Isolation of the biosynthetic gene cluster for tautomycetin, a linear polyketide T cell-specific immunomodulator from *Streptomyces* sp. CK4412

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The bacterial genus *Streptomyces* has long been appreciated for its ability to produce various kinds of medically important secondary metabolites, such as antibiotics, anti-tumour agents, immunosuppressants and enzyme inhibitors. Tautomycetin (TMC), which is produced by *Streptomyces* sp. CK4412, is a novel activated T cell-specific immunosuppressive compound with an ester bond linkage between a terminal cyclic anhydride moiety and a linear polyketide chain bearing an unusual terminal alkene. Using a *Streptomyces* polyketide methylmalonyl-CoA acyltransferase gene as a probe, three overlapping cosmids were isolated from the genomic library of TMC-producing *Streptomyces* sp. CK4412. Sequence information of an approximately 70 kb contiguous DNA region revealed two multi-modular type I polyketide synthases (PKSs), and 12 additional gene products presumably involved in TMC biosynthesis. The deduced roles for most of the TMC PKS catalytic domains were consistent with the expected functions necessary for TMC chain elongation and processing. In addition, disruption of a putative TMC acyl-CoA transferase gene, located upstream of the PKS gene locus, completely abolished TMC biosynthesis. Taken together, these data provide strong supporting evidence that the cloned gene cluster identified in this study is responsible for TMC biosynthesis in *Streptomyces* sp. CK4412, and set the stage for detailed genetic and biochemical studies of the biosynthesis of this important metabolite.

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INTRODUCTION

The bacterial genus *Streptomyces* is widely known for its ability to produce a variety of secondary metabolites, including valuable antibiotics, anti-tumour agents, immunosuppressants and enzyme inhibitors (Chater 1990; Hopwood, 1987; Strauch *et al.*, 1991; Myles, 2003; Hranueli *et al.*, 2005). A secondary metabolite produced by *Streptomyces* sp. CK4412, originally isolated in Jeju Island, Korea, has been identified as an activated T cell-specific immunosuppressive compound with a novel mode of pharmacological action, both *in vivo* and *in vitro*, and

whose chemical structure has been shown to be identical to that of a previously reported antifungal compound, tautomycetin (TMC), produced by *Streptomyces griseochromogenes*. (Cheng *et al.*, 1989; Fig. 1). Inhibition of T cell proliferation with TMC has been observed at concentrations 100-fold lower than those needed to achieve maximal inhibition with cyclosporin A (CsA) (Shim *et al.*, 2002). TMC is believed to specifically block tyrosine phosphorylation of intracellular signal mediators downstream of Src tyrosine kinases in a T cell-specific manner, leading to apoptosis due to cleavage of Bcl-2, caspase-9, caspase-3 and poly(ADP-ribose) polymerase, but not caspase-1 (Shim *et al.*, 2002). Thus, it has been proposed that TMC, whose mechanism of action is different from that of CsA or FK506, is a novel, potent, T cell-specific immunosuppressive agent (Shim *et al.*, 2002).

TMC is classified as a type I polyketide-derived metabolite based on its chemical structure, specifically the presence of a linear branched-chain fatty acid-like moiety (Fig. 1). The genes, enzymes and mechanism for type I polyketide biosynthesis have been thoroughly reviewed (Katz & Donadio, 1993; Rawlings, 2001; Floss, 2006), and this has highlighted the enzyme complexes known as modular polyketide synthases (PKSs). The catalytic sites of type I

Abbreviations: ACP, acyl carrier protein; AT, acyl transferase; CsA, cyclosporin A; DH, dehydratase; eAT, ethylmalonyl-AT; ER, enoyl reductase; KR, β -ketoacyl reductase; KS, β -ketoacyl synthase; mAT, malonyl-specific AT; mmAT, methylmalonyl-specific AT; PKS, polyketide synthase; TE, thioesterase; TMC, tautomycetin.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is DQ983361.

A multiple sequence alignment using CLUSTALW of the substrate-specificity motifs of the AT domains of 10 modules from *tmcA* and *tmcB*, and sequence alignments between the key motifs of deduced TMC gene products and the conserved motifs of several PKS domains, are available as supplementary data with the online version of this paper.

Fig. 1. Structure of TMC.

PKSs are encoded by a series of catalytic domains within large multifunctional proteins. A simple carboxylic acid starter unit including acetyl-CoA or propionyl-CoA is typically transferred to the cysteine active site of a β -ketoacyl synthase (KS) in the PKS complex. A specific extender unit determined by a unique acyl transferase (AT) domain in the PKS complex, such as a malonyl-CoA, methylmalonyl-CoA or ethylmalonyl-CoA, is transferred to the thiol group of the phosphopantetheine arm of an adjacent acyl carrier protein (ACP). These subunits are joined by a decarboxylative condensation catalysed by KS and remain covalently attached to ACP, followed by a partial or full series of keto group processing reactions, including β -ketoacyl reductase (KR), dehydratase (DH) and enoyl reductase (ER). The growing chain is transferred from the ACP to a downstream KS, and further rounds of elongation and processing occur to achieve the full-length polyketide chain. The completed chain is typically released from the PKS by a terminal thioesterase (TE) to form a macrocyclic lactone ring or a linear product.

In many modular PKS systems, the arrangement of specific catalytic domains encoded in the PKS gene cluster is usually well correlated to the final structure of the corresponding polyketide compound. This collinearity of DNA sequence information from the PKS gene is usually sufficient to predict the final structure of the corresponding polyketide metabolite. However, despite the power of bioinformatic analysis in secondary metabolism, the isolation and characterization of key biosynthetic genes and enzymes is essential to understand the details of natural product assembly and tailoring. This information also provides opportunities to develop new natural product derivatives through combinatorial biosynthesis, chemoenzymic synthesis or heterologous expression approaches (Floss, 2006; Grunewald & Marahiel, 2006; Wenzel & Muller, 2005). The unique chemical structure of TMC, which includes an ester bond linkage between a cyclic C8 dialkylmaleic anhydride at one terminus, and a linear polyketide chain bearing a terminal alkene at the other, indicates that the corresponding biosynthetic pathway features a number of unique biochemical steps with significant potential for generating novel TMC derivatives (Li *et al.*, 2006). Specifically, the genes and enzymes that specify biosynthesis of the cyclic C8 dialkylmaleic anhydride and subsequent linkage with the linear polyketide have not yet been investigated. Moreover, unlike most macrolactonization reactions catalysed by PKS TE domains, the TMC polyketide moiety is presumably released as a linear chain following a

putative decarboxylative dehydration resulting in a terminal alkene residue.

Here we report the isolation and initial characterization of the TMC biosynthetic gene cluster. Using a type I PKS methylmalonyl-CoA AT-specific PCR screening strategy, three overlapping cosmids were isolated as a contiguous 110 kb sequence from a genomic DNA library of the TMC-producing strain *Streptomyces* sp. CK4412. Complete sequencing of an approximately 70 kb DNA region and subsequent bioinformatic analysis revealed two putative type I PKSs and 12 additional gene products, presumably involved in TMC biosynthesis. Most of the deduced functions of TMC PKS domains correlate well with their expected roles in TMC polyketide backbone biosynthesis. In addition, disruption of a putative TMC acyl-CoA transferase gene, located just upstream of the PKS gene in the *tmc* cluster, completely abolished natural product biosynthesis. Taken together, these results demonstrate that the cloned gene cluster identified in this study is responsible for TMC biosynthesis in *Streptomyces* sp. CK4412.

METHODS

Bacterial strains and culture conditions. *Streptomyces* sp. CK4412, a TMC-producing strain (Shim *et al.*, 2002), was kindly provided by ForHumanTech Ltd and used as the source of DNA for the construction of the genomic DNA library. The strain was cultivated at 28 °C in either R2YE or YEME liquid medium (Kieser *et al.*, 2000). *Escherichia coli* DH5 α strain was used for DNA cloning and plasmid propagation. *E. coli* XL-1 Blue MR strain was used for cosmid library construction. *E. coli* ET12567/pUZ8002 (*dam*⁻, *dcm*⁻, *hrdM*) was used as the transient host for *E. coli*-*Streptomyces* conjugation. All *E. coli* strains were cultured at 37 °C in Luria broth or on Luria agar, supplemented with the appropriate antibiotics when needed (Kieser *et al.*, 2000).

Cloning and sequence analysis of the TMC gene cluster.

Total genomic DNA of *Streptomyces* sp. CK4412 grown on YEME medium was prepared by lysozyme digestion, phenol/chloroform/isoamyl alcohol extraction (25:24:1) and spooling from ethanol, as described by Kieser *et al.* (2000). A cosmid library was prepared using *Streptomyces* sp. CK4412 genomic DNA partially digested with *Sau*3AI and a commercially available Supercos-1 cosmid system (Stratagene), according to the manufacturer's protocol. The cosmid library was then screened by PCR using a type I PKS-specific primer pair. The PCR primer pair [forward primer 5'-TS(C/G)AAGTCS(C/G)AACATCGGB(C/G/T)CA-3' and reverse primer 5'-CGCAGG-TTS(C/G)CS(C/G)GTACCAGTA-3'] was designed based on the conserved sequences found in a KS domain and a methylmalonyl AT domain of type I PKS genes (Ayuso-Sacido & Genilloud, 2005). PCR was performed in a final volume of 20 μ l containing 0.4 μ M each primer, 0.25 mM of each of the four dNTPs (Roche), 1 μ l extracted DNA, 1 U *Ex Taq* polymerase (TaKaRa) with its recommended reaction buffer, and 10% DMSO. Subsequent amplifications were then performed in a Rapid Cycler (Idaho Technology), according to the following profile: 30 cycles of 30 s at 95 °C, 30 s at 48 °C and 40 s at 68 °C. Amplification products were analysed by electrophoresis in 1% (w/v) agarose gels and verified by sequencing using T7 promoter primer/T3 primer in a pSupercos-1 vector. Complete sequencing of the three positive cosmid clones was performed at Genotech. DNA sequences were assembled using BLAST searches on the National Center for Biotechnology Information (NCBI) server, and

also analysed using the web-based program Frameplot 2.3.2 (<http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl>). In addition, PKS amino acid sequence domain data were analysed using the Modular Polyketide Synthase Database (<http://linux1.nii.res.in/~pkssdb/DBASE/page.html>).

Inactivation of a putative TMC biosynthetic gene. The *tmcD* biosynthetic gene encoding a putative acyl-CoA transferase located upstream of the PKS *tmcA* gene was inactivated using a PCR-targeted gene-disruption system (Gust *et al.*, 2003). An apramycin-resistance gene/*oriT* cassette for the replacement of *tmcD* was amplified using the following primers: TFredF (5'-tcgtcggtgctcatgctcggtctctcctgtgaattcctcaATTCCGGGGATCCGTCGACC-3') and TFredR (5'-ttcggtcacgcccagaacgacgtctcgtgctgtggaagggtGTAGGCTGGAGC-TGCTTC-3'). Lower-case type represents 39 nt homologous extensions to the DNA regions inside *tmcD*. This cassette was introduced into *E. coli* BW25113/pIJ790 containing pTMC2982. The gene replacement in *tmcD* was confirmed by restriction analysis of the mutated pTMC2982 (pTMC2982Δ*tmcD*). pTMC2982Δ*tmcD* was introduced into *Streptomyces* sp. CK4412 by conjugation from *E. coli* ET12567/pUZ8002. After incubation at 28 °C for 16 h, each plate was overlaid with 1 ml sterile water containing apramycin at a final concentration of 50 µg ml⁻¹ and nalidixic acid at a final concentration of 25 µg ml⁻¹. Incubation continued at 28 °C until conjugants appeared. The double-crossover recombinants were first selected by PCR and then confirmed by Southern blot hybridization of *Streptomyces* sp. CK4412 genomic DNA.

HPLC quantification and antifungal bioassay for TMC. For HPLC analysis, culture broth supernatants were extracted with equal volumes of chloroform. The extracts were dried using a rotoevaporator and then resuspended in methanol. Extracts were fractionated by HPLC using isocratic conditions of methanol:water:buffer (1 % diethylamine/formic acid, pH 7.3) (75:15:10) on a Genesis C18 4 µm column with UV detection at 273 nm. TMC production was also evaluated by biological assay against *Aspergillus niger* as an indicator using the agar-plug diffusion method (Isaacson & Kirschbaum, 1986). The agar plug from the 7-day-old *Streptomyces* solid agar culture was placed on top of *A. niger* that had been incubated on GY medium for 6 h at 30 °C, and this was followed by the measurement of the inhibition zone after overnight incubation at 30 °C.

RESULTS AND DISCUSSION

Cloning and sequencing of the TMC biosynthetic gene cluster

To isolate the *tmc* biosynthetic gene cluster, a total genomic DNA library from the TMC-producing *Streptomyces* sp. CK4412 was constructed. Due to the absence of genetic information for biosynthesis of the cyclic anhydride of TMC, the highly conserved PKS KS-AT regions were chosen to develop PCR primers for cosmid library screening. TMC has several branched methyl groups along the linear polyketide chain, so an AT-specific PCR primer was designed to hybridize to the methylmalonyl-CoA-specific AT sequences. After screening >1000 cosmids, several candidates were initially identified that contained PKS gene-homologous sequences. After further analysis, a single cosmid (pTMC2290) was developed for additional library screening and provided two additional overlapping cosmids (pTMC2395 and pTMC2982). Complete sequencing of cosmid pTMC2395 and both of its flanking regions (a total of 70 kb contiguous DNA) revealed two ORFs encoding a

typical modular PKS gene as well as 12 ORFs located on both flanking regions, whose deduced functions were consistent with TMC biosynthesis (Table 1, Fig. 2). Although the 70 kb contiguous DNA cluster appears to contain most of the genes involved in TMC biosynthesis, we cannot rule out the possibility that additional genes are necessary for TMC biosynthesis.

Overall organization of the TMC-encoded PKS genes and catalytic domains

Biosynthesis of the TMC polyketide moiety is believed to be catalysed by two ORFs (*tmcA* and *tmcB*) that encode multi-modular PKSs. Both *tmcA* and *tmcB*, organized in the same transcriptional direction, have a total of 10 PKS modules: six modules including a loading module in *tmcA*, and four modules in *tmcB*. Within the 10 PKS modules are various functional domains, including 10 KS domains, seven DH domains, 10 AT domains, three ER domains, 10 ACP domains and one TE domain (Table 2). All active-site motifs within individual catalytic domains were highly conserved in each of the 10 TMC PKS modules (Zirkle *et al.*, 2004). TE was found to be the final catalytic domain encoded by *tmcB*. TmcB TE contains the highly conserved sequence GxSxG and GdH motifs (Zirkle *et al.*, 2004), and is presumed to play a key role in the formation of the terminal alkene residue of TMC. Interestingly, several marine cyanobacterial natural products have been characterized that contain terminal alkene residues, including kalkitoxin (Wu *et al.*, 2000), somocystinamide (Nogle & Gerwick, 2002) and the linear polyketide curacin A (William *et al.*, 1994). Although there is limited amino acid sequence similarity between the terminal module of the curacin A biosynthetic pathway (CurM) and TmcB module 10, it is possible that the terminal decarboxylative dehydration of the linear TMC polyketide operates in a manner analogous to that recently proposed for the curacin A produced by *Lyngbya majuscula* (Chang *et al.*, 2004). In the unique polyketide termination catalysed by TmcB module 10, the TMC TE domain is presumed to catalyse hydrolysis of the final chain-elongation intermediate to the corresponding carboxylic acid. Whether TMC TE is responsible for the additional steps resulting in terminal alkene formation, or whether a separate decarboxylase and dehydratase are involved, remains unclear. Detailed biochemical studies will be required to dissect the formation of this unusual terminal functional group.

Organization of AT domains in TMC PKS genes

All 10 AT domains in TMC PKS modules possess the expected set of conserved consensus sequences. It has been reported that the conserved amino acid sequence pattern in AT domains determines the extender unit specificity such as malonyl-specific AT (mAT), methylmalonyl-specific AT (mmAT) and ethylmalonyl-AT (eAT) (Schwecke *et al.*, 1995; Haydock *et al.*, 1995; Del Vecchio *et al.*, 2003). Based on the analysis of conserved sequence patterns, all 10 AT domains in TMC PKS genes were classified: AT domains in

Table 1. Deduced functions of ORFs identified in TMC gene cluster

ORF	Accession no. of best match	Identity (%)	Species	Proposed function
<i>tmcA</i>			<i>Streptomyces</i> sp.	Type I PKS
<i>tmcB</i>			<i>Streptomyces</i> sp.	Type I PKS
<i>tmcC</i>	CAB93516	28	<i>Bacillus</i> sp. BP-7	Type B carboxylesterase
	BAD77334	26	<i>Geobacillus kaustophilus</i>	Thermostable carboxylesterase
<i>tmcD</i>	YP_440415	60	<i>Burkholderia thailandensis</i>	CAIB/BAIF family protein
	ZP_00492720	60	<i>Burkholderia pseudomallei</i>	Predicted acyl-CoA transferase/carnitine dehydratase
<i>tmcE</i>	ZP_00492721	44	<i>B. pseudomallei</i>	Citrate lyase beta subunit
	YP_440414	44	<i>B. thailandensis</i>	Probable citrate lyase beta subunit
<i>tmcF</i>	ZP_00567646	47	<i>Frankia</i> sp. EAN1pec	Hypothetical protein (AMP-binding protein)
	CAG75597	41	<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> SCRI 1043	Putative phenazine antibiotic biosynthesis protein
<i>tmcG</i>	YP_480914	28	<i>Frankia</i> sp. CcI3	Hypothetical protein Francci3_1809
	AAR38130	13	Uncultured bacterium 578	Gamma-butyrobetaine, 2-oxoglutarate dioxygenase
<i>tmcH</i>	AAL17933	41	<i>Mycobacterium smegmatis</i>	Hypothetical protein
	YP_412473	28	<i>Nitrosospora multiformis</i>	YbhB and YbcL
<i>tmcI</i>	BAD57584	34	<i>Nocardia farcinica</i>	Hypothetical protein
	AAU34200	33	<i>Streptomyces hygroscopicus</i>	Polyprenyl phosphomannosyltransferase MppI
<i>tmcJ</i>	CAD19469	63	<i>Kluyvera cryocrescens</i>	Putative Pad1 protein
	CAD06027	62	<i>Salmonella enterica</i>	Possible decarboxylase
<i>tmcK</i>	ZP_00140684	37	<i>Pseudomonas aeruginosa</i>	3-Polyprenyl-4-hydroxybenzoate decarboxylase and related decarboxylases
	XP_718068	31	<i>Candida albicans</i>	Carboxylase UbiD-like protein
<i>tmcL</i>	AAR16523	74	<i>Streptomyces diastaticus</i>	RimJ
	ZP_00569161	75	<i>Frankia</i> sp. EAN1pec	Crotonyl-CoA reductase
<i>tmcM</i>	CAD16864	36	<i>Ralstonia solanacearum</i>	Hypothetical protein
	ZP_00946460	37	<i>R. solanacearum</i>	CaiB, predicted acyl-CoA transferase/carnitine dehydratase
<i>tmcN</i>	AAD28307	32	<i>Rhodococcus erythropolis</i>	ThcG
	ZP_00566813	34	<i>Frankia</i> sp. EAN1pec	Regulatory protein, LuxR

modules 1, 3, 5, 8 and 10 as malonyl extenders; AT domains in modules 2, 4, 6 and 7 as methylmalonyl extenders; and the AT domain in module 9 as an ethylmalonyl extender unit (Supplementary Fig. S1A). The order of AT domains in the TMC PKS was exactly matched with the structure of the expected extender unit for the corresponding TMC polyketide (Supplementary Fig. S1B). Although the AT domain in module 9 showed significant overall homology with mmATs, it is believed to load an ethylmalonyl-CoA, because the active-site recognition sequences (SVATH)

are unique in comparison to those of mmATs (DYASH) (Supplementary Fig. S1A). Moreover, most mmATs closely resemble one another (Wu *et al.*, 2000).

Organization of DH/ER/KR domains in TMC PKS genes

Within 10 TMC PKS modules, seven DH domains, three ER domains and nine KR domains were identified. All the TMC DH domains exhibited 57–76 % amino acid homology

Fig. 2. Distribution of sequenced cosmids and organization of the TMC biosynthetic gene cluster in the sequenced ~70 kb DNA fragment. The upper portion shows the entire cluster, and the lower portion shows the flanking regions. The orientation of the arrows indicates the direction of transcription; the length indicates the approximate size of the ORF. Letters within arrows indicate the ORFs in Table 1.

Table 2. Sequence coordinates of the TMC PKS gene cluster

ORF	Proposed function of sequence similarities detected					
<i>tmcA</i>						
Module 1 (loading)	KS	mAT				ACP
Module 2	KS	mmAT	DH*		KR	ACP
Module 3	KS	mAT			KR	ACP
Module 4	KS	mmAT	DH*		KR*	ACP
Module 5	KS	mAT	DH*		KR	ACP
Module 6	KS	mmAT	DH	ER	KR	ACP
<i>tmcB</i>						
Module 7	KS	mmAT	DH	ER	KR	ACP
Module 8	KS	mAT	DH	ER	KR	ACP
Module 9	KS	eAT	DH		KR	ACP
Module 10	KS	mAT			KR	ACP
						TE

*Predicted to be inactive.

to each other. Unlike a typical DH active-site motif LXX-HXXXGXXXXP (Zirkle *et al.*, 2004), however, the two DH domains in TMC PKS modules were predicted to be inactive due to aberrant active-site sequences, including LXXPXX-GXXXXP in TmcA DH1 and LXXYXXXGXXXXP in TmcA DH2 (Supplementary Fig. S2A). Although TmcA DH3 in module 5 seems to be functional, based on active-site-motif sequence alignment, this domain might also be non-functional, based on the absence of a corresponding double bond in the final TMC structure. A similar discrepancy regarding sequence-based structure prediction has also been reported in other DH domains in PKS systems (Aparicio *et al.*, 1996).

The three ER domains in TMC PKS modules showed 64–74 % amino acid homology to each other, and contained the conserved active motif GGVGXAAXQXA (Supplementary Fig. S2B). All nine KR domains in TMC PKS modules showed the conserved active site motifs GXGXX-G(A)XXXA and LXS(G)RXG(T,A). The TmcA KR3 domain in module 4, which was significantly different from other KRs, seemed to be inactive due to a 16-amino-acid deletion in the catalytic domain (Keatinge-Clay & Stroud, 2006; Supplementary Fig. S2C).

TMC genes involved in post-PKS tailoring functions

There are seven ORFs organized as a transcriptional unit located upstream of *tmcA*. The deduced amino acid sequences showed significant similarity to enzymes that catalyse tailoring functions expected in TMC biosynthesis, including a carboxylesterase and citrate lyase, consistent with a proposed biosynthetic origin of the cyclic C8 dialkylmaleic anhydride moiety. The product of *tmcC* is believed to be responsible for the linking of the cyclic C8 dialkylmaleic anhydride moiety to the linear polyketide moiety (Gandolfi *et al.*, 2001). Thus, *tmcD*, which exhibits significant homology to an acyl-CoA transferase/carnitine

dehydratase, is believed to encode an enzyme that activates the cyclic C8 dialkylmaleic anhydride moiety (Engemann *et al.*, 2005) as the corresponding CoA ester. Based on previous studies to determine the biosynthetic origin of TMC, it is evident that the cyclic anhydride is generated by condensation of one molecule of propionate with 2-oxoglutarate (Ubukata *et al.*, 1995). Thus, the *tmcE*–*tmcI*-encoded gene products might be responsible for the aldol condensation, dehydration and subsequent hydration of the allylic position at C3' (Fig. 1). However, these biochemical steps remain to be explored in detail and confirmed. Prior to esterification, the polyketide moiety initially synthesized by TmcA and TmcB might be further modified by several enzymes, such as a putative decarboxylase encoded by *tmcJ* (and/or *tmcK*), or a dehydratase encoded by *tmcM*. The ketone group located close to the right-hand end of TMC in Fig. 1 is believed to be introduced after polyketide biosynthesis by a separate cytochrome P450 hydroxylase enzyme, whose gene has been identified outside the cloned TMC cluster (S.-S. Choi and others, unpublished data). Since *tmcL*, encoding a putative crotonyl-CoA reductase, and *tmcM*, encoding a putative L-carnitine dehydratase, are located downstream of *tmcB* and show significant homologies with the genes responsible for the biosynthesis of ethylmalonate, these two gene products might be responsible for the synthesis of ethylmalonyl-CoA, an extender unit for TMC PKS AT9 (Table 1; Wu *et al.*, 2000). A 3 kb ORF (*tmcN*) located downstream of *tmcB* could be a pathway-specific regulatory gene due to its chromosomal location within the cluster as well as its homology to LuxR-family regulatory genes, often found in secondary-metabolite gene clusters of Gram-positive bacteria (Haydock *et al.*, 2005). In addition, a rare UUA codon was found in *tmcN*, suggesting that this gene encodes a pathway-specific regulatory protein whose expression is regulated by the *blaA* gene that encodes UUA-specific tRNA (Haydock *et al.*, 2005). The current proposed mechanism for TMC biosynthesis is shown in Fig. 3.

Fig. 3. Proposed TMC polyketide biosynthetic pathway in *Streptomyces* sp. CK4412. A schematic representation of the modular organization of PKS is shown, with catalytic domains represented by circles. The proposed post-PKS reaction is indicated by arrows.

Confirmation of the identity of the TMC biosynthetic gene cluster by gene disruption

The organization of the *tmc* gene cluster, and especially that of the PKS domain motif arrangement that was isolated and sequenced from *Streptomyces* sp. CK4412, is consistent with its role in TMC biosynthesis. To verify its identity

experimentally, we sought to confirm the function of this locus using a gene-disruption approach. Inactivation of *tmcD*, presumably involved in the biosynthesis of cyclic C8 dialkylmaleic anhydride-CoA, was performed using a PCR-targeted gene-disruption system (see Methods). pTMC2982 was replaced by an apramycin-resistance/*oriT* cassette generating pTMC2982Δ*tmcD* that was introduced into

Fig. 4. Gene replacement of TMC gene cluster. (A) Schematic representation of the PCR-targeted gene replacement disruption of *tmcD* and apramycin-resistance (*apr*)*oriT*. (B) Confirmation of the constructed *tmcD* mutant by PCR. Lanes: M, 100 bp ladder; 1, wild-type genomic DNA; 2, *Streptomyces* sp. CK4412-001 genomic DNA; 3, pTMC2982 DNA; 4, pTMC2982Δ*tmcD* DNA. Upper and lower panels in (B) show the results of PCR with two alternative primer pairs.

Fig. 5. Confirmation of the TMC biosynthetic gene cluster by gene disruption. (A) Comparison of antifungal activity against *A. niger* of TMC between *Streptomyces* sp. CK4412-001 (right) and *Streptomyces* sp. CK4412 (left). (B) HPLC analysis of chloroform extracts of *Streptomyces* sp. CK4412 and *Streptomyces* sp. CK4412-001. The dashed rectangles highlight the location of TMC peaks on the HPLC chromatograms.

Streptomyces sp. CK4412 by conjugative gene transfer (Fig. 4A). Construction of the *tmcD* mutant (*Streptomyces* sp. CK4412-001) generated by PCR-targeted disruption was confirmed by PCR analysis. The expected 1.3 kb PCR-amplified bands were observed in genomic DNA samples isolated from *Streptomyces* sp. CK4412 and pTMC2982 DNA, while the expected size (1.5 kb) PCR-amplified bands were observed in genomic DNA samples isolated from *Streptomyces* sp. CK4412-001 and pTMC2982Δ*tmcD* (Fig. 4B, top). In addition, the expected size (0.5 kb) PCR-amplified fragment, using an alternative PCR primer pair designed to detect an apramycin-resistance gene/*oriT* cassette, was observed only in *Streptomyces* sp. CK4412-001 and pTMC2982Δ*tmcD* (Fig. 4B, bottom), implying that *tmcD* was specifically disrupted, as expected. Both *Streptomyces* sp. CK4412 wild-type and *Streptomyces* sp. CK4412-001 strains were grown under conditions optimal for TMC production, followed by antifungal bioassay and HPLC quantification. Although a very weak antifungal activity was detected for the *Streptomyces* sp. CK4412-001 strain, probably due to the yet-to-be-confirmed linear polyketide precursor, the significantly reduced antifungal activity against *Aspergillus niger* (Fig. 5A), as well as the absence of TMC in extracts of the *Streptomyces* sp. CK4412-001 strain under the same culture conditions (Fig. 5B), provides strong evidence that *tmcD* (encoding a putative acyl-CoA transferase) plays an essential role in TMC biosynthesis.

Concluding remarks

TMC is a structurally unique secondary metabolite produced by *Streptomyces* sp. CK4412, with an ester bond linkage between a terminal cyclic C8 dialkylmaleic anhydride moiety and a linear polyketide chain bearing an unusual terminal alkene. Moreover, TMC exhibits a novel activated T cell-specific immunosuppressive activity with a mechanism of action unique in comparison with that of previously known T cell-specific immunosuppressors, such as CsA and FK506. The isolation and domain characterization of two multi-modular type I TMC PKS genes described here provide a key first step toward detailed characterization of the biosynthetic steps involved in the assembly and tailoring of this linear polyketide bearing an unusual terminal alkene. In addition, the 12 additional TMC gene products that flank the PKS will provide valuable insights into the elaboration of the previously uncharacterized cyclic C8 dialkylmaleic anhydride moiety. In conclusion, isolation of the biosynthetic gene cluster for TMC sets the stage for detailed genetic and biochemical studies of the biosynthesis of this important metabolite, providing opportunities to develop new TMC derivatives through combinatorial biosynthesis, chemoenzymic synthesis and heterologous expression approaches.

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