Identification of TmcN as a pathway-specific positive regulator of tautomycetin biosynthesis in *Streptomyces* sp. CK4412

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Tautomycetin (TMC) is a novel activated T-cell-specific immunosuppressive compound with a unique structure, containing an ester bond linkage between a terminal cyclic anhydride moiety and a linear polyketide chain bearing an unusual terminal alkene. A 3 kb gene, *tmcN*, with a deduced product of 1029 amino acid residues, located on the 3'-terminus of an approximately 70 kb contiguous TMC biosynthetic gene cluster, was found to have amino acid sequence homology with bacterial regulatory proteins. *In silico* database comparisons revealed that TmcN belongs to the large ATP-binding regulators of the LuxR protein family. Gene disruption of *tmcN* from the *Streptomyces* sp. CK4412 chromosome resulted in significantly reduced antifungal activity against *Aspergillus niger*, as well as the absence of TMC. In addition, complementation by an integrative plasmid carrying *tmcN* restored TMC biosynthesis, strongly suggesting that TmcN is a positive regulator of TMC biosynthesis. Gene expression analysis by RT-PCR of the TMC biosynthetic genes revealed that a TmcN mutant strain exhibited reduced expression levels for most of the biosynthetic genes except for its own *tmcN*. It is thus suggested that TmcN is a pathway-specific positive regulator that activates transcription of the TMC biosynthetic pathway genes in *Streptomyces* sp. CK4412.

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INTRODUCTION

Streptomycetes are Gram-positive filamentous soil bacteria with a complex life cycle that involves morphological differentiation such as sporulation. They are also widely known for their ability to produce a variety of commercially valuable enzymes and secondary metabolites, including antibiotics, anti-tumour agents, immunosuppressants and enzyme inhibitors (Chater, 1989; Hopwood, 1988; Strauch *et al.*, 1991; Myles, 2003; Hranueli *et al.*, 2005). It has been well documented that secondary metabolite production generally occurs at the onset of the stationary phase of growth of *Streptomyces* spp. and correlates temporally with the formation of aerial mycelium in cultures grown on the surface of solid media (Chater & Bibb, 1997; Martin *et al.*, 2000; Bibb, 2005). Previous

studies have revealed that the regulation of secondary metabolite production in *Streptomyces* spp. involves a complex regulatory network in response to nutritional and environmental factors, operating at several layers of control. Some of these affect only antibiotic production, whereas others affect both antibiotic production and morphological differentiation, suggesting that the two processes share some elements of genetic control (Chater & Bibb, 1997), while others are unique.

Among this regulatory network, the genes working at the proximal level usually reside within the respective biosynthetic gene cluster and are pathway-specific regulatory genes that only affect a single secondary metabolite biosynthetic pathway. The best-characterized pathway-specific regulatory proteins, including ActII-ORF4 for actinorhodin biosynthesis from *Streptomyces coelicolor* A3(2) (Arias *et al.*, 1999; Wietzorrek & Bibb, 1997) and DnrI for doxorubicin biosynthesis from *Streptomyces peucetius* (Sheldon *et al.*, 2002), belong to the so-called *Streptomyces* antibiotic regulatory proteins (SARPs) family (Wietzorrek & Bibb, 1997). These regulatory proteins contain a unique OmpR-like DNA-binding domain that is quite different from the typical domain with a helix–turn–helix (HTH) motif (Sheldon *et al.*, 2002; Wietzorrek &

Abbreviations: CsA, cyclosporin A; HTH, helix–turn–helix; LAL, large ATP-binding regulators of the LuxR family; PKS, polyketide synthetase; SARP, *Streptomyces* antibiotic regulatory protein; TMC, tautomycetin; wt, wild-type.

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A supplementary figure showing real-time RT-PCR analysis of the TMC gene cluster and a supplementary table of RT-PCR primer sequences are available with the online version of this paper.

Bibb, 1997). Unlike the SARP family (typically between 277 and 665 aa), another important, less-studied transcriptional family of regulators has recently been found in several macrolide antibiotic pathways (De Schrijver & De Mot, 1999), which are relatively large in size (872 to 1 159 aa) and have an N-terminal ATP-binding domain represented by discernible Walker A and B motifs (Walker *et al.*, 1982) and a C-terminal LuxR-type DNA-binding domain (Henikoff *et al.*, 1990). Regulators belonging to this so-called LAL (large ATP-binding regulators of the LuxR) family, whose prototype member is the *Escherichia coli* MalT, involved in the uptake and catabolism of maltodextrins (Richet & Raibaud, 1989), have been identified and characterized in several macrolide antibiotic pathways, including PikD for pikromycin from *Streptomyces venezuelae* (Wilson *et al.*, 2001), RapH for rapamycin from *Streptomyces hygroscopicus* (Aparicio *et al.*, 1996; Molnár *et al.*, 1996) and NysRI/RIII for nystatin from *Streptomyces noursei* (Brautaset *et al.*, 2000).

Tautomycetin (TMC), a secondary metabolite 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, whose chemical structure is identical to a previously reported antifungal compound produced by *Streptomyces griseochromogenes* (Cheng *et al.*, 1989). Inhibition of T cell proliferation by TMC was observed at concentrations 100-fold lower than those needed to achieve maximal inhibition with cyclosporin A (CsA) (Shim *et al.*, 2002). Due to its promising characteristics, TMC, whose mechanism of action is different from CsA or FK506, has been identified as a new drug candidate with potent T-cell-specific immunosuppressive activity (Shim *et al.*, 2002). Previously, we isolated and characterized the entire TMC biosynthetic gene cluster from *Streptomyces* sp. CK4412 originating from Jeju Island, Korea, and demonstrated its identity by gene disruption analysis (Choi *et al.*, 2007). The TMC biosynthetic gene cluster revealed two ORFs encoding a typical modular polyketide synthetase (PKS) gene as well as 12 ORFs located at both flanking regions, the deduced functions of which were consistent with TMC biosynthesis (Fig. 1).

Here, we describe the cloning and characterization of *tmcN*, a TMC pathway-specific regulatory gene located at the downstream end of the TMC biosynthetic gene cluster. We also demonstrate its role as a LAL-family pathway-specific transcriptional activator for TMC biosynthesis via *tmcN* gene disruption–complementation as well as RT-PCR-driven TMC gene expression analysis in *Streptomyces* sp. CK4412.

METHODS

Bacterial strains and culture conditions. *Streptomyces* sp. CK4412, kindly provided by ForHumanTech Ltd, Korea, was used as a TMC-producing strain as well as the source of DNA for *tmcN* gene disruption and its complementation (Table 1) (Shim *et al.*, 2002). The strain was cultivated at 28 °C in either R2YE or YEME liquid medium (Kieser *et al.*, 2000). *E. coli* DH5 α was used for DNA cloning and plasmid propagation. *E. coli* XL-1 Blue MR was used for cosmid library construction. *E. coli* ET12567/pUZ8002 (*dam2 dcm2 hrdM*) was used as the transient host for *E. coli*–*Streptomyces* conjugation (Choi *et al.*, 2004). 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 *tmcN*. The *tmcN* gene was identified in a previously isolated pTMC2290 cosmid (GenBank accession number, DQ983361) after the complete sequencing of the three positive cosmid clones, performed at Genotech, Korea (Table 1) (Choi *et al.*, 2007). DNA sequences for *tmcN* were assembled using BLAST searches on the National Center for Biotechnology Information (NCBI) server, and the ORF was subsequently predicted using the web-based program FramePlot 2.3.2 (<http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl>).

Construction of a *tmcN* mutant via chromosomal gene disruption. The *tmcN* gene encoding a putative LuxR family regulator with an ATP-binding site located downstream of the PKS *tmcB* gene was inactivated using a PCR-targeted gene-disruption system (Gust *et al.*, 2003). An apramycin-resistance gene/*oriT* cassette for the replacement of *tmcN* was amplified using the pIJ773 as a template (Gust *et al.*, 2003) using the following primers: forward primer (5'-tcagatccgtctcgttcg tgtgctcgtcaccgtgccgttATCCGGGG-ATCCGTCGACC-3') and reverse primer (5'-acgaatatcgcggcatctgaccacgttcgaggtcacggtGTAGGCTGGAