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Biosynthesis of 3-Hydroxy-5-Methyl-*O*-Methyltyrosine in the Saframycin/Safracin Biosynthetic Pathway

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The biosynthesis study of antibiotics saframycin (SFM) in *Streptomyces lavendulae* and safracin (SAC) in *Pseudomonas fluorescens* demonstrated that 3-hydroxy-5-methyl-*O*-methyltyrosine (3h5m*O*mTyr), a nonproteinogenic amino acid, is the precursor of the tetrahydroisoquinoline molecular core. In the biosynthetic gene cluster of SAC/SFM, *sacD*/*sfmD* encodes a protein with high homology to each other but no sequence similarity to other known enzymes; *sacF*/*sfmM2* and *sacG*/*sfmM3* encode methyltransferases for *C*-methylation and *O*-methylation; and *sacE*/*sfmF* encodes a small protein with significant sequence similarity to the MbtH-like proteins, which are frequently found in the biosynthetic pathways of nonribosomal peptide antibiotics and siderophores. To address their function, the biosynthetic cassette of 3h5m*O*mTyr was heterologously expressed in *S. coelicolor* and *P. putida*, and an in-frame deletion and complementation *in trans* were carried out. The results revealed that (i) SfmD catalyzes the hydroxylation of aromatic rings; (ii) *sacD*/*sacF*/*sacG* in the SAC gene cluster and *sfmD*/*sfmM2*/*sfmM3* in the SFM cluster are sufficient for the biosynthesis of 3h5m*O*mTyr; and (iii) the *mbtH*-like gene is not required for the biosynthesis of the 3h5m*O*mTyr precursor.

Keywords: 3-Hydroxy-5-methyl-*O*-methyltyrosine, biosynthesis, saframycin, safracin, MbtH-like protein, heterologous expression

One reason for the enormous structural diversity in non-ribosomal peptide natural products is that the biosynthetic machinery for these compounds is not limited to the 22 proteinogenic amino acids found in ribosomally generated peptides. To date, more than 300 nonproteinogenic amino acids have been found in natural products including a variety of hydroxyl amino acids, which contribute to a wide range of biological activity [12]. The unusual, non-proteinogenic amino acid moieties may arise either from the incorporation of a natural, proteinogenic amino acid into the growing peptidyl chain by the nonribosomal peptide synthetases (NRPSs) followed by downstream modification [5, 12, 25, 28]. Alternatively, they may be synthesized by a dedicated biosynthetic pathway, to provide the precursors for the particular NRPS assembly line [10].

The nonproteinogenic amino acid 3-hydroxy-5-methyl-*O*-methyltyrosine (3h5m*O*mTyr, **7**; Fig. 1), is found in most of the tetrahydroisoquinoline alkaloids [26], including saframycin A (SFM-A, **1**), safracin B (SAC-B, **2**), ecteinascidin 743 (ET743, **3**), renieramycin H (**4**), naphthyridinomycin (**5**), and lemonomycin (**6**) (Fig. 1). The synthesis, activity and mechanism of action of these natural products have attracted significant attention over the past 30 years because of their potent antitumor and antimicrobial activities [26]. Ecteinascidin-743 (Et743, Trabactedin, Yondelis), which is isolated from marine invertebrates, was approved by the EMEA in 2007 for the treatment of advanced soft tissue sarcoma [1, 7] and is currently in Phage III clinical trials in the U.S. Mechanistically, these compounds interact with DNA and form covalent bounds that are thought to be necessary for their cytotoxicity [26]. For members of the tetrahydroiso-quinoline group, 3h5m*O*mTyr residues play crucial roles in the structure and function of the antibiotics by creating their characteristic rigid core scaffold. Additionally, 3h5m*O*mTyr is involved in further oxidation and alkylation modification to contribute to more structural diversity.

Labeling studies have demonstrated that the non-proteinogenic amino acid 3h5m*O*mTyr of the tetrahydroisoquinoline group of antibiotics is derived from tyrosine [18, 33]. Further evidence for this has been derived from recent sequencing and characterization of the SAC-B biosynthetic gene cluster in *Pseudomonas fluorescens* A2-2 [30] and the SFM-A gene cluster in *Streptomyces lavendulae* NRRL 11002 [14]. The *in vitro* amino-acid-dependent ATP-PPi exchange assay, which determines the substrate specificity

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of SfmC toward *L*-3h5m*O*mTyr, strongly supports the notion that the nonproteinogenic amino acid moieties are synthesized by a dedicated biosynthetic pathway to provide the precursors for NRPS assembly lines [14]. In the SAC-B gene cluster, four open reading frames (ORFs), SacD, SacE, SacF, and SacG, have their high sequence homology counterparts, SfmD, SfmF, SfmM2, and SfmM3, in the SFM-A biosynthetic pathway (Fig. 2A), can be organized into a biosynthetic pathway that includes *C*-methylation, *O*-methylation, and hydroxylation. SfmF, with high similarity to SacE, belongs to a family of MbtH-like proteins that are encoded by genes found in many, but not all, NRPS-encoding gene clusters.

Here, we present the heterologous expression, in-frame deletion, and complementation of *sfmD* and *sfmF* in a heterologous host and define their roles in the biosynthesis of 3h5m*O*mTyr.

Materials and Methods

Strains, Plasmids, and Reagents

Bacterial strains and plasmids used in this study are summarized in Table 1. Compounds 3h5m*O*mTyr and 3-methyl-*O*-methyltyrosine (3m*O*mTyr) were synthesized as described previously [14]. Biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were from standard commercial sources.

DNA Manipulation

DNA isolation and manipulation in *E. coli* and *Pseudomonas* were carried out according to standard methods [8, 23]. PCR amplification was carried out in an Eppendorf AG 22331 Thermal Cycler (Hamburg, Germany) using either *Taq* DNA polymerase or PfuUltra High-Fidelity DNA polymerase. Primer synthesis and DNA sequencing were performed at Invitrogen Shanghai Center.

Construction of a Shuttle Plasmid for Heterologous Expression in *S. coelicolor*

The following primers were used to amplify the individual genes: for *sfmD*, 5'-GGAAGCTTAATTAA**GGAGG**AGCCAGCATGACG-GCACCGGCCGACAC-3' (HindIII/PacI) and 5'-GGTCTAGAATC-ACGAGGGTTCTCCCTGCTG-3' (XbaI); for *sfmF-M2*, 5'-GGAA-GCTTACTAGT**GGAGG**AGCCAGCATGACAACGAAAGGAGTG ACAG-3' (HindIII/SpeI) and 5'-GGTCTAGAATTACTTGGTGGCG-ATGATGACG-3' (XbaI); for *sfmM3*, 5'-GGAAGCTTACTAGT**GG-AGG**AGCCAGCATGAGACGGATGCTGTATGCC-3' (HindIII/SpeI) and 5'-GGTCTAGAATCAGGCGGCGCGGACCGC-3' (XbaI). The restriction sites are underlined, and the optimal ribosome binding site (RBS) introduced at the 5' end of each gene is shown in bold. The multicistronic cassette was constructed using the compatible XbaI/SpeI cohesive ends, and the entire cassette was cloned into the PacI/XbaI sites of pYT315 [32] to yield pTL2027.

Construction of the Shuttle Plasmids for Heterologous Expression in *P. putida*

For heterologous expression of *SacDEFG*, a 3.6-kb fragment amplified by PCR using primers 5'-ATATGGTACCCATATGGAATCGATAG-

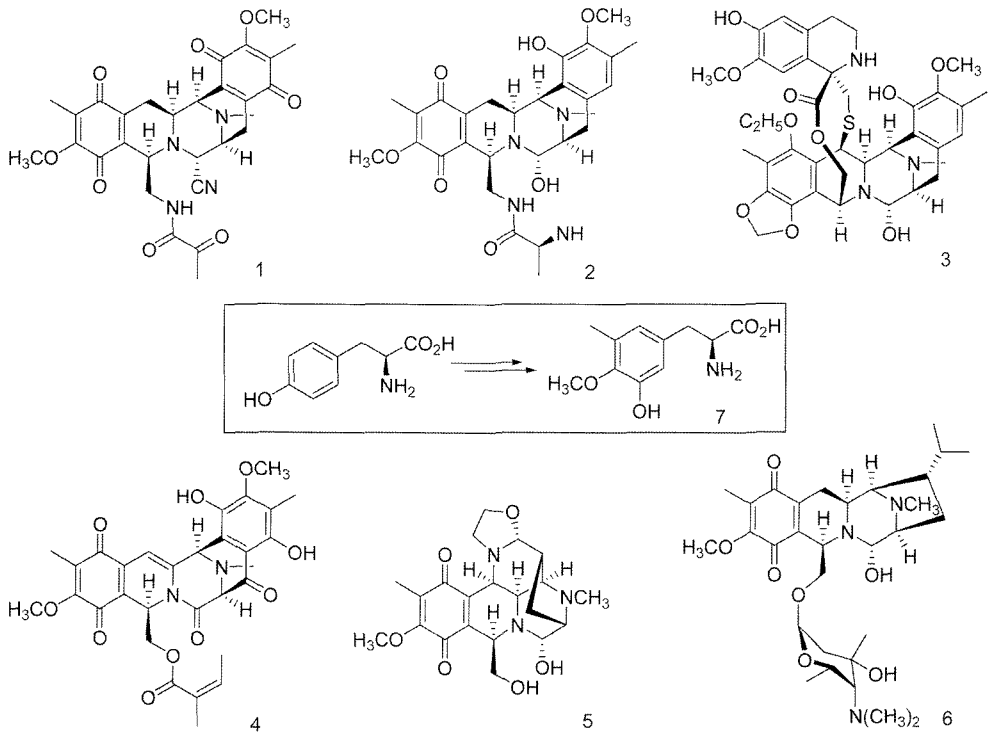

Fig. 1. Structures of saframycin A (SFM-A, **1**), safracin B (SAC-B, **2**), ecteinascidin 743 (ET743, **3**), renieramycin H (**4**), naphthyridinomycin (**5**), lemonomycin (**6**), and nonproteinogenic amino acid 3-hydroxy-5-methyl-*O*-methyltyrosine (3h5m*O*mTyr, **7**) derived from tyrosine.

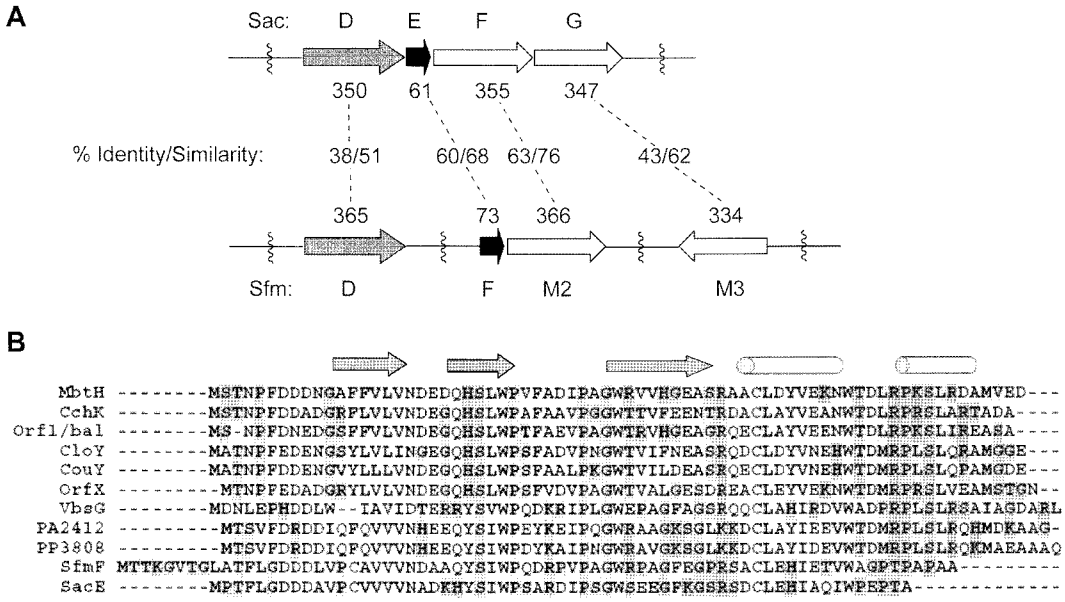

Fig. 2. Comparison of proteins involved in the biosynthesis of 3h5m*O*mTyr.

A. Number of amino acids used for comparisons are given above and below the number of % identity/similarity. **B**. Amino acid sequence alignment of several family members of MbtH-like proteins. The sequences shown are mainly from *mbtH*-like genes found in the biosynthetic gene clusters of different antibiotics and siderophores, recently characterized: MbtH, mycobactin [22], CchK, coelichelin [13], Orf1/bal, balhimycin [29], CloY, clorobiocin [31], CouY, coumermycin A1 [31], OrfX, CDA [13], VbsG, vicibactin [2], PA2412, pyoverdine [4]. The secondary structural elements were depicted according to the published structure of PA2412 [4], sequence with sheets as arrows and helices as rods.

CCTTTCCC-3' (KpnI/NdeI) and 5'-ATATACTAGTCAGCGTCGA-CATGTGTCTTCC-3' (SpeI) was cloned into the KpnI/SpeI site of pANT841 to yield pTL2029, in which the inserted fragment was confirmed by sequencing. With digestion of NdeI and HindIII, a 3.6-kb inserted fragment was cloned into the same site of pET28a to yield pTL2030, in which an XbaI site and an RBS were fused to the 5' terminus of *sacDEFG*. Consequently, a 3.7-kb XbaI/HindIII fragment that contained the intact *sacDEFG* with an RBS was recloned into the same site of pVLT33 to yield the expression construct pTL2031. Using a similar cloning strategy, plasmids pTL2032, pTL2033, pTL2037, and pTL2038 were constructed for heterologous expression of *SacEFG*, *SfmD-SacEFG*, *SacE*, and *SfmF*, respectively.

Production, Analysis, and Identification of the Tyrosine Derivatives

P. putida cells were grown at 28°C in LB medium until OD_{600} up to 0.6, and then they were induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, and further incubated at 28°C for overnight. The supernatant of the culture broth was analyzed by HPLC at room temperature using an analytical Inertsil ODS-EP column (5 μm, 4.6×250 mm; GL Sciences) on an Agilent 1100 Series HPLC instrument. A series of linear gradients was developed from solvent A (0.1% TFA) to solvent B (0.1% TFA in acetonitrile) in the following program: 0–3 min, 0% B; 3–18 min, 40% B; 18–20 min, 95% B; 20–23 min, constant 95% B; 23–25 min, 0% B; 25–28 min, constant 0% B. The flow rate was kept constant at 1.0 ml/min and elution was monitored at 275 nm. The eluted compound was confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis performed on an LCMS-2010 A (Liquid Chromatograph Mass Spectrometer, Shimadzu, Japan).

S. coelicolor strains were grown on solid R5 plates with 25 μg/ml thiostrepton at 30°C for 10 days. Then, a well-pigmented plate was chopped into fine pieces and extracted with 50 ml of ethyl acetate-methanol-acetic acid (89%–9.8%–1.2%). The extracts were dried over anhydrous Na_2SO_4, the resultant solvent was removed *in vacuo*, and the residue was dissolved in 0.25 ml of methanol, and then analyzed by HPLC and LC-MS.

Suicide Mutagenesis of *PP3808* in *P. putida*

To inactivate the *mbtH*-like gene *PP3808* of *P. putida* KT2440Tc and heterologously express *sacDFG in cis*, a 1.6-kb HindIII/SpeI fragment [5'-ATATACTAGTGTATTGCTCTTCGTGGTTG-3' (SpeI) and 5'-ATATAAGCTTGCTGCGACTTGTGCGTAG-3' (HindIII) as PCR primers], a 1.5-kb SpeI/BamHI fragment that contained the *lacI*^{q}-*Ptac* promoter region [5'-ATATACTAGTGCGCGAAGGCGA-AGCG-3' (SpeI) and 5'-ATATGGATCCTCTGTTTCCTGTGTGAA-ATTG-3' (BamHI) as primers], and a 1.2-kb BamHI/EcoRI fragment that contained *sacD* and part of *sacE* [5'-ATATGGATCCACACCG-GCTTCATGGC-3' (BamHI) and 5'-TATAGAATTCTCTAGACAC-TGCGTCGTCGTCTC-3' (EcoRI/XbaI) as primers] were successively ligated and cloned into the HindIII/EcoRI sites of pANT841 to yield pTL2034. Then, a 3.0-kb SpeI/BamHI fragment that contained part of *sacE* and *sacFGH* [5'-ATATACTAGTCATATCGCGCAAATC-TGG-3' (SpeI) and 5'-TATAGGATCCATAACAGCCAACAAACA-TAAAC-3' (BamHI) as primers], and a 1.65-kb BglII/KpnI fragment [5'-ATATAGATCTATCGATGAAGTCTGGACC-3' (BglII) and 5'-ATATGGTACCCAGTGAAGGTCGACTCC-3' (KpnI) as primers] were ligated and cloned into the SpeI/KpnI sites of pANT841 to yield pTL2035. Finally, a 4.3-kb HindIII/XbaI fragment from pTL2034 and a 4.7-kb SpeI/KpnI fragment from pTL2035 were cloned into the HindIII/KpnI sites of pTL2028 to yield the construct pTL2036, in which *sacE* was inactivated by in-frame deletion, and *sacDFGH* was under the control of the *lacI*^{q}-*Ptac* promoter. The resultant

Table 1. Strains and plasmids used in this study.

