC family transcriptional regulator	PF00165, bacterial regulatory helix−turn−helix proteins, AraC family
ptlJ	SAV3001	3757659	3758093	putative lyase	PF00903, glyoxalase/bleomycin resistance protein, dioxygenase superfamily
ptlL	SAV3002	3758163	3758933	hypothetical protein	Nfa37730 of *Nocardia farcinica* IFM 10152

[a] For detailed annotation and alignments, see the *S. avermitilis* Genome Project website (http://avermitilis.ls.kitasato-u.ac.jp/). [b] PF number is the NCBI PFAM reference.

pressed with the corresponding genes for self-resistance, the 13 ORFs were proposed to make up an operon for the biosynthesis of pentalenolactone. We report below the results of combined molecular genetic and biochemical experiments that provide compelling evidence for the identity of the *ptl* cluster.

EXPERIMENTAL PROCEDURES

Materials. *S. avermitilis* cosmids CL_216_D07 and CL_214_C12, from the *S. avermitilis* genomic DNA library (http://avermitilis.ls.kitasato-u.ac.jp/) (*30*), were used to transform *E. coli* ElectroTenBlue (Stratagene). Oligonucleotide primers were purchased from Integrated DNA Technologies. *S. arenae* TÜ469 was obtained from the DMSZ Gmbh, Braunschweig, Germany. The pET28a expression vector was purchased from Stratagene. pET28e was prepared from pET28a by replacing the *Xba*I site with an *Eco*RI site. Restriction enzymes and ligases were purchased from Promega. Pentalenene synthase from *Streptomyces* UC5319 was purified from *E. coli*/pZW05 as previously described (*17*), omitting the DNase treatment. Pentalenolactone was isolated from *S. arenae* TÜ469 (*3*) and converted to the benzylamine salt, PLBA (*11*). HiTrap Fast Flow columns were purchased from GE Healthcare. [1-^{3}H]Farnesyl diphosphate triammonium salt (16.1 Ci/mmol), obtained from Perkin-Elmer Life Sciences, Boston, MA, was mixed with unlabeled FPP, prepared as previously described (*32*, *33*), to a final specific activity of 44 μCi/μmol. All other chemicals and other buffer components were purchased from Sigma-Aldrich and were of the highest grade available.

Methods. Electrocompetent cells for electroporation were prepared from *E. coli* BL21(DE3) and *E. coli* BL21(DE3) RP Codon Plus (Stratagene) by standard methods. DNA sequencing of plasmid constructs was performed by the Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine, New Haven, CT, using fluorescently labeled dideoxynucleotides. Polymerase chain reactions were carried out using Pfu Turbo polymerase (Stratagene) as recommended by the manufacturer. Qiaquick PCR purification kits, Qiaprep spin miniprep kits, and Ni-NTA resin were used as suggested by the supplier (Qiagen). The concentration of D-G-3-P was determined from the D/L mixture as suggested by Sigma. Standard molecular biological procedures were performed as described (*34*). Generation of *Streptomyces* exoconjugants and homologous recombination utilized standard methodology (*35*). Protein concentrations were determined by the method of Bradford (*36*) using the Bio-Rad kit. Steady-state kinetic and enzyme inactivation parameters were calculated by direct fitting to the Michaelis−Menten or other appropriate equations by nonlinear least-squares regression using Kaleidagraph V 3.6 (Adelbeck Software, Reading, PA). UV spectra were recorded on a HP 8452A photodiode array spectrophotometer. GC-MS analysis of sesquiterpenes was carried out on a Hewlett-Packard (Agilent) GC-MSD Series 2. Liquid scintillation was measured with a Beckman LS6500 in 7 mL of Opti-Fluor.

