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Heterologous Expression and Manipulation of Three Tetracycline Biosynthetic Pathways**

Peng Wang, Woncheol Kim, Lauren B. Pickens, Xue Gao, and Yi Tang*

Department of Chemical and Biomolecular Engineering, Department of Chemistry and Biochemistry, University of California, Los Angeles Los Angeles, CA 90095 (USA)

Abstract

Three and one: Three tetracycline biosynthetic pathways have been overexpressed and manipulated in heterologous host *Streptomyces lividans* K4-114. New tetracycline modifying enzymes have been identified through a series of gene inactivation and intermediate characterization. The collection of newly discovered tailoring enzyme and the heterologous platform will promote our understanding of tetracycline biosynthesis, as well as our performance to engineer tetracycline biosynthesis in an efficient manner.

Keywords

heterologous expression; polyketide; tetracycline; biosynthesis

Engineered biosynthesis of natural products is a powerful approach towards generating structural diversity.^[1, 2] To fruitfully manipulate a biosynthetic pathway, it is desirable to have a robust heterologous host^[3] and a set of enzymatic tools that can modify the target compound. Analogs of numerous important natural products, including erythromycin,^[4] novobiocin,^[5] and daptomycin,^[6] have been obtained through shuffling of biosynthetic genes in genetically amendable heterologous hosts. The tetracycline family of natural products has been highly important to human health, as evident in the continual development of semisynthetic tetracyclines as new antibiotics in the last forty years.^[7, 8] The 2-carboxamide tetracyclic structure universal to all tetracyclines has been recognized as a privileged scaffold for drug discovery.^[9] We report here the heterologous expression and genetic manipulation of three structurally diverse natural tetracyclines: the classic antibiotic oxytetracycline **1**,^[10] the antitumor SF2575 **2**,^[11] and dactylocycline **3** that is active against tetracycline resistant strains.^[12] Numerous new tetracycline compounds are obtained from

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*Fax: (+1) 310-206-4107, Phone: (+1) 310-825-9538, yitang@ucla.edu.

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the engineered host/pathway pairs, which lead to the revelation of a new set of tailoring enzymes that can modify the tetracycline scaffold at different positions.

Our first objective was to establish a heterologous host for reconstitution of the *oxy* cluster and biosynthesis of **1**, which would circumvent genetic manipulation in its native *Streptomyces rimosus*.^[13] While systematic build-up of the *oxy* genes in *Streptomyces coelicolor* CH999 has provided key insights into the earlier steps of the pathway,^[14,15] biosynthesis of **1** using this approach has not been accomplished due to the incomplete understanding of the last few steps of the pathway, namely from anhydrotetracycline **5** to **1**. Despite previous reports of production of **1** from *Streptomyces lividans*^[16] and *Myxococcus Xanthus*,^[17] we did not detect the biosynthesis of **1** in the model heterologous host *S. lividans* K4-114^[18] upon insertion of the *oxy* cluster (*oxyA-T*, 25 kb) into its chromosome using the integrative vector pOTC. To enhance the transcription of the *oxy* pathway, a *Streptomyces* antibiotic regulatory protein (SARP), encoded by *ctc11* from the chlorotetracycline gene cluster,^[19] was placed under the constitutive *ermE** promoter^[20,21] in pCTC11 and co-transformed into K4-114 with pOTC, resulting in the strain K4/pOTC-Ctc11 (Table S1). We chose *ctc11* because no corresponding SARP is found in the vicinity of the *oxy* cluster. HPLC-MS analysis showed the production of **1** as a predominant product at ~20 mg/L (Figure 1C). Using pOTC as the template, the λ -Red-mediated recombination method^[22,23] was employed to delete *oxyS* encoding the anhydrotetracycline oxygenase (Scheme 1A). Cotransformation of the resulting plasmid with pCTC11 into K4-114 led to the accumulation of **5** and disappearance of **1** in K4/pSKO-Ctc11 (Figure 1D). While OxyS has been linked to the C6 hydroxylation of **5** to yield **5a**, 11a-dehydrotetracycline **4**,^[24,25] previous knockout studies in *S. rimosus* did not lead to the isolation of **5**, presumably due to off-pathway modifications by endogenous enzymes.^[26] Therefore, the availability of the “clean” heterologous host will enable the complete understanding of the enzymology between **5** and **1**, a critical requirement for generating tetracycline-like analogs.

The highly modified **2** produced from *Streptomyces* sp. 2575 contains unique modifications around its tetracycline aglycon, including the C4O salicylate, C9 D-olivose, C18O angelate, and methoxy groups at C12a and C6. Unveiling the biochemistry behind these reactions can therefore lead to a new group of tetracycline-specific transferases. However, due to genetic difficulties in manipulating the *ssf* pathway in *S. sp.* 2575, only limited in vitro experiments have been performed on the *ssf* tailoring enzymes such as the salicylate transferase SsfX3.^[11] To construct a K4-114 host for the production of **2**, pSF2575 encoding the entire *ssf* gene cluster (40 genes, 47 kb)^[27] was integrated into the chromosome and the SARP SsfT1 was overexpressed under the *ermE** promoter yielding K4/pSF2575-T1 (Table S1). HPLC-MS analysis showed the production of **2** and two intermediates **13** and **14** with a combined titer of ~127 mg/L (Figure 2A and Scheme 1B).

Starting with K4/pSF2575-T1, the roles of four *ssf* SAM-dependent methyltransferases were first probed through individual gene inactivation of *ssfM1*, *ssfM2*, *ssfM3*, and *ssfM4* using λ -Red recombination as described for *oxyS* (Table S1). HPLC-MS analysis showed that *ssfM1* produced three major compounds **15**, **16** and **17**, the masses of which correspond to the desmethyl versions of **13**, **14** and **2**, respectively (Figure 2B). Isolation and full NMR

characterization of **17** revealed the compound to be the previously isolated TAN-1518A (Table S3 and Figure S5),^[28] which is the C12a hydroxyl version of **2**. Similarly, inactivation of *ssfM2* led to the isolation of three different desmethyl compounds **18**, **19** and **20** (Figure 2C). Compound **20** was characterized by NMR to be the C6 hydroxyl version of **2** (Table S4 and Figure S6). Complementation of *ssfM1* and *ssfM2* strains with plasmid-borne copies of *ssfM1* and *ssfM2*, respectively, each restored the production of **2** in the host (Figure S4). Therefore, SsfM1 and SsfM2 are assigned as the C12aO and C6O O-methyltransferases, respectively, both activities are new to the tetracycline family.

Inactivation of *ssfM3* did not abolish the production of **2** (Figure 2D). Sequence analysis of SsfM3 shows significant homology (39% identity) to MtmMII, which is a C9 C-methyltransferase in the mithramycin biosynthetic pathway.^[29] Considering the same position in **2** is decorated with D-olivose, it is likely that SsfM3 is rendered inactive in this pathway. Indeed, the conserved histidine serving as general base in the catalytic pocket is substituted with Phe in SsfM3 (Figure S17).^[30]

SsfM4 was identified to catalyze C6 C-methylation by heterologous reconstitution in CH999.^[27] To examine the effect of removing this enzyme on the *ssf* pathway, the *ssfM4* mutant K4/pM4KO-T1 was generated. HPLC-MS analysis revealed **21** and **22** were biosynthesized (Figure 2E and Scheme 2B), both exhibiting identical UV spectra to that of **5** (Figure S7, S8). Following full NMR analyses (Tables S5–6 and Figures S7–8), **21** is confirmed to be an analog of **5** that differs in C9 D-olivose, C12a methoxy and C4 hydroxyl functional groups. Hence, inactivation of the C6 methyl transfer, which takes place early in the pathway to generate 6-methylpretetramid **6**, does not affect the functions of the glycosyltransferase and tailoring reactions on rings B and A. The unexpected isolation of **22** suggests that **21** can undergo oxidation in ring C, and the resulting phenol can be methylated by SsfM2. This is confirmed by the further inactivation of *ssfM2* in the *ssfM4* mutant, which produced only **21** (Figure 2F).

The attachment of D-olivose in **21** also suggests that glycosylation of C9 takes place on the naphthacene intermediate, instead of on the matured tetracycline scaffold. To confirm this timing, inactivation of *ssfS6* that encodes the only glycosyltransferase in the *ssf* cluster was performed. A single metabolite **9** was found in the extract of the mutant (Figure 2G), which was characterized to be the C4 hydroxyl, C12a methoxy version of **5**. The Friedel-Crafts C-glycosylation of **9** with D-olivose is analogous to that catalyzed by glycosyltransferase UrdGT2 in the urdamycin pathway.^[31] Hence, SsfS6 represents the first enzyme identified that can regioselectively modify the anhydrotetracycline-like molecules at C9, which is a position that has been fruitfully modified to yield newer generations of tetracyclines.^[8]

Elucidating the timing of SsfS6 suggests that hydroxylation of the C6 position by an OxyS homolog in the *ssf* pathway likely acts on a glycosylated substrate. Indeed, inactivation of *ssfO1*, which encodes a flavin-dependent monooxygenase that shows 54% sequence similarity to OxyS, led to the disappearance of **2** and the emergence of a single compound **10** (Figure 2J). Complementation of *ssfO1* restored the biosynthesis of **2** in the K4 host (Figure S4). Structural characterization revealed **10** is the glycosylated version of **9** (Table S8 and Figure S10), which indicates that SsfO1 functions immediately following SsfS6. The

stereochemistry of the C6 methoxy group in **2** was previously determined to be in the *R* configuration by X-ray structure,^[32] which is opposite to the *S* configuration in **1**. Therefore, we expect the binding of **5** and **10** in OxyS and SsfO1, respectively, to be significantly different in order to facilitate the hydroxylation from opposite faces of the naphthacenedione ring.

An interesting structural feature shared by both **9** and **10** is the C4 hydroxyl group, which is the site of salicyl-transfer catalyzed by SsfX3 much later in the pathway.^[11] In the *oxy* pathway, the C4 ketone group in **7** is reductively aminated and *N,N*-dimethylated to yield **5**.^[15] The presence of the *R*-C4-hydroxyl group in **9** indicates that ketoreduction at C4 by a ketoreductase should take place early in the pathway. This is further verified in the *ssfS6/ssfM1* double knockout, which produced a single product **8** (Figure 2K) that was verified by NMR to be 4-hydroxy-anhydrotetracycline (Table S9 and Figure S11). To identify which of the two unassigned ketoreductases (SsfK and SsfF) is involved in the reduction of **7** to **8**, inactivation of both genes were performed independently followed by metabolite analysis. Since **7** can spontaneously undergo rapid retro-Claisen cleavages to yield the shunt product WJ135 **24**,^[15] inactivation of the C4 ketoreductase should yield **24** as a major product. Whereas *ssfK* continued to produce the compounds **13**, **14** and **2** (Figure 2H), *ssfF* led to complete abolishment of **2** along with the emergence of **24** (Figure 2I). Complementation of *ssfF* in K4/pFKO-T1 restored the production of **2** (Figure S4), confirming the unique role of SsfF in catalyzing the *R*-C4-ketoreduction that serves as the diverging point between the *oxy* and *ssf* pathways.

To obtain an additional analog of **5** using the *ssf* pathway, we constructed a *ssfS6/ssfM4* double knockout, which predicatively afforded **23**, the C6-desmethyl analog of **9** (Figure 2L, Scheme 1B). To evaluate the antibiotic activities of these new compounds, we measured the minimum inhibitory concentration (MIC) values against both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Salmonella enterica*, *Pseudomonas fluorescens* Pf0-1, and *E. coli* DH10B) bacteria (Table S10). MIC assays showed that **8**, **9**, and **23** exhibited comparable antimicrobial activities as **5**, while the C9 glycosylation in **21** and **22** dramatically increased the MIC values towards all strains. **16**, **17**, and **20** showed decreased MIC values towards the Gram-positive *B. subtilis*, which reconfirms that hydroxylation at the C6 position is important for antibiotic activities. Moreover, attachment of angelic acid at C18O in **20** led to a further decrease in MIC values towards *B. subtilis* implying the C18O position is a promising site to generate active tetracycline analogs.

To further utilize the K4-114 strain as a heterologous host for tetracycline biosynthesis, we targeted the biosynthesis of **25**, which is the aglycon of **3** (Scheme 1C). **3** is a heavily modified tetracycline isolated from *Dactylosporangium* sp. SC 14051^[33] and is active against *Staphylococcus aureus* strains that are resistant towards **1**.^[12] However, **3** was shown to be overly acid labile to be developed as an antibiotic.^[12] Therefore, understanding the biosynthesis of **3** may lead to the development of better analogs. However, this is particularly challenging in the native host due to its exceedingly slow growth rate and complete genetic inalcitrance. Reconstituting the biosynthesis of **25** (and ultimately **3**) in K4-114 may serve as a promising alternative. Toward this end, we sequenced the genome of

the producing strain and identified the putative *dac* gene cluster (38 genes, 45 kb) (Table S11, Figure S1). The *dac* gene cluster contains homologs to numerous *oxy* and *ssf* genes that perform similar functions in the generation of the tetracycline scaffold. Unique tailoring genes are also present, in accordance with the structural features of **3**, including those encoding putatively a C7 halogenase DacE, a C4a monooxygenase DacO3, a C8O methyltransferase DacM3, a C6O glycosyltransferase DacS8 and a set of enzymes that synthesize the hydroxylamino deoxysugar. A proposed biosynthetic pathway of **3** is shown in Scheme 1C and Figure S2.

To transplant the *dac* pathway into K4-114, two plasmids encoding all the necessary genes proposed for the formation of **25** and overexpression of the SARP DacT1 (Figure S2) were constructed and transformed into K4-114 to generate K4/pDac-T1O3E. HPLC-MS analysis showed the emergence of two major compounds with same mass and UV absorption spectrum as **25** at a titer of ~1 mg/L (Figure 1E). Proton NMR analysis confirmed one of the compounds to be **25**, while the other compound was assigned to be the C4-epimer of **25**.^[34] The isolation of **25** confirms the role of the *dac* gene cluster in the biosynthesis of **3**, and affords a third set of enzymatic tools that can modify the tetracycline scaffold. This also represents the first report of reconstituting natural products originating from *Dactylosporangium* in *Streptomyces*.

In conclusion, we have reconstituted three tetracycline pathways in the genetically superior host *S. lividans* K4-114. Using the heterologous platforms and newly discovered tailoring enzymes, we will be able to complete our understanding of tetracycline biosynthesis, as well as to perform engineered biosynthesis of tetracycline analogs in an efficient manner.

Supplementary Material

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Figure 1.

HPLC profiles of extracts from cultures of different K4 strains. $\lambda=358$ nm (A, C, and E),
 $\lambda=430$ nm (B and D).

Figure 2.

HPLC profiles of extracts from cultures of K4-114 integrated with different knockouts of the *ssf* pathways. $\lambda=358$ nm (A–D and H), $\lambda=430$ nm (E–G and J–K), and $\lambda=400$ nm (I).

A

B

C

Scheme 1.

Biosynthetic pathways of (A) oxytetracycline; (B) SF2575; (C) Dactylocycline.