Strain/Plasmid	Characteristics[a]	Reference
Strains		
E. coli DH5α	Host for general cloning	Invitrogen
E. coli S17-1(λ*pir*)	Donor strain for conjugation between *E. coli* and *Pseudomonas*	[16]
S. coelicolor CH999	Wild-type strain	[17]
S. coelicolor TL2103	*S. coelicolor* CH999 containing pTL2027, expression of *sfmD-FM2-M3*, producing 3h5m*O*mTyr	This work
P. putida KT2440Tc	Wild-type strain	[20]
P. putida TL2104	*P. putida* KT2440Tc containing pTL2031, expression of *sacDEFG*, producing 3h5m*O*mTyr	This work
P. putida TL2105	*P. putida* KT2440Tc containing pTL2032, expression of *sacEFG*, producing 3m*O*mTyr	This work
P. putida TL2106	*P. putida* KT2440Tc containing pTL2033, expression of *sfmD* and *sacEFG*, producing 3h5m*O*mTyr	This work
P. putida TL2107	*ΔPP3808* gene replacement mutant derived from *P. putida* KT2440Tc, in which *PP3808* gene (*mbtH*-like) was replaced by *sacDFGH(ΔsacE)* with in-frame deletion of *sacE*	This work
P. putida TL2108	TL2107 derivative containing pTL2037, expression of *sacE*	This work
P. putida TL2109	TL2107 derivative containing pTL2038, expression of *sfmF*	This work
P. putida TL2110	TL2107 derivative containing pVLT33	This work
Plasmids		
pSP72	Ap^R, *E. coli* subcloning vector	Promega
pANT841	Ap^R, *E. coli* subcloning vector	AF438749
pET28a	Km^R, heterologous expression vector in *E. coli*	Invitrogen
pVLT33	Km^R, heterologous expression vector in *Pseudomonas*	[16]
pYT315	Tsr^R, heterologous expression vector in *S. coelicolor*	[32]
pTL2027	3.5-kb fragment containing *sfmD-FM2-M3* under control of *actI* in pYT315	This work
pTL2028	Km^R, *oriT-RP4* , *sacB* , gene replcement vector with MCS from pSP72	This work
pTL2029	3.6-kb fragment containing *sacDEFG* in pANT841	This work
pTL2030	3.6-kb fragment containing *sacDEFG* in pET28a	This work
pTL2031	3.7-kb fragment containing *sacDEFG* under control of $lacI^q$-*Ptac* in pVLT33	This work
pTL2032	2.6-kb fragment containing *sacEFG* under control of $lacI^q$-*Ptac* in pVLT33	This work
pTL2033	3.7-kb fragment containing *sfmD* and *sacEFG* under control of $lacI^q$-*Ptac* in pVLT33	This work
pTL2034	4.3-kb fragment containing part of upstream sequence of *PP3808*, $lacI^q$-*Ptac* promoter, and *sacD* in pANT841	This work
pTL2035	4.7 kb fragment *sacFGH* and part of downstream sequence of *PP3808* in pANT841	This work
pTL2036	9.0-kb fragment containing *sacDFGH*, *sacE* gene in-frame deletion in pTL2028	This work
pTL2037	0.3-kb fragment containing *sacE* under control of $lacI^q$-*Ptac* in pVLT33	This work
pTL2038	0.3-kb fragment containing *sfmF* under control of $lacI^q$-*Ptac* in pVLT33	This work

[a]Abbreviations: Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tsr^R, thiostrepton resistance.

plasmid pTL2036 was introduced into *P. putida* KT2440Tc and colonies with kanamycin resistance were identified as single crossover mutants. Further incubation and screening for colonies that were sucrose-resistant and kanamycin-sensitive were selected as the double-crossover mutant TL2107.

Results and Discussion

Analysis of Genes Involved in the Biosynthesis of 3h5m*O*mTyr

Nature usually synthesizes the same compound using the similar biosynthetic machinery, exemplified with the biosynthetic pathway of 3h5m*O*mTyr in the biosynthesis of SFM-A and SAC-B from *S. lavendulae* and *P. fluorescens*. In the biosynthetic gene cluster of SAC-B, a four-gene cassette, *sacDEFG*, was proposed to encode the biosynthesis of the nonproteinogenic amino acid 3h5m*O*mTyr. This was based on heterologous expression of this cassette and cocultivation with mutant strains *ΔsacG* or *ΔsacF*, which results in SAC-B production [30]. The sequencing and characterization of the SFM-A gene cluster has revealed four genes, *sfmD*, *sfmF*, *sfmM2*, and *sfmM3*, with significant overall similarity to *sacDEFG* (Fig. 2A). Intriguingly, SfmM2 exhibited higher sequence similarity to SacF (63% identity) than to other SAM-dependent methyltransferases (usually 25%–47% identity), and might have acted as a *C*-methyltransferase to introduce a methyl

group at the *C*-3 position of Tyr. SfmM3 shared high sequence homology to SacG (43% identity) and various *O*-methyltransferases (typical *O*-methyltransferases have 30%–37% identity), which supports its role in *O*-methylation. However, in the biosynthesis of SFM-MX1 from *Myxococcus xanthus*, a member of the same family of natural products with a very closely related chemical structure to SFM-A and SAC-B, the *O*-methyltransferase homolog SafC (with no obvious homology to SfmM3 or SacG) has recently been characterized as a catechol 4-*O*-methyltransferase [19]. This indicates that the biosynthetic pathways of the same nonproteinogenic amino acids sometimes differ between microorganisms. SfmD, with no other homologous proteins reported, has a relatively high sequence similarity to SacD (38% identity and 51% similarity), which was deduced to be responsible for the hydroxyl group substitution at the *C*-5 position that converts 3m*O*mTyr into 3h5m*O*mTyr.

In the biosynthetic gene cassette of 3h5m*O*mTyr in the SFM-A and SAC-B pathways, there are two genes, *sfmF* and *sacE*, which encode small proteins with high similarity to each other (60% identity and 68% similarity), and obvious homology to a family of MbtH-like proteins [22]. These *mbtH*-like genes are frequently found in the biosynthetic gene clusters of peptide antibiotics and siderophores. Sequence alignment of selected MbtH-like proteins (Fig. 2B) revealed an overall remarkable sequence conservation, which was most obvious from the conservation of the sequence S(L/I/V)WPX$_3$PXGWX$_{12}$CLX$_6$WX$_4$P with three conserved tryptophan (W) residues. *S. coelicolor* possesses two homologs of *mbtH*-like genes, including *cchK* of the Cch cluster that directs coelichelin biosynthesis, and *cdaX* (*orfX*) within the CDA biosynthetic gene cluster. *In vivo* gene disruption and complementation have revealed that one of the two genes is required for biosynthesis of coelichelin or CDA, and they can cross-talk between the two biosynthetic pathways [13]. *In vitro* enzymic studies on enterochelin formation [3, 6] have suggested that the MbtH-like proteins may not have an essential catalytic function; therefore, it has been speculated that these small proteins are involved in transport processes, protein-protein interactions, or precursor formation. More recently, using homologous expression of the clorobiocin biosynthetic gene cluster in *S. coelicolor* and modification of the host genome, the *mbtH*-like gene *cloY* has been shown to be necessary for efficient formation of the aminocoumarin moiety of clorobiocin [31]. Therefore, it would be interesting to investigate whether *sfmF*/*sacE* is involved in the biosynthesis of 3h5m*O*mTyr, the precursor of the tetrahydroisoquinoline family of alkaloids.

Heterologous Expression of the Biosynthetic Pathway of 3h5m*O*mTyr and the Necessary Role of *sfmD* in Hydroxylation

To further investigate the biosynthetic pathway of 3h5m*O*mTyr, multicistronic cassette *sfmD-FM2-M3* of the SFM-A gene

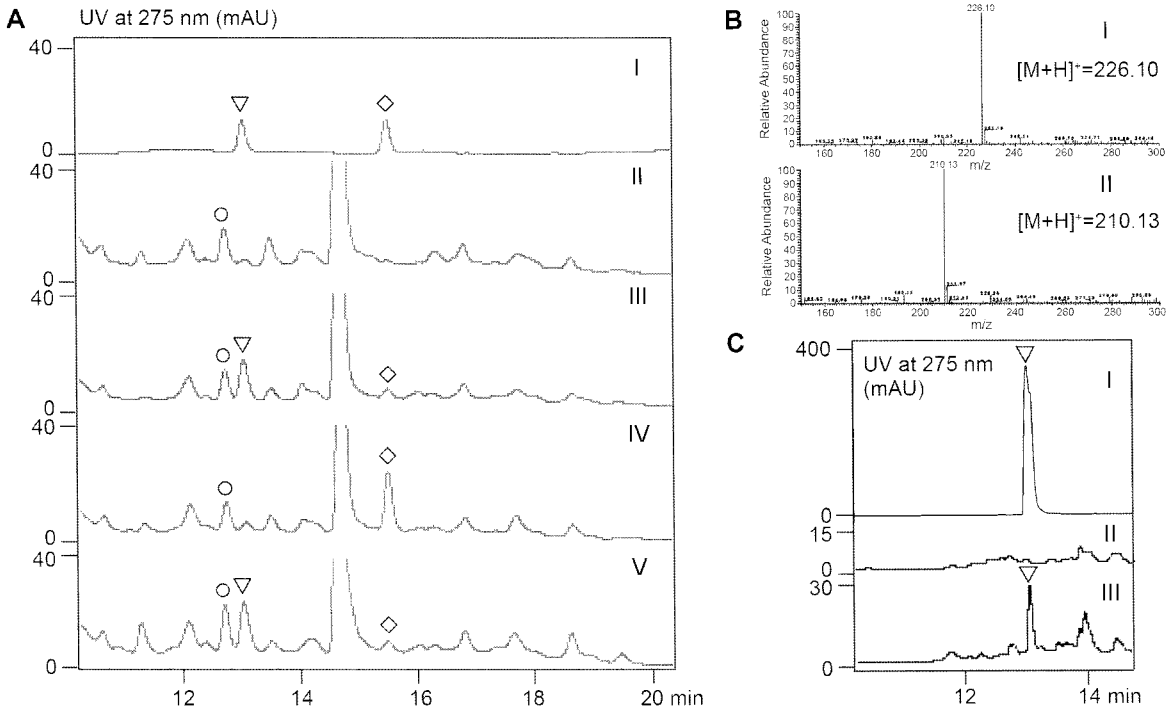

Fig. 3. HPLC analysis of tyrosine derivative production in heterologous hosts.

A. *P. putida* KT2440Tc as host: standards (I), control, *P. putida* TL2110 (II), TL2104 (III), TL2105 (IV), and TL2106 (V). **B**. Mass spectrum of 3h5m*O*mTyr (I) and 3m*O*mTyr (II) recording in positive-ion mode showing the characteristic *m*/*z*=226 and 210 [M+H] peak. **C**. *S. coelicolor* CH999 as host: standards (I), control (II), TL2103 (III). (▽) 3h5m*O*mTyr; (◇) 3m*O*mTyr; (○) unidentified metabolite whose production is independent of tyrosine derivative production.

cluster was constructed under the control of the *actI* promoter and heterologously expressed in *S. coelicolor* CH999 [17, 32]. Similarly, the four-gene cassette of the SAC-B gene cluster, *sacDEFG*, was expressed under the control of the *lacI*^{q}-*Ptac* promoter [16] in *P. putida* KT2440Tc. Both resultant recombinant strains produced an obvious amount of 3h5m*O*mTyr (Figs. 3AIII and 3CIII) compared with that in control strains (Figs. 3AII and 3CII). As determined by HPLC analysis and confirmed by LC-MS analysis, the compound showed $(M+H)^+$ ion at *m/z* = 226.1 (Fig. 3BI), consistent with the molecular formula $C_{11}H_{15}NO_4$. When the *sacD* gene was deleted, the resultant recombinant strain TL2105 produced no 3h5m*O*mTyr, but it did produce 3m*O*mTyr (Fig. 3AIV), which recorded in $(M+H)^+$ ion at *m/z*=210.1 (Fig. 3BII), consistent with the molecular formula $C_{11}H_{15}NO_3$. However, when the hybrid four-gene cassette *sfmD-sacEFG* was constructed under the constitutive *lacI*^{q}-*Ptac* promoter and heterologously expressed in *P. putida*, the resultant recombinant strain TL2106 produced a similar amount of 3h5m*O*mTyr (Fig. 3AV) as TL2104 did, as determined by HPLC analysis. These results demonstrate that the four-gene cassette is essential for 3h5m*O*mTyr production, and SfmD/SacD may catalyze the transformation of 3m*O*mTyr into 3h5m*O*mTyr.

Enzymatic hydroxylation of the aromatic ring of Tyr derivatives exists widely in nature. Mechanistically, this family of enzymes can be divided into three groups: (i) Tyr hydroxylases, distributed throughout the phylogenetic scale, are non-heme Fe-dependent enzymes that require reduced pteridine cofactor (*6R*)-5, 6, 7, 8-tetrahydrobiopterin as an electron donor [11]; (ii) tyrosinases, which are involved in the biosynthesis of melanin pigments, contain two copper atoms [24]; and (iii) SgcC in the biosynthetic pathway of enediyne antibiotic C-1027, has recently been characterized as a two-component, FAD-dependent monooxygenase that requires carrier protein-tethered substrates [15]. However, SfmD/SacD showed no obvious homology to any of these three groups, nor to the conserved cofactor-binding amino acid residues. Other Tyr hydroxylation enzymes that exhibit characteristics different from the above groups have also been reported in lincomycin [21] and benzodiazepine [9] biosynthetic pathways, but the exact nature of their catalytic mechanism remains to be determined.

Deletion of *mbtH* Homologs in *P. putida*

Genome sequencing data show that *mbtH* homologs are usually present in many bacteria, and the functional complementation of *cdaX* and *cchK* in *S. coelicolor* [13] reveals that gene disruption cannot determine whether the *mbtH*-like genes are necessary for NRPS-directed biosynthesis. Therefore, heterologous expression of the biosynthetic pathway in a completely sequenced host is a good option for trying to solve this problem. *P. putida* KT2440 was deemed suitable for this purpose because its genome has been published [20], it is a preferred host for gene manipulation, and it allows successful expression of the *sacDEFG* gene cassette. Analysis of the *P. putida* KT2440 genome sequence yielded one gene, *PP3808* (AAN69402), which encoded a 73-amino-acid protein with high similarity

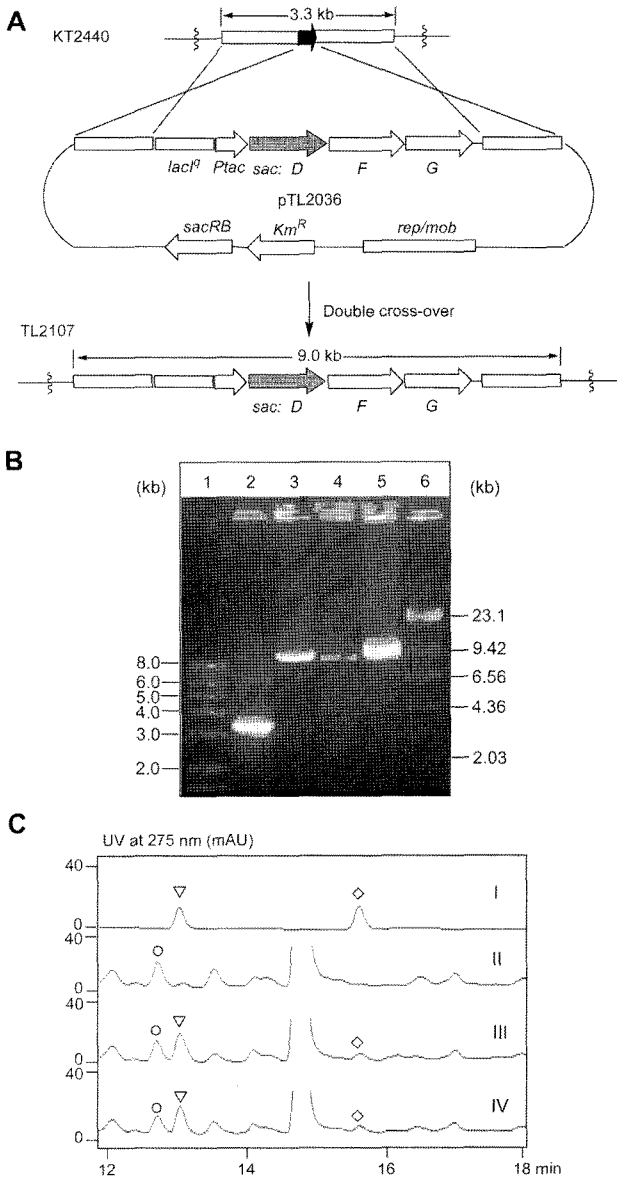

Fig. 4. Deletion of *mbtH* homologs in *P. putida*, and heterologous expression of the biosynthetic pathway of 3h5m*O*mTyr *in cis*.
A. Construction of *mbtH*-like gene deletion and *sacDFG* heterologous expression strain *P. putida* TL2107 (*ΔPP3808, ΔsacE, and sacDFG*$^+$) *via* homologous recombination. **B.** PCR analysis of the genotype using 5'-ATATGGTACCCAGTGAAGGTCGACTCC-3' and 5'-ATATAAGCTTG-CTGCGACTTGTGCGTAG-3' as primers. Lanes 1 and 6, DNA marker; lane 2, *P. putida* KT2440; lanes 3–5, *P. putida* TL2107. **C.** HPLC analysis of tyrosine derivative production of mutants for deletion of *mbtH* homologs in *P. putida*, heterologous expression, and complementation. Standards (I), *P. putida* KT2440Tc (II), TL2104 (III), TL2107 (IV). (▽) 3h5m*O*mTyr; (◇) 3m*O*mTyr; (○) unidentified metabolite whose production is independent of tyrosine derivative production.

to other MbtH-like proteins (Fig. 2B). To delete this *mbtH* homolog in *P. putida* KT2440, a gene replacement plasmid (pTL2036) was constructed, in which *sacE* was inactivated by in-frame deletion, and *sacDFG* was under the control of the *lacI*^{q}-*Ptac* promoter (Fig. 4A). This construction also contained two DNA fragments amplified from upstream and downstream of *PP3808* for homologous recombination, and the *sacRB* gene as a positive selection marker for double crossover [27]. The plasmid pTL2036 was introduced into *P. putida* KT2440Tc by conjugation to select for the resulting double-crossover mutant *P. putida* TL2107, which was sucrose-resistant and kanamycin-sensitive (Fig. 4A). The genotype of the *P. putida* TL2107 mutant strain, in which the *mbtH*-like gene *PP3808* was substituted with the *lacI*^{q}-*Ptac* promoter and *sacDFG* gene cassette, was confirmed by PCR analysis. Genomic DNA from both the *P. putida* wide type and TL2107 mutant strain was used as template, respectively. The wide-type strain yielded a distinct signal at 3.3 kb, whereas this fragment was shifted to 9.0 kb in the TL2107 mutant strain, as would be predicted for the replacement of the 0.2-kb fragment of the *PP3808* gene by the 5.8-kb *lacI*^{q}-*Ptac-sacDFG* cassette (Fig. 4B).

Heterologous Expression of *sacDFG* and Complementation of the *ΔmbtH* Double Mutant with *sacE* and *sfmF*

The *mbtH*-like gene was deleted, and mutant strains TL2107 with heterologous expression of *sacDFG* was fermented under standard conditions, with *P. putida* wide-type as a negative control and strain TL2104 with heterologous expression of plasmid pTL2031 as a positive control. Fermentation cultures were analyzed by HPLC for 3h5m*O*mTyr production. However, the recombinant strain TL2107, with inactivation of two MbtH-like proteins by deletion of both *PP3808* and *sacE* genes, produced a similar amount of 3h5m*O*mTyr (Fig. 4CIV) to that of TL2104 (Fig. 4CIII), which possessed normal MbtH-like proteins. Further complementation of the *mbtH*-like gene, *sacE* or *sfmF*, *in trans* did not markedly change the production level of 3h5m*O*mTyr for either system. These results suggest that the *mbtH*-like gene, *sacE*, *sfmF*, or *PP3808*, is not required for the biosynthesis of the nonproteinogenic amino acid 3h5m*O*mTyr in the SAC-B or SFM-A biosynthetic pathways.

Although several studies have provided some insights into MbtH-like proteins for nonribosomal peptide biosynthesis, the precise function of this type of small proteins still remains unclear. Previous *in vivo* gene inactivation studies have given conflicting conclusions, because of possible polar effects and the homologs mediating cross-talk [2, 13, 29]. More recently, heterologous expression and functional complementation in *S. coelicolor* have defined an auxiliary role for MbtH-like proteins in the biosynthesis of nonproteinogenic amino acids [31], and transcriptional analysis has ruled out any possible regulatory effects on transcription [13]. The genes *sfmF*/*sacE* of the SFM-A/SAC-B biosynthetic gene cluster encode typical MbtH-like proteins, which have high sequence homology and conserved amino acids, although they have a slight defect at the C terminus and the final α helix, compared with the structure of PA2412 [4], a MbtH-like protein in *P. aeruginosa* (Fig. 2B). However, heterologous expression mutants with multicopies or without *mbtH*-like homologs produced a similar amount of 3h5m*O*mTy, implying that the *mbtH*-like gene is not required for the biosynthesis of 3h5m*O*mTyr in the SAC-B or SFM-A biosynthetic pathway.

In conclusion, the significant overall similarity between the four-gene cassette, *sacDEFG* in the SAC-B cluster and *sfmDFM2M3* in the SFM-A cluster, strongly indicates the existence of a relatively close evolutionary relationship. The functionally cross-complementation of *sacD* and *sfmD* supports a common step of 3h5m*O*mTyr biosynthesis, where the transformation of 3m*O*mTyr into 3h5m*O*mTyr defines the encoding proteins to catalyze the hydroxylation of the aromatic ring of Tyr derivatives, and the *mbtH*-like gene does not impair the biosynthesis of 3h5m*O*mTyr. These results enrich the current knowledge about nonproteinogenic amino acid biosynthesis, put into context for future efforts in combinatorial biosynthetic manipulations.

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References

1. Carter, N. J. and S. J. Keam. 2007. Trabectedin: A review of its use in the management of soft tissue sarcoma and ovarian cancer. *Drugs* **67:** 2257–2276.
2. Carter, R. A., P. S. Worsley, G. Sawers, G. L. Charllis, M. J. Dillworth, K. C. Carson, *et al.* 2002. The *vbs* genes that direct synthesis of the siderophore vicibactin in *Rhizobium leguminosarum*: Their expression in other genera requires ECF sigma factor RpoI. *Mol. Microbiol.* **44:** 1153–1166.
3. Crosa, J. H. and C. T. Walsh. 2002. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiol. Mol. Biol. Rev.* **66:** 223–249.

4. Drake, E. J., J. Cao, J. Qu, M. B. Shah, R. M. Straubinger, and A. M. Gulick. 2007. The 1.8 Å crystal structure of PA2412, an MbtH-like protein from pyoverdine of *Pseudomonas aeruginosa*. *J. Biol. Chem.* **282:** 20425–20434.
5. Fischbach, M. A. and C. T. Walsh. 2006. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: Logic, machinery, and mechanisms. *Chem. Rev.* **106:** 3468–3496.
6. Gehring, A. M., I. Mori, and C. T. Walsh. 1998. Reconstitution and characterization of the *Escherichia coli* enterobactin synthetase from EntB, EntE, and EntF. *Biochemistry* **37:** 2648–2659.
7. Grosso, F., R. L. Jones, G. D. Demetri, I. R. Judson, J.-Y. Blay, A. L. Cesne, *et al.* 2007. Efficacy of trabectedin (ecteinascidin-743) in advanced pretreated myxoid liposarcomas: A retrospective study. *Lancet Oncol.* **8:** 595–602.
8. Herrero, M., De V. Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* **172:** 6557–6567.
9. Hu, Y., V. Phelan, L. Ntai, C. M. Farnet, E. Zazopoulos, and B. O. Bachmann. 2007. Benzodiazepine biosynthesis in *Streptomyces refuineus*. *Chem. Biol.* **24:** 691–701.
10. Hubbard, B. K. and C. T. Walsh. 2003. Vancomycin assembly: Nature's way. *Angew. Chem. Int. Ed.* **42:** 730–765.
11. Kappock, T. J. and J. P. Caradonna. 1996. Pterin-dependent amino acid hydroxylases. *Chem. Rev.* **96:** 2659–2756.
12. Konz, D. and M. A. Marahiel. 1999. How do peptide synthetases generate structural diversity? *Chem. Biol.* **6:** R39–R48.
13. Lautru, S., D. Oves-Costales, J.-L. Pernodet, and G. L. Challis. 2007. MbtH-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in *Streptomyces coelicolor* M145. *Microbiology* **153:** 1405–1412.
14. Li, L., W. Deng, J. Song, W. Ding, Q.-F. Zhao, C. Peng, W.-W. Song, G.-L. Tang, and W. Liu. 2008. Characterization of the saframycin A gene cluster from *Streptomyces lavendulae* NRRL 11002 revealing a NRPS system for assembling the unusual tetrapeptidyl skeleton in an iterative manner. *J. Bacteriol.* **190:** 251–263.
15. Lin, S., S. G. Van Lanen, and B. Shen. 2008. Characterization of the two-component, FAD-dependent monooxygenase SgcC that requires carrier protein-tethered substrates for the biosynthesis of the enediyne antitumor antibiotic C-1027. *J. Am. Chem. Soc.* **130:** 6616–6623.
16. de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis. 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. *Gene* **123:** 17–24.
17. McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla. 1993. Engineered biosynthesis of novel polyketides. *Science* **262:** 1546–1550.
18. Mikami, Y., K. Takahashi, K. Yazawa, T. Arai, M. Namikoshi, S. Iwasaki, and S. Okuda. 1985. Biosynthetic studies on saframycin A, a quinone antitumor antibiotic produced by *Streptomyces lavendulae*. *J. Biol. Chem.* **260:** 344–348.
19. Nelson, J. T., J. Lee, J. W. Sims, and E. W. Schmidt. 2007. Characterization of SafC, a catechol 4-*O*-methyltransferase involved in saframycin biosynthesis. *Appl. Environ. Microbiol.* **73:** 3575–3580.
20. Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. P. Martins dos Santos, *et al.* 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4:** 799–808.
21. Neusser, D., H. Schmidt, J. Spizèk, J. Novotnà, U. Peschke, S. Kaschabeck, P. Tichy, and W. Piepersberg. 1998. The genes *lmbB1* and *lmbB2* of *Streptomyces lincolnensis* encode enzymes involved in the conversion of L-tyrosine to propylproline during the biosynthesis of the antibiotic lincomycin A. *Arch. Microbiol.* **169:** 322–332.
22. Quadri, L. E., J. Sello, T. A. Keating, P. H. Weinreb, and C. T. Walsh. 1998. Identification of a *Mycobacterium tuberculosis* gene cluster encoding the biosynthetic enzymes for assembly of the virulence conferring siderophore mycobactin. *Chem. Biol.* **5:** 631–645.
23. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
24. Sánchez-Ferrer, Á., J. N. Rodríguez-López, F. García-Cánovas, and F. García-Carmona. 1995. Tyrosinase: A comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1247:** 1–11.
25. Schwarzer, D., R. Finking, and M. A. Marahiel. 2003. Nonribosomal peptides: From gene to products. *Nat. Prod. Rep.* **20:** 275–287.
26. Scott, J. D. and R. M. Williams. 2002. Chemistry and biology of the tetrahydroisoquinoline antitumor antibiotics. *Chem. Rev.* **102:** 1669–1730.
27. Selbitschka, W., S. Niemann, and A. Pühler. 1993. Construction of gene replacement vectors from Gram^{-} bacteria using a genetically modified *sacRB* gene as a positive selection marker. *Appl. Microbiol. Biotechnol.* **38:** 615–618.
28. Sieber, S. A. and M. A. Marahiel. 2005. Molecular mechanisms underlying nonribosomal peptide synthesis: Approaches to new antibiotics. *Chem. Rev.* **105:** 715–738.
29. Stegmann, E., C. Rausch, S. Stockert, D. Burkert, and W. Wohlleben. 2006. The small MbtH-like protein encoded by an internal gene of the balhimycin biosynthetic gene cluster is not required for glycopeptide production. *FEMS Microbiol. Lett.* **262:** 85–92.
30. Velasco, A., P. Acebo, A. Gomez, C. Schleissner, P. Rodríguez, T. Aparicio, *et al.* 2005. Molecular characterization of the safracin biosynthetic pathway from *Pseudomonas fluorescens* A2-2: Designing new cytotoxic compounds. *Mol. Microbiol.* **56:** 144–154.
31. Wolpert, M., B. Gust, B. Kammerer, and L. Heide. 2007. Effects of deletions of *mbtH*-like genes on clorobiocin biosynthesis in *Streptomyces coelicolor*. *Microbiology* **153:** 1413–1423.
32. Zhang, W., B. D. Ames, S.-C. Tsai, and Y. Tang. 2006. Engineered biosynthesis of a novel amidated polyketide, using the malonamyl-specific initiation module from the oxytetracycline polyketide synthase. *Appl. Environ. Microbiol.* **72:** 2573–2580.
33. Zmijewski, M. J., Jr. M. Mikolajczak, V. Viswanatha, and V. J. Hruby. 1982. Biosynthesis of the antitumor antibiotic naphthyridinomycin. *J. Am. Chem. Soc.* **104:** 4969–4971.