Isolation of Pentalenolactone F. A mutant of *S. avermitilis* (ΔaveR ΔolmA5), blocked in the production of both avermectins and oligomycins, was used to examine the production of pentalenolactones. The spores of the mutant were transferred to a 250 mL flask containing 20 mL of vegetative medium [glucose (0.5 g), soy flour (1.5 g), and yeast extract (0.5 g) per 100 mL, pH 7.2] and allowed to grow while being shaken at 30 °C for 2 days. A 1 mL portion was removed and used to inoculate 20 × 500 mL flasks containing 100 mL of production medium [soluble starch (40 g), soy flour (20 g), $FeSO_4 \cdot 7H_2O$ (0.5 g), K_2HPO_4 (1 g), and KCl (0.3 g) per liter, pH 6.5]. After incubation while being shaken at 28 °C for 4 days, the culture was filtered. The mycelium was washed with tap water and extracted with 500 mL of methanol. The concentrated supernatant was diluted with an equal volume of water and acidified with 2 N H_2SO_4 to pH 2.5. The products were then extracted twice with a half volume of chloroform. The organic fraction was concentrated under reduced pressure and subjected to flash silica gel chromatography (30:1 chloroform/methanol). Pentalenolactone-rich fractions were pooled and concentrated to an oil. This residue was dissolved in THF/diethyl ether (1:1) and treated with a solution of diazomethane in diethyl ether and 50% KOH for 10 min at 0 °C. After being quenched by addition of ethereal acetic acid, the ether layer was washed with saturated bicarbonate solution. Methyl ester metabolites were further purified by preparative silica TLC, and final

purification was performed by ODS-HPLC using *n*-hexane/ethyl acetate (1:1), resulting in the isolation of 8 mg of pentalenolactone F methyl ester (**8**-Me).

Construction of a ptl Deletion Mutant of S. avermitilis. Two DNA segments were amplified by PCR from cosmid CL_216_D07. A segment upstream of *gap1* (nt 3743485−3745499) was amplified using the forward (5′-CTCGAG-**CAATTG**CAACGCCTATCTGGACACCTCGAC-3′) and reverse (5′-CTCGAG**ACTAGT**CATGGATCCCTCTCCTAG-CGCTG-3′) primers to introduce *Mfe*I and *Spe*I restriction sites (in bold), respectively. The forward (5′-CTCGAG**AC-TAGT**CCTACCGGTCCGGGTCAGCCGTC-3′) and reverse (5′-CTCGAG**CAATTG**CTCGCCCTCGCCGAGT-TCGTGAC-3′) primers were used for the amplification of a second segment downstream of *ptlL* (nt 3758936−3760758), with introduction of *Spe*I and *Mfe*I sites (in bold), respectively. The two amplified DNA segments were ligated together into *Eco*RI-cut pKU250 and transformed into *E. coli* DH5α. The resultant plasmid was digested with *Spe*I, and the linearized plasmid was ligated with the *Spe*I fragment of the *aphII* kanamycin resistance cassette. The plasmid pKU250::upstream-*aphII*-downstream construct in *E. coli recA dcm*/pUB307*aph*::Tn*7* was introduced into *S. avermitilis* by conjugation. After exoconjugants were selected by vector marker (thiostreptone resistance), *ptl* deletion mutants, generated by double homologous recombination upstream of *gap1* and downstream of *ptlL*, were obtained by selection for thiostreptone sensitivity and neomycin resistance. The deletion was confirmed by PCR. Neither pentalenolactones nor pentalenic acid could be detected in cultures of the *S. avermitilis ptl* deletion mutant.

Transfer of the ptl Cluster to Streptomyces lividans 1326. Cosmid CL_216_D07 was digested with *Avr*II and *Sna*BI, and the 14.9-kb segment was purified by agarose gel electrophoresis and ligated with the *Xba*I/*Ecl*136II-cut φC31-based integrating vector carrying *aphII* as selectable marker. The resulting plasmid was used to transform *E. coli* S17.1 and then transferred into *S. lividans* 1326 by conjugation. The *S. lividans*::*ptl* exoconjugants, obtained by selection for neomycin resistance, produced ∼1 mg/L pentalenic acid (**9**) along with traces of pentalenolactone metabolites. The insertion of the intact *ptl* gene cluster was verified by PCR.

Expression and Purification of S. avermitilis Pentalenene Synthase. The *ptlA* gene was amplified by PCR from template DNA from cosmid CL_216_D07 using the forward (5′-GCGCGC**CATATG**GCAATGCCCCAGGACGTCG-3′) and reverse (5′-GGCCGG**AAGCTT**CGGCTACAGCGTGCT-GCC-3′) primers to introduce *Nde*I and *Hin*dIII restriction sites (in bold) flanking the normal start and stop codons, respectively. The resulting amplicon and the pET28a vector were digested separately with *Nde*I and *Hin*dIII and purified using a PCR purification kit before ligation with T4 DNA ligase and transformation of *E. coli* ElectroTenBlue cells. The resultant pET28a/SAV2998 plasmid was then used to transform *E. coli* BL21(DE3), which was grown at 37 °C in LB media (1 L) supplemented with kanamycin (80 mg/L) to an OD_{600} of ∼0.6, and then induced by the addition of IPTG (0.5 mM). After a further 5 h at 30 °C, the cells were harvested by centrifugation. The cell pellet was resuspended in lysis buffer [30 mL, 20 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl, 5 mM $MgCl_2$, 2.7 mM β-mercaptoethanol, 10% glycerol (v/v), pH 8.0] and disrupted by sonication. After removal of the cellular debris by centrifugation (20 min, 30000*g*, 4 °C), the recombinant protein was purified by HiTrap Ni affinity column chromatography using a linear gradient of imidazole (10−400 mM) in lysis buffer. Fractions were analyzed by SDS−PAGE, and those containing the desired protein were concentrated by ultrafiltration (10 kDa NMWL) (9.4 mg of protein). N-Terminal His_6-tagged PtlA eluted at 90−118 mM imidazole.

His_6-Tagged Pentalenene Synthase from Streptomyces UC5319. DNA from plasmid pZW05, harboring the pentalenene synthase gene of *Streptomyces* UC5319 (*17*), was amplified by PCR using the forward (5′-CCGCGCGC-**CATATG**CCCCAGGACGTCGACTTCC-3′) and reverse (5′-GGCCGG**AAGCTT**ACTAGTCAATTGCTAGTGGG-CGTGCTGCCGAG-3′) primers. The first ten PCR cycles employed an annealing temperature of 60 °C while the next 25 cycles used an annealing temperature of 71 °C. The resulting amplicon and pET28e vector were digested separately with *Nde*I and *Hin*dIII and then purified by the PCR purification kit before ligation with T4 DNA ligase overnight at 4 °C and transformation of *E. coli* XL1-Blue. The resultant pET28e/PS2HIS plasmid was used to transform *E. coli* BL21(DE3). The protein was expressed in LB media (1 L) supplemented with kanamycin (80 mg/mL). The cells were shaken at 37 °C to an OD_{600} of 0.6, at which point they were induced by the addition of IPTG (1 mM) and further incubated at 25 °C for 6 h. Recombinant protein was isolated and purified as above by Ni affinity chromatography (10−500 mM imidazole in lysis buffer). The fractions containing pentalenene synthase (0.6 mg) which eluted between 97 and 118 mM imidazole were pooled and concentrated by ultrafiltration (10 kDa NMWL).

GC-MS Analysis of Pentalenene Synthase Extracts. The formation of pentalenene was verified by GC-MS analysis using the protocol previously described to analyze the pentane-soluble, hydrocarbon product of the incubation of FPP with pentalenene synthase (*37*). GC-MS was performed using a chiral 25 m × 0.25 mm hydrodex-β-6-TBDM column (Macherey-Nagel) with a temperature ramp of 45−180 °C at 2 °C/min and an inlet temperature of 150 °C. Under these conditions pentalenene eluted as a single peak at $R_t = 45.13$ min. An authentic sample of pentalenene was generated by parallel incubations with recombinant *Streptomyces* UC5319 pentalenene synthase. Pentalenene: *m*/*z* (observed) 204.36 Da; *m*/*z* (calculated for $C_{15}H_{24}$) 204.35.

Pentalenene Synthase Assay. Assays were performed as previously described using [1-^{3}H]FPP (*17*). Assays with *S. avermitilis* pentalenene synthase and *Streptomyces* UC5319 His_6-tagged pentalenene synthase used 15−80 μM FPP. The pentane extracts were passed through a 2 cm silica gel Pasteur pipet column before quantitation by liquid scintillation counting. The steady-state kinetic parameters, k_{cat} and K_m, were calculated by fitting the liquid scintillation data to the Michaelis−Menten equation.

Preparation of Pentalenolactone-Resistant GAPDH (Gap1). *S. avermitilis gap1* DNA was amplified by PCR from the cosmid CL_216_D07. The forward and reverse primers (5′-CCGCGCGC**CATATG**ACTGTTCGTGTCGGCATC-AATGGC-3′ and 5′-GGCCGG**AAGCTT**ACTAGTCAAT-TGTCAGAGGGTGTCGCCGACCAGC-3′) were used to introduce *Nde*I and *Hin*dIII sites (in bold), respectively, flanking the coding region of the gene. The resulting

amplicon and pET28a vector were digested separately with *Nde*I and *Hin*dIII, and the digests were purified by a PCR purification kit before ligation with T4 DNA ligase overnight at 4 °C and transformation of *E. coli* XL-1 Blue. The resultant plasmid, pET28/SAV2990, was used to transform *E. coli* BL21(DE3) RP Codon Plus. The culture was grown in LB medium (1 L) supplemented with kanamycin (50 mg/L) at 37 °C to an OD_{600} of 0.5, then induced with IPTG (0.9 mM), and incubated for an additional 3 h at 37 °C. The cell pellet after centrifugation was resuspended in lysis buffer [20 mL, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10% glycerol (v/v), 2.7 mM β-mercaptoethanol, 5 mM imidazole, 300 mM NaCl, 1.5 mM benzamidine, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 0.16 mM NAD^+] and disrupted with a French press at 10000 psi. Cellular debris was removed by centrifugation, and purification was carried out using Ni-NTA resin. GAPDH-containing fractions (0.7 mg of protein, eluting at 40 mM imidazole) were pooled, concentrated by ultrafiltration (10 kDa NMWL), and exchanged into storage buffer [20 mM Tris-HCl, pH 8.5, 2.7 mM β-mercaptoethanol, 20% glycerol (v/v), 20 mM NaCl, 0.16 mM NAD^+] via a PD-10 column (Amersham).

Preparation of Pentalenolactone-Sensitive GAPDH (Gap2). *S. avermitilis gap2* was amplified by PCR from the cosmid CL_214_C12, using the forward and reverse primers, 5′-GCGCGC**CATATG**ACGATCCGCGTAGGCATCAAC-3′ and 5′-GGCCGG**AAGCTT**TTAGAGCTGGTTGCCGAC-GAAGAC-3′, respectively, to introduce *Nde*I and *Hin*dIII sites (in bold) flanking the coding region. After ligation into pET28a, the Gap2 protein was expressed in *E. coli* BL21-(DE3)/pET28a/SAV6296 and purified by Ni affinity chromatography using same protocols used for *S. avermitilis* Gap1. The desired protein (4.2 mg) eluted at 60 mM imidazole.

GAPDH Assay. GAPDH activity was assayed at room temperature, with substitution of arsenate for inorganic phosphate, as previously described (*11*, *38*, *39*). Under these conditions, the initially generated product 1-arseno-3-phosphoglycerate undergoes spontaneous hydrolysis to 3-phosphoglycerate, thereby avoiding interfering product inhibition by 1,3-diphosphoglycerate. To the assay buffer (480 μL, 133 mM Tris-HCl, pH 8.75, 0.2 mM EDTA, 17 mM $Na_2HAsO_4 \cdot 7H_2O$, 3 mM DTT, 0.4 mM NAD^+ and 0.098 μM Gap1 or 1.2 mM NAD^+ and 0.067 μM Gap2) was added a 20 μL solution of D-G-3-P at concentrations of 25.5−956 μM. The formation of NADH was monitored by UV at 340 nm. The initial rates of each reaction were calculated using the UV−visible ChemStation software (HP), and the data were fit to the Michaelis−Menten equation to determine k_{cat} and K_m.

Time-Dependent Inactivation of GAPDH. Recombinant Gap2 (2.1 μM) was preincubated with a range of fixed concentrations of PLBA (0, 74.5, 149, and 224 μM) in 500 μL of buffer containing Tris-HCl (133 mM, pH 8.75), EDTA (0.2 mM), $Na_2HAsO_4 \cdot 7H_2O$ (17 mM), DTT (3 mM), and NAD^+ (0.61 mM) at 23 °C. Aliquots of 20 μL were withdrawn from the incubation mixtures and diluted 25-fold into GAPDH assay buffer at periodic intervals, and the residual activity was assayed at 23 °C. The enzymatic reactions were initiated by adding solutions of G-3-P (10 μL) to achieve an initial D-G-3-P concentration of 0.29 mM. The assays were performed in triplicate, and the initial rates were measured as described above. The maximum rate of inactivation, k_{inact}, and the PLBA inhibition constant, K_I, were calculated by direct fitting of the observed rates of inactivation k_{obs}, at individual concentrations of PLBA [I], to the standard equation for active site directed irreversible inactivation: $k_{obs} = k_{inact}[[I]/(K_I + [I])]$ (*40*).

Analogous experiments were also carried out with recombinant Gap1 (4.55 μM) in the presence of PLBA (0, 24.4, 61, 183, and 610 μM). A preincubation temperature of 4 °C was used to compensate for slight thermal instability of the protein. Aliquots of 20 μL were withdrawn at periodic intervals (0.5, 1.5, 3, 5, and 8 min) and diluted in 480 μL of the assay buffer and assayed in triplicate for residual GAPDH activity at 23 °C. Under these conditions there was no detectable decrease in GAPDH activity even after 8 min preincubation in the presence of 610 μM PLBA.

RESULTS

Isolation of Pentalenolactone F and Characterization of a ptl Deletion Mutant. Although pentalenolactones occur widely in *Streptomyces*, there have been no prior reports of their isolation from *S. avermitilis*. A 4 L culture of *S. avermitilis* (Δ*aveR* Δ*olmA5*), blocked in the formation of both avermectins and oligomycins, was grown for 4 days at 28 °C. Extraction of the harvested mycelium with methanol followed by chromatographic fractionation of the methylated extract gave 8 mg of purified pentalenolactone F methyl ester (**8**-Me) whose ^{1}H NMR, IR, and mass spectra were identical in all respects with those previously reported for **8**-Me (*24*). The microorganism also produced the shunt metabolite, pentalenic acid (**9**). Pentalenolactone (**1**) itself was not detected in the organic extract of *S. avermitilis*. An engineered *S. avermitilis ptl* deletion mutant, lacking the 13.4 kb segment containing the entire *ptl* cluster (*gap1* to *ptlL*; SAV2990−3002), produced neither pentalenolactone F (**8**) nor pentalenic acid (**9**). In a complementary experiment, when a 14.9 kb *Avr*II−*Sna*BI segment (nt 3744874−3759745) containing the *ptl* cluster was introduced by conjugation into *S. lividans* 1326, which normally does not produce pentalenolactones, the resultant exoconjugants were found to produce ∼1 mg/L pentalenic acid (**9**), accompanied by traces of pentalenolactone metabolites.

S. avermitilis Pentalenene Synthase. Using DNA from *S. avermitilis* cosmid CL_216_D07 as template (*30*), the 1011 bp presumptive coding region for pentalenene synthase was amplified by PCR while introducing *Nde*I and *Hin*dIII restriction sites at the 5′- and 3′-termini, respectively, of the ORF. The amplified DNA was ligated into the expression vector pET28a. The resultant plasmid, pET28a/SAV2998, was purified from *E. coli* ElectroTenBlue and used to transform the T7 RNA polymerase-based expression host *E. coli* BL21(DE3). The resulting soluble protein was subjected to Ni^{2+} affinity purification. The recovered recombinant SAV2998 (9.4 mg from 1 L of culture) was judged to be >90% pure by SDS−PAGE. Incubation of the purified SAV2998 protein with 80 μM FPP and analysis of the pentane-soluble extract by capillary chiral GC-MS revealed the formation of a single sesquiterpene product identical to pentalenene by direct comparison with an authentic sample, thereby confirming the predicted pentalenene synthase activity of PtlA (SAV2998) (see Supporting Information, Figure S3).

Table 2: Steady-State Kinetic Parameters for Pentalenene Synthase (PS) from *S. avermitilis* and *Streptomyces* UC5319

protein	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
PtlA (SAV2998)	0.065 ± 002	36.0 ± 3.6	1.8×10^3
His-tagged PS	0.40 ± 0.02	28.6 ± 3.6	1.4×10^4
PS[a]	0.3	0.3	1.0×10^6

[a] Data from ref *37*.

The steady-state kinetic parameters were determined for *S. avermitilis* pentalenene synthase as well as for N-terminal His_6-tagged pentalenene synthase from *Streptomyces* UC5319 using [1-^{3}H]FPP as substrate (Table 2). By direct comparison, the k_{cat} for the *S. avermitilis* enzyme of 0.065 s^{-1} was ~6-fold lower than the value of 0.40 s^{-1} determined for the *Streptomyces* UC5319 His_6-tagged synthase, while the K_m values for FPP for each enzyme were very similar (36 μM compared to 29 μM). The addition of the N-terminal His tag has little apparent effect on the k_{cat} when compared to the native recombinant *Streptomyces* UC5319 pentalenene synthase (k_{cat} 0.3 s^{-1}) but results in an marked 100-fold increase in the K_m for FPP, compared to a K_m of 0.3 μM for the native cyclase (*37*).

Pentalenolactone-Insensitive GAPDH. The *gap1* gene (SAV2990), harbored within cosmid CL_216_D07, was amplified by PCR, ligated as an *Nde*I−*Hin*dIII fragment into pET28e, and expressed in *E. coli* BL21(DE3) RP Codon Plus. The resultant protein, carrying an N-terminal His_6 tag was purified by Ni^{2+} affinity chromatography to yield 0.7 mg of Gap1 protein/L of culture. The NAD^+-dependent GAPDH activity was confirmed by standard arsenate-decoupled assay (*11*, *38*, *39*), giving a k_{cat} for D-glyceraldehyde 3-phosphate (G-3-P) of 33 s^{-1} and a K_m of 0.33 mM. To assess the inhibitory effect of pentalenolactone, Gap1 (4.5 μM) was preincubated with concentrations up to 0.6 mM of the benzylamine salt of pentalenolactone (PLBA) (*11*). Aliquots were periodically withdrawn after 0.5−8 min, diluted 25-fold into arsenate assay buffer, and monitored for GAPDH activity. Exposure of Gap1 to 0.6 mM pentalenolactone for as long as 8 min at 4 °C had no detectable effect on the measured GAPDH activity. The Gap1 protein is thus insensitive to pentalenolactone.

Pentalenolactone-Sensitive GAPDH. S. avermitilis harbors a second apparent GAPDH gene, *gap2* (SAV6296), located between nt 7559711 and nt 7560718 of the *S. avermitilis* chromosome and assigned to PFAM PF02800 (GAPDH).

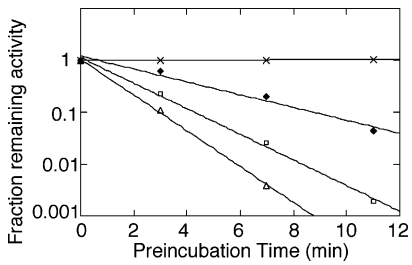


FIGURE 2: Time-dependent inactivation of *S. avermitilis* Gap2 by pentalenolactone. Semilogarithmic plot of the inactivation of Gap2 (2.1 μM) as a function of preincubation time and varied PLBA concentrations: 0 (×), 74.5 (◆), 149 (□), and 223.5 μM (△). Aliquots of 20 μL were withdrawn at periodic intervals and assayed for residual GAPDH activity in a total volume of 500 μL of assay buffer, as described in Methods. Each data point represents the mean of triplicate values.

The *gap2* gene was amplified by PCR from *S. avermitilis* cosmid CL_214_C12, and the derived *Nde*I−*Hin*dIII fragment was inserted into the corresponding cloning sites of pET28a. The resultant plasmid, pET28a/SAV6296, was used to transform *E. coli* BL21(DE3). IPTG-induced expression and Ni^{2+} affinity purification gave 4.2 mg of N-terminal His_6-tagged Gap2 whose GAPDH activity was confirmed, giving a k_{cat} 165 s^{-1} and K_m for D-G-3-P of 0.33 mM. Incubation of *S. avermitilis* Gap2 at 23 °C with increasing concentrations of PLBA up to 225 μM resulted in time-dependent, irreversible inactivation. Semilogarithmic plots of the fraction of remaining GAPDH activity as a function of PLBA concentration showed the expected pseudo-first-order inactivation (*11, 40*) (Figure 2). The rate of inactivation by pentalenolactone was saturable, with a maximum rate of inactivation, k_{inact}, of 5.3 ± 1.6 min^{-1} ($t_{1/2}$ = 0.13 min) and a K_I for PLBA of 1.3 ± 0.4 mM.

DISCUSSION

The increasing availability of complete microbial genome sequences has recently opened up the possibility of the top-down characterization of the molecular genetics and biochemistry of entire biosynthetic pathways whose genes are frequently organized in discrete clusters. As an essential first step in the elucidation of the enzymology and genetics of pentalenolactone biosynthesis in *S. avermitilis*, we have established that *ptlA* (SAV2998), one of 13 ORFs within a 13.4 kb cluster, encodes a 336 aa protein that catalyzes the cyclization of FPP to pentalenene (**3**), the well-established sesquiterpene hydrocarbon precursor of the pentalenolactone family of metabolites. The pentalenolactone biosynthetic pathway is functional in *S. avermitilis*, as confirmed by the isolation of both pentalenolactone F (**8**) and the shunt metabolite pentalenic acid (**9**). Deletion of the entire *ptl* cluster from *S. avermitilis* abolished the production of pentalenolactone metabolites, while transfer of this same cluster to the naive host, *S. lividans* 1326, endowed the transformants with the ability to generate pentalenic acid (**9**), accompanied by trace amounts of pentalenolactones. We have also demonstrated that *gap1*, located at the 5′-end of the biosynthetic gene cluster, corresponds to the essential locus for self-resistance to the antibiotic pentalenolactone, which acts as an active site directed, irreversible inhibitor of the glycolytic enzyme GAPDH. Recombinant Gap1 retained full activity even after exposure to 0.6 mM pentalenolactone for 8 min at 4 °C, conditions under which rabbit GAPDH is completely inactivated (*11*). Gap1 shows considerable sequence identity to the homologous, pentalenolactone-insensitive GAPDH that confers resistance to pentalenolactone to *S. arenae* (*15*). Similar to *S. arenae*, *S. avermitilis* also harbors a second, pentalenolactone-sensitive, isoform of GAPDH. Interestingly, although the maximum rate of inactivation of Gap2 by PLBA is 2−3 times that previously observed for rabbit muscle GAPDH (k_{inact} 5.3 min^{-1} compared to 1.3−3.3 min^{-1}) (*11*), *S. avermitilis* Gap2 is considerably less sensitive to pentalenolactone than is the rabbit enzyme, exhibiting a K_I of 1.3 mM that is nearly 100-fold higher than that for rabbit GAPDH (K_I of 6−12 μM). In preliminary experiments on expression of *gap1* and *gap2* in *S. avermitilis*, *gap2* was constitutively expressed during mycelial growth while expression of *gap1* from the ptl gene cluster started after at least 15 h of growth. Thus prior to

antibiotic production, Gap2 is utilized for the glycolytic pathway in *S. avermitilis*.

Within the *ptl* cluster, there are eight ORFs that appear to encode redox enzymes, as deduced from extensive sequence comparisons (Table 1). Cytochrome P450s are known to be able to mediate the three-step oxidative conversion of methyl groups to the corresponding carboxylic acids (*41*). It is therefore likely that CYP183A1, encoded by *ptlI* (SAV2999) (*42*), catalyzes the conversion of pentalenene (**3**) to 1-deoxypentalenic acid (**4**) (Scheme 1). Indeed, we have already obtained preliminary experimental evidence in support of this hypothesis (D. E. Cane and R. Quaderer, unpublished results). PtlH (SAV2991), which resembles a non-heme iron, α-ketoglutarate-dependent hydroxylase, can oxidize 1-deoxypentalenic acid (**4**), with the resultant product undergoing oxidation to the corresponding ketone mediated by the dehydrogenase PtlF (SAV2993). Intriguingly, the putative monooxygenase, PtlE (SAV2994), shows a strong resemblance (49% identity and 62% similarity over 591 aa) to the monooxygenase of *Rhodococcus ruber* (Genbank AY052630.1) that catalyzes the Baeyer−Villiger-like oxidation of cyclodecanone to lauric acid. The latter three enzymes could therefore convert 1-deoxypentalenic acid (**4**) in three steps to the known metabolite pentalenolactone D (**6**). From pentalenolactone D, formation of pentalenolactones E (**7**) and F (**8**) would involve straightforward enzymatic desaturation adjacent to the lactonic carbonyl and epoxidation, possibly mediated by some combination of PtlD (SAV2995) and PtlJ (SAV3001). Formation of pentalenolactone (**1**) itself would require oxidative rearrangement of pentalenolactone F (**8**) (*21*). These and related possibilities are currently under active study.

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SUPPORTING INFORMATION AVAILABLE

Sequence alignments for *S. avermitilis* PtlA (SAV2998) and for Gap1 (SAV2990) and Gap2 (SAV6296) and GC-MS identification of pentalenene. This material is available free of charge via the Internet at http://pubs.acs.org.

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