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Functional Characterization of the Genes *tauO*, *tauK*, and *tauI* in the Biosynthesis of Tautomycetin[§]

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Tautomycetin is a specific protein phosphatase I inhibitor. In an effort to elucidate the biosynthetic mechanism of tautomycetin, we inactivated genes of the tautomycetin biosynthetic gene cluster, *tauI*, *tauO*, and *tauK*, which encode for putative P450 oxidase, citryl-CoA lyase, and esterase enzymes, respectively. The mutant STQ0606 (ΔtauO) did not produce any detectable amount of tautomycetin intermediates but could convert dialkylmaleic anhydride to tautomycetin, strongly indicating that TauO was involved in dialkylmaleic anhydride biosynthesis. STQ1211 (*ΔtauK*) accumulated dialkylmaleic anhydride, whereas the cofermentation of STQ1211 (*ΔtauK*) and STQ0606 (*ΔtauO*) restored the production of tautomycetin. Together, these results suggest that TauK was responsible for the conjugation of dialkylmaleic anhydride and the polyketide moiety in tautomycetin biosynthesis. The disruption of *tauI* resulted in the accumulation of 5-des-keto-tautomycetin, revealing that TauI was responsible for the oxidation at C5 as the last step. Although the shunt pathways were involved in the biosynthesis of tautomycetin, the main post-polyketide synthase tailoring steps were dehydration, decarboxylation and oxidation, taking place consecutively. This study allowed us to predict the biosynthesis of tautomycetin more accurately and provided novel insights into the mechanism of the biosynthesis of tautomycetin.

Keywords: tautomycetin biosynthesis, gene inactivation, dialkymaleic anhydride, post-PKS modification

Introduction

Tautomycetin (TMC) is a specific protein phosphatase I inhibitor that plays an important role in the regulation of the cell cycle (Mitsuhashi *et al.*, 2001; Honkanen and Golden, 2002). TMC is first isolated from *Streptomyces griseochromogenes* (Cheng *et al.*, 1990), and the structure consists of a distinctive dialkylmaleic anhydride (DA) moiety joined to a long polyketide chain via an ester bond (Fig. 1). In addition to exhibiting a unique chemical structure, TMC possesses medicinally important activities, including antifungal, antitumor and immunosuppressive properties (Mitsuhashi *et al.*, 2001; Honkanen and Golden, 2002; Kim *et al.*, 2005), emphasizing the importance of the investigation of the TMC biosynthetic mechanism.

TMC is a dialkylmaleic anhydride antibiotic, the polyketide backbone of which is synthesized by type I polyketide synthase (PKS). The gene cluster involved in the biosynthesis of TMC has been reported (Choi *et al.*, 2007; Li *et al.*, 2009). Within the TMC biosynthetic gene cluster (Fig. 1), *tauA* and *tauB* are responsible for encoding type I PKS. TauCDFI are proposed to be four post-PKS tailoring enzymes; TauK is proposed to be the carboxyl-esterase that catalyzes the conjugation of DA and the polyketide moiety, and TauLMNOPRS is responsible for the biosynthesis of DA (Li *et al.*, 2009). Among the proposed enzymes for biosynthesis of DA, TauP, TauR, TauS, and TauM, have been characterized as the functional enzymes involved in the biosynthesis of TMC (Li *et al.*, 2009). However the function of TauO, a putative citryl-CoA lyase, has not been described.

Compared to the putative product of PKS, the structure of TMC has three functional groups, including a DA moiety,

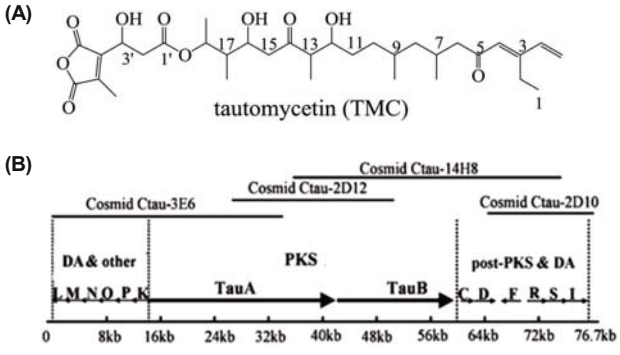

Fig. 1. The structure of TMC (A) and the organization of the primary genes in TMC biosynthesis gene cluster (B). (B) The 76.7 kb segment of TMC biosynthesis gene cluster from *S. griseochromogenes* chromogenes encodes two modular type I PKS (TauAB), four tailoring enzymes (TauCDFI) and DA biosynthesis & other enzymes (TauLMNOPRSK). The *tauA* gene encodes the loading module and extension module 1-5, whereas *tauB* encodes extension modules 6-9 with a C-terminal thioesterase domain for release of the full-length polyketide chain. Proposed founction of genes: *tauA*, PKS modules 0-5; *tauB*, PKS modules 6-9; *tauC*, Flavoprotein decarboxylase; *tauD*, UbiD family decarboxylase; *tauF*, Dehydrase; *tauI*, P450; *tauK*, Carboxylesterase; *tauM*, hydroxylase; *tauN*, unknown; *tauO*, Citryl-CoA lyase; *tauP*, Acyl-CoA transferase; *tauR*, dehydratase; *tauS*, unkown. And *tauI*, *tauO*, and *tauK* showed in magenta were inactivated here.

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a carbonyl group at the C5 position and a diene structure (Fig. 1). Therefore, the linear polyketide moiety should be subjected to several tailoring steps to produce an active TMC molecule, and this process should include the incorporation of the DA moiety and dehydration, decarboxylation, and oxidation at the C5 position. It has been reported that TauF is likely to perform the dehydration of the nascent polyketide, as indicated by the isolation of TTN F-1 from the ΔtauF mutant, and that TauD is directly responsible for the decarboxylation, as indicated by the isolation of TTN D-1 to TTN D-4 in the *ΔtauD* mutant (Luo *et al.*, 2010). However, the enzymes that catalyze the formation of the C5-ketone and ester bond linkage between DA and the linear polyketide moiety have not been verified; thus, the TMC biosynthesis pathway remains unclear. To resolve the functions of the candidate genes putatively responsible for the biosynthesis of TMC, the *tauI*, *tauK*, and *tauO* genes, which encode a cytochrome P450 oxidase (TauI), a carboxyl-esterase (TauK), and a citryl-CoA lyase (TauO), were inactivated. Using *aac(3)*IV/oriT gene cassette replacement via PCR targeting strategies (Gust *et al.*, 2003), we introduced constructs into *S. griseochromogenes* to replace the wild-type genes by sequential homologous recombination. The most prominent metabolites that accumulated in each mutant were then isolated and characterized. This study reports significant novel properties that aid in the further understanding of the TMC biosynthetic mechanism.

Materials and Methods

Materials

The cosmids Ctau-3E6 and Ctau-2D10 were used to knockout the genes *tauO*, *tauI* or *tauK*, which were cloned and characterized from *S. griseochromogenes* by Tang (Reid and Tang, 2005). We further analyzed the cosmids that contain the entire gene cluster of TMC biosynthesis, consistent with the data reported by Li *et al.* (2009). *Escherichia coli* (*E. coli*) BW25113/pIJ790 and *E. coli* DH5α/pIJ773 were provided by the John Innes Centre as part of the REDIRECT Technology kit (Gust *et al.*, 2003).

The fermentation medium (FM) consisted of 2% soluble starch, 1% glucose, 2.5% soybean powder, 0.4% dry yeast, 0.1% meat extract, 0.2% polypeptone, 0.5% $CaCO_3$, 0.2% NaCl, 0.005% K_2HPO_4, and 0.0001% $CoCl_2$, pH 7.0.

Conjugation procedure

Conjugation between *E. coli* and *S. griseochromogenes* was performed as previously described by Kieser *et al.* (2000), with modifications. *S. griseochromogenes* spores were subjected to heat shock at 45°C for 10 min, followed by incubation at 37°C for 2 h to allow the spores to germinate. The germinated spores were then mixed with the *E. coli* ET12567/pUZ8002/cosmid and spread onto modified MS plates freshly supplemented with 30 mM Mg^{2+}, 50 mM Ca^{2+}, and 0.2% glycine. The plates were incubated at 30°C for 16–18 h and then overlaid with apramycin and nalidixic acid to a final concentration of 33 μg/ml, and the plates were then incubated until exconjugants appeared.

Gene inactivation

TauO, *tauK*, and *tauI* were inactivated using REDIRECT Technology according to a previously described protocol (Gust *et al.*, 2003). Briefly, the apramycin resistance gene cassette for the replacement of target genes, *aac(3)*IV/oriT, was amplified from pIJ773 with the following primer pairs: IJKF/IJKR, IJOF/IJOR, and IJPF/IJPR (Supplementary data Table S1). The mutated cosmids, Ctau-3E6-O (*ΔtauO*), Ctau-3E6-K (*ΔtauK*), and Ctau-2D10-I (*ΔtauI*), were constructed and introduced into the wild-type *S. griseochromogenes* by conjugation. The desired double crossover mutants, selected by an apramycin-resistant and kanamycin-sensitive phenotype, were isolated as STQ0606 (*ΔtauO*), STQ1211 (*ΔtauK*), and STQ1010 (*ΔtauI*) by PCR analysis (Supplementary data Figs. S1–S3 and Table S2).

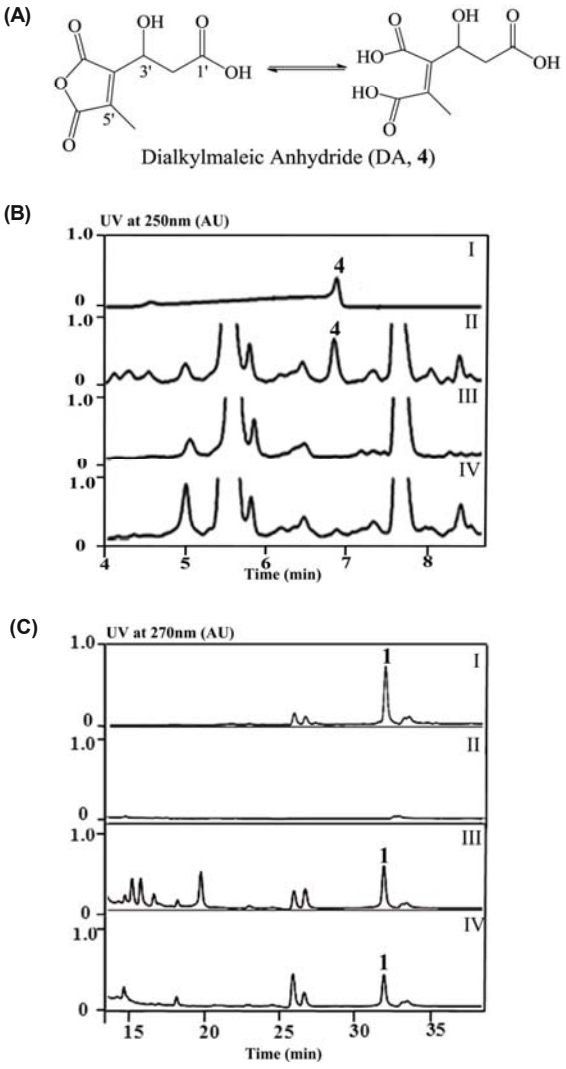

Fig. 2. The structure of DA (4) (A), the HPLC analysis of 4 (B) and the analysis of TMC from STQ0606 (*ΔtauO*) (C). B-I, DA control compound; B-II, STQ1211 (*ΔtauK*) fermentation broth; B-III, STQ0606 (*ΔtauO*) fermentation broth; B-IV, wild-type fermentation broth. C-I, wild-type; C-II, STQ0606 (*ΔtauO*); C-III, STQ06O (*tauO* complemented); C-IV, STQ0606 (*ΔtauO*) could transform DA to TMC.

To select the recombinant strains bearing the complementation plasmids construct, a plasmid pSET1521 was constructed by inserted a thiostrepton-resistance gene into the parental pSET152. The genes *tauO*, *tauK*, and *tauI* were amplified, digested with *Spe*I and *Eco*RI, and cloned into pSET1521 to yield pSET152O (for *tauO* expression), pSET152I (for *tauI* expression) and pSET152K (for *tauK* expression). Introduction of the expression constructs into the corresponding mutants by conjugation yielded strains STQ06O (*tauO*), STQ12K (*tauK*), and STQ10I (*tauI*). In these strains, the mutations of Δ*tauO*, *ΔtauK*, and *ΔtauI* were complemented by the constitutive expression of functional copies of *tauO*, *tauK*, and *tauI*, respectively, under the control of the *ErmE** promoter (Supplementary data Table S3).

Preparation of DA as a control compound

3-(4-methyl-2,5–dioxo-2,5-dihydrofuran) propanoic acid (**4,** DA) was produced by a chemical degradation method. A solution of 20% Cs_2CO_3 was added drop-wise (with stirring) to a solution of compound **1** in CH_3CN until it reached pH 10. It was then stirred at ambient temperature for several hours. The resulting solution was adjusted to pH 4.0 with 0.1 M HCl and then concentrated to remove the CH_3CN. The remaining aqueous solution was extracted with EtOAc and then evaporated to dryness under vacuum. The residue was subjected to semi-preparative chromatography to yield **4**, as monitored by UV detection at 250 nm, 270 nm, 285 nm, 235 nm and 220 nm (one representative peak is shown in Fig. 2B). The structure of **4** was validated by MS, ^{1}H-NMR and ^{13}C-NMR (Supplementary data Fig. S11, S12 and Table S5).

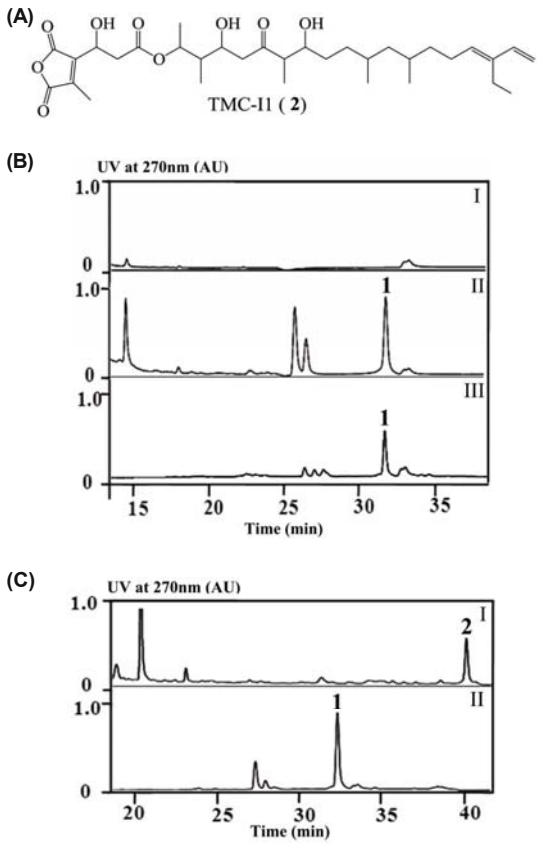

Fig. 3. The structure of 2 (A) and the HPLC analysis of TMC and 2 from STQ1211 (*ΔtauK*) (B) and STQ1010 (*ΔtauI*) (C). B-I, STQ1211 (*ΔtauK*); B-II, STQ12K (*tauK* complemented); B-III, mixed fermentation of STQ1211 (*ΔtauK*) and STQ0606 (*ΔtauO*). C-I, STQ1010 (*ΔtauI*); C-II, STQ10I (*ΔtauI* complemented).

Production and purification of TMC and analogs from wild-type and mutant strains

Wild-type *S. griseochromogenes* and STQ1010 (*ΔtauI*) were cultivated in FM medium. The fermentation broth, adjusted to pH 4.0 with 1 N HCl, was extracted twice with an equal volume of EtOAc and then evaporated to yield an oily residue. The oily residue was applied to a silica gel column (1.5 cm × 40 cm) and was eluted with a stepwise gradient consisting of $CHCl_3$-MeOH (100:1, 75:1, 50:1, 30:1, 20:1, and 10:1). The eluent of the TMC analogs was subjected to a final purification using semi-preparative chromatography (ZORBAX SB-C18, 250 mm × 9.4 mm, 5 μm).

For the preparation of the TMC analogs, the sample was subjected to HPLC analysis involving elution with a linear gradient from 20% to 80% CH_3CN/H_2O over 40 min, followed by 100% CH_3CN for 5 min at a flow rate of 1 ml/min. The eluent was monitored by UV detection at 250 nm, 270 nm, 285 nm, 235 nm, and 220 nm.

Three compounds were isolated: TMC (**1**) from the wild-type strain; **2** from STQ1010 (*ΔtauI*); and **3** from the biotransformation broth of STQ1010 (*ΔtauI*). The structures of **2** and **3** were characterized by MS and NMR analyses (Supplementary data Figs. S3 and S4).

Compound **2** had a molecular formula of $C_{33}H_{52}O_9$ and a mass of m/z 591.4201 ([M-H]$^-$), and it was 14 mass units less than compound **1**. The characteristic ^{1}H-NMR signal of the proton at C4 was a triplet at δH 5.40 (1H, t, J=7.3) instead of the singlet signal observed in **1**. The ^{13}C-NMR signal of the C5 carbon at δC 201.48 was not observed in **2**. Thus, C5 of **2** was a methylene group and not a ketone. The methylene group at C5 was deduced based on the NMR signals at δH 2.11 (2H, m) and δC 25.44. The other NMR signals were consistent with those of **1** and were assigned on the basis of 2D-NMR cross signals (Supplementary data Figs. S4–S8 and Table S4).

The molecular formula of **3**, $C_{33}H_{52}O_{10}$, was established from m/z 607.3538 ([M-H]$^-$), two mass units greater than that of **1**, and required one more oxygen atom than **2**. The only difference between **3** and **2** was that the characteristic ^{1}H-NMR signal of the proton of C4 is a doublet at δH 5.36 (1H, d, J=9.0) instead of the triplet singlet signal observed in **2** and the singlet signal in **1**. Thus, C5 was defined as a methine group. The oxygenation at C5 was deduced in view of the NMR signals at δH 4.56 (1H, dt, J=8.9, 6.7). However, the stereochemical configuration of C5 was not confirmed in the present study. The other NMR signals were consistent with those of **2** (Supplementary data Figs. S9–S10 and Table S4).

Biotransformation of intermediates by STQ1010 (*ΔtauI*) and STQ0606 (*ΔtauO*)

STQ1010 (*ΔtauI*) or STQ0606 (*ΔtauO*) was inoculated in

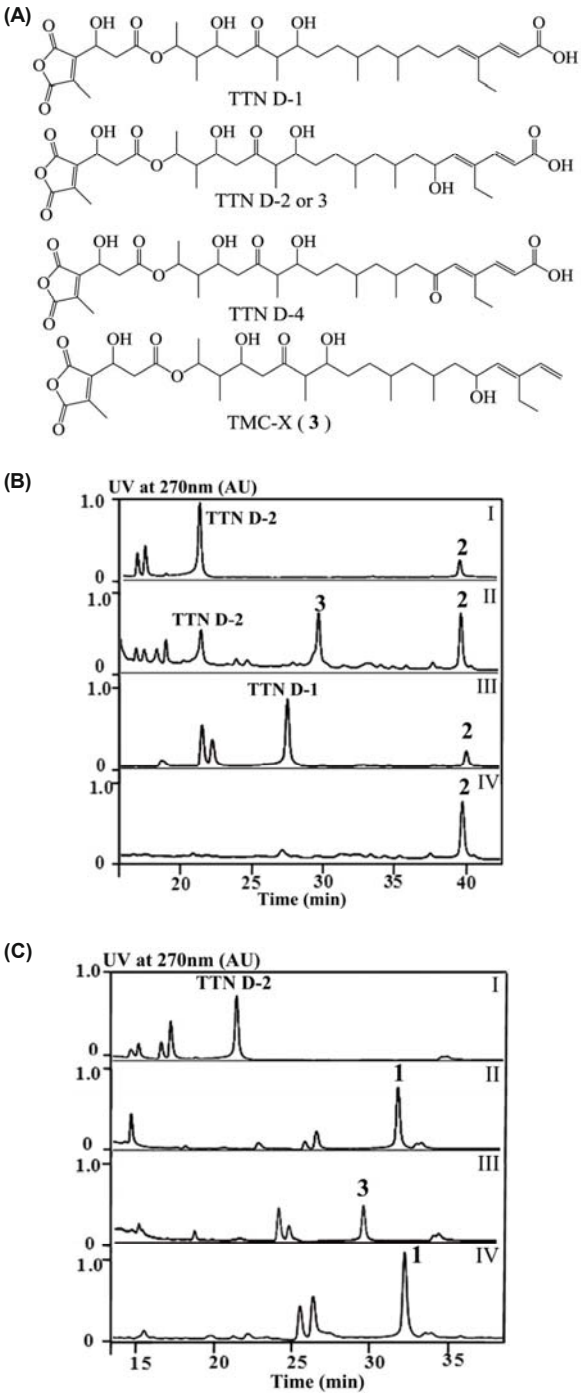

Fig. 4. **The structure of the TMC analogs (A) and the HPLC analysis of the biotransformation of TMC analogs by STQ1010 (*ΔtauI*) (B) or STQ0606 (*ΔtauO*) (C).** B-I, TTN D-2 added to STQ1010 (*ΔtauI*); B-II, TTN D-2 was converted to **3** by STQ1010 (*ΔtauI*); B-III, TTN D-1 added to STQ1010 (*ΔtauI*); B-IV, TTN D-1 was converted to **2** by STQ1010 (*ΔtauI*); C-I, TTN D-2 added to STQ0606 (*ΔtauO*); C-II, TTN D-2 was transformed to TMC by STQ0606 (*ΔtauO*); C-III, **3** added to STQ0606 (*ΔtauO*); c-IV, **3** was transformed to TMC by STQ0606 (*ΔtauO*).

50 ml FM medium and allowed to grow at 28°C with shaking at 250 rpm for 24 h. Approximately 5 mg of target compound, TTN D-1 or TTN D-2 or **3** was added to the fermentation broth, and the culture was grown for 24 h. The conversion of the target compound in the culture was monitored by HPLC and LC/MS.

Results

The preparation of cyclic C8 dialkylmaleic anhydride

According to the gene sequence analysis of the gene cluster for TMC biosynthesis, TauLMNOPRS plays an important role in the construction of DA (**4**). To determine the gene functions for DA biosynthesis, we adapted the chemical degradation method to prepare **4** as the control compound.

As there are two chemical forms of **4** under neutral conditions (Fig. 2), anhydride and diacide, we choose CH_3CN adjusted to pH 4.0 with 0.1 M HCl as the solution, thus avoiding the esterification of DA by methanol. Moreover, the analysis of **4** using HPLC was very difficult due to its high polarity. Subsequently, the analysis of **4** was subjected to HPLC analysis involved elution with a linear gradient from 5% to 80% CH_3CN/H_2O over 40 min at a flow rate of 0.8 ml/min.

The citryl-CoA lyase, TauO, is involved in the biosynthesis of dialkylmaleic anhydride

To understand the biological function of *tauO*, which encodes a citryl-CoA lyase, we first confirmed the inactivation of TauO in the STQ0606 (*ΔtauO*) mutant by an apramycin-resistant and kanamycin-sensitive phenotype. Furthermore, STQ0606 (*ΔtauO*) and the wild-type strain were cultivated under optimal conditions for TMC production; yet both of **4** and TMC were not found in the fermentation broth of STQ0606 (*ΔtauO*) (Fig. 2), indicating a key function for TauO in TMC biosynthesis. However, **4** can be biotransformed to TMC by STQ0606 (*ΔtauO*) at a high level (Fig. 2), indicating that the failure to produce TMC in STQ0606 (*ΔtauO*) was due to the lack of TauO-mediated production of **4**. Therefore, we concluded that TauO is involved in the biosynthesis of **4**.

The carboxylesterase, TauK, is involved in the incorporation of DA

To clarify the role of *tauK*, a carboxylesterase, in TMC biosynthesis, we obtained the desired mutant, STQ1211 (Δ*tauK*), by inactivating *tauK*, with a confirmed phenotype of apramycin resistance and kanamycin sensitivity. Although the analysis of the fermentation broth of STQ1211 (*ΔtauK*) detected a small amount of **4** (Fig. 3), it was insufficient to elucidate the structure.

Remarkably, TMC was produced at a significant level (Fig. 3) by the combined fermentation of STQ1211 (*ΔtauK*) and STQ0606 (*ΔtauO*), similar to the results of adding **4** to the culture of STQ0606 (*ΔtauO*). This suggested that STQ1211 (*ΔtauK*) accumulated **4** and that TauK was a functional enzyme in the biosynthesis of TMC.

Taken together, our data have demonstrated that the inactivation of *tauK* abolished the production of TMC but led to the accumulation of **4**. In other words, our data in-

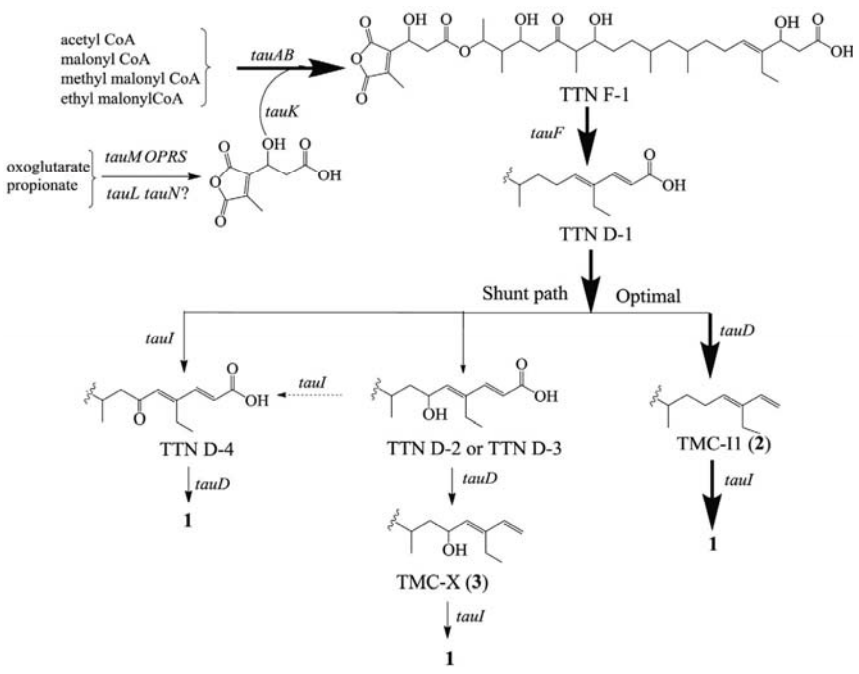

Fig. 5. Proposed biosynthesis of TMC, as predicted on the basis of metabolite 2 from ST1010 (Δ*tauI*), 4 from STQ0606 (Δ*tauO*) and the biotransformation experiment of the TMC analogs by ST1211 (Δ*tauK*) and STQ1010 (Δ*tauI*). TauMOPRS were characterized as the functional enzymes acting in biosynthesis of DA, which was coupled with a polyketide moiety by TauK; the putative TauL and TauN have not been characterized. The optimal post-PKS tailoring steps of TMC is in the scheme of TauF and TauD, followed by TauI for wild-type *S. griseochromogenes* (bold arrows in the figure). However, the accumulation of diverse metabolites by the knock-out mutant strains indicates a considerable degree of promiscuity of some of the post-PKS modification enzymes.

dicated a critical role for TauK in the enzyme-dependent events related to incorporation of DA with the polyketide moiety.

TauI as a post-PKS tailoring enzyme for the C5-ketone construction

The putative P450 enzyme, TauI, was inactivated to yield the desired mutant, STQ1010 (Δ*tauI*), with a confirmed apramycin-resistant and kanamycin-sensitive phenotype. STQ1010 (Δ*tauI*) and the wild-type strain were cultivated under the same conditions, and, as shown in Fig. 3, STQ1010 (Δ*tauI*) failed to produce **1** but rather produced a distinct compound, 5-des-keto-TMC (**2**), which lacked the C5 ketone moiety found in **1**.

Compared to TMC, from a structural perspective, **2** has a DA moiety and a diene group without the C5-ketone group. This indicates that **2** has been dehydrated and decarboxylated by the post-PKS enzymes, TauF and TauD. Consequently, the inactivation of TauI does not have a significant impact on the activity of TauD and TauF. Therefore, we have demonstrated that TauI, a cytochrome P450 oxidase, is responsible for the C5 oxidation that is the last step of the post-PKS tailoring during the biosynthesis of TMC.

The C5-hydroxyl in TMC analogs is transformed to C5-ketone via oxidation by TauI

Analogous to the method reported by Luo *et al.* (2010), we have also isolated and prepared a TauD mutant to produce analogs TTN D-1~TTN D-4 (Fig. 4). To probe the relationship between the C5-hydroxyl group of TTN D-2 and the C5-ketone group of TMC, the biotransformation of TTN D-2 by STQ1010 (Δ*tauI*) was performed, with the biotransformation of TTN D-1 as a control. A very interesting observation was made where TTN D-2 was transformed by STQ1010 (Δ*tauI*) to an analog, TMC-X (**3**), instead of TMC. Similar to TTN D-2, **3** maintains a hydroxyl group at C5. Furthermore, both TTN D-2 and **3** were converted to TMC by STQ0606 (Δ*tauO*), which had an active TauI. These results strongly suggest that the methine or methylene group at C5 in the TMC analogs could be oxidized to form a ketone bond (Fig. 4) when the P450 enzyme, TauI, is active. In addition, TMC-producing strain of *S. griseochromogenes* might possess a shunt pathway to synthesize TMC (Fig. 5).

Rescue of TMC production by TauK, TauI, and TauO complementation

To verify that TauK, TauO, and TauI were truly responsible for TMC production, we performed genetic complementation of *S. griseochromogenes* by expressing *tauO* or *tauI* or *tauK* under the control of the *ErmE** promoter. For this purpose, we used the integrating conjugative vector, pSET1521, into which the coding region of *tauI* or *tauO* or *tauK* was cloned, resulting in pSET152I or pSET152O or pSET152K. These plasmids were used to transform the corresponding mutant strain, STQ1010 (Δ*tauI*), STQ0606 (Δ*tauO*), or STQ1211 (Δ*tauK*), to select the thiostrepton-resistant complemented strains, STQ10I, STQ06O, and STQ12K. Both HPLC and LC/MS confirmed that TMC production was restored to significant levels (Figs. 2 and 3). Our results indicate that the absence of TMC production by STQ1010 (Δ*tauI*) was due to the lack of a TMC-specific oxidation enzyme, TauI, whereas STQ0606 (Δ*tauO*) lacked TauO and STQ1211 (Δ*tauK*) lacked TauK.

Discussion

The polyketide chain of TMC has two different groups that

are absent from the molecule just liberated from PKS, including a carbonyl group at the C-5 position and the terminal diene structure. The nascent polyketide needs to undergo the following three post-PKS steps to complete the synthesis of TMC: 1) dehydration to incorporate the carboxyl-diene; 2) decarboxylation to form the terminal diene; and 3) oxidation at C5-site for the establishment of the ketone oxidation state.

It was proposed that the biosynthetic pathway of TMC invokes the sequential actions of dehydration (TauF), oxidation (TauI) and decarboxylation (TauD or TauC). Concerning the function of the two candidate decarboxylase genes, *tauC* and *tauD*, in the TMC gene cluster, the inactivation of *tauC*, which encodes a flavoprotein decarboxylase, resulted in the production of TMC at the wild-type levels (unshown in the paper), whereas the inactivation of *tauD*, an UbiD family decarboxylase, resulted in the accumulation of TTN D-1 to TTN-D4 (Luo *et al*., 2010). This observation identified TauD as the decarboxylase for the post-PKS modification steps in TMC biosynthesis, whereas the function of TauC remains unknown. Based on the accumulation of TTN F-1 by the *ΔtauF* mutant strain, TauF, as a dehydratase, is responsible for the incorporation of the carboxyl-diene and TauD alone is not sufficient to realize the decarboxylation step (Luo *et al*., 2010). And we also isolated the new analogs TTN D-1 to TTN D-4 from *ΔtauD* mutant and TTN F-1 from *ΔtauF* mutant. Based on the accumulation of TTN D-2 to TTN D-4, Luo have proposed two putative pathways for the biosynthesis of TMC and concluded that the C5-hydroxyl group might be formed by the reduction of the C5-ketone (Luo *et al*., 2010).

So the function of TauI and the role of TTN D-2 or TTN D-3 in the biosynthesis of TMC in the biosynthesis of TMC still need to be verified.

The function of the putative oxidase, TauI, was evaluated using a gene inactivation strategy, followed by the examination of metabolites and complementation of the mutant. The disruption of *tauI* abolished the production of TMC, showing that TauI plays an essential role as a post-PKS modification enzyme in TMC biosynthesis. Our results also showed that STQ1010 (*ΔtauI*) predominantly accumulated **2** alone (Fig. 3). However, no trace of any compound with an acrylic acid moiety attached to C3, as described in the TauD mutant (Luo *et al*., 2010), was found in STQ1010 (*ΔtauI*), indicating that the oxidation at C5 to form a ketone may be the last step of TMC biosynthesis. The non-oxidation of C5 does not have a significant impact on the activity of the TauD enzyme, whereas the abolishment of the decarboxylation in TMC biosynthesis has a significant impact on the activity of the TauI enzyme (Luo *et al*., 2010). These results indicated that TauI was involved in the post-PKS modifications during the late stage of TMC biosynthesis.

Regarding the shunt pathway for TTN D-2 described by Luo *et al*. (2010), we suggest that uncertainties remain and that these uncertainties will cause concerns about the results of biotransformation experiments of TTN D-2 by STQ1010 (*ΔtauI*) or STQ0606 (*ΔtauO*). TTN D-2 was biotransformed to **3,** which retains the C5-hydroxyl group, showing that the methine or methylene group at C5 was not converted when TauI was inactive. However, both **3** and TTN D-2 were transformed to TMC by STQ0606 (*ΔtauO*), in which TauI was active, indicating that the C5-hydroxyl in the TMC analogs could be oxidized to C5-ketone by TauI. Thus, there is a considerable degree of promiscuity of some of the post-PKS modification enzymes in the biosynthesis of TMC in *S. griseochromogenes* (Fig. 5). Furthermore, the shunt pathway may act in the mutant strain, but this pathway is different from the shunt pathway described by Luo *et al*. (2010). Nevertheless, in wild-type *S. griseochromogenes*, the consecutive post-PKS tailoring steps of TMC appear to be dehydration (TauF), decarboxylation (TauD), and oxidation (TauI) (bold arrows in Fig. 5).

The biosynthesis of TMC consists of the polyketide moiety by PKS, the post-PKS tailoring and the construction of DA. Based on prior experiments (Ubukata *et al*., 1995) using ^{13}C-labeled precursors, DA was determined to be derived from α-ketoglutarate and propionate of the Krebs cycle during TMC biosynthesis. The prevailing hypothesis is that TauLMNOPRS is responsible for the construction of **4**. However, the detailed enzymatic mechanism to produce **4** remains unclear. Recently, it was reported that TauP, TauR, and TauS have been elucidated as the functional enzymes for the biosynthesis of TMC (Li *et al*., 2009). Moreover, the ΔtauM mutant accumulated 3'-dehydroxy TMC, showing that TauM was responsible for the hydroxylation of C3' (Li *et al*., 2009).

However, the mechanism associated with the biosynthesis of DA remains unclear and there is a strong need to identify the biological function of TauO and TauK during TMC biosynthesis. Therefore, we have assessed the function of the putative citryl-CoA lyase, TauO, and the putative carboxylesterase, TauK, by a gene inactivation strategy. STQ0606 (*ΔtauO*) failed to produce any detectable DA and TMC intermediates. Thus, the results implicated that TauO was a key enzyme for the biosynthesis of TMC. In addition, the biotransformation of **4** by STQ0606 (*ΔtauO*) convinced us that TauO was really involved in the biosynthesis of DA. Interestingly, we have found that STQ1211 (*ΔtauK*) could not produce TMC and only minor amounts of DA. Furthermore, the combined fermentation (Pettit, 2009) of STQ1211 (*ΔtauK*) and STQ0606 (*ΔtauO*) resulted in TMC reproduction, consistent with the biotransformation of DA by STQ0606 (*ΔtauO*). These data suggested that STQ1211 (*ΔtauK*) actually accumulated DA for the TMC biosynthesis. More importantly, TauK was a key enzyme in the biosynthesis of TMC but not for the biosynthesis of DA and the inactivation of TauK led to the failure of DA linkage with the polyketide moiety.

In summary, our work has demonstrated that TauO plays an important role in the biosynthesis of DA and that TauK is crucial in the enzyme-dependent events related to the biosynthesis of TMC. Unfortunately, the putative biological function of the TauL and TauN enzymes remains unclear. Recently, Ju's group has determined that the anhydride-to-polyketide coupling step occurs prior to the release of the polyketide from polyketide synthase (Ju *et al*., 2009). However, the detailed mechanism of the incorporation of **4** with the polyketide moiety remains debatable and warrants further investigation.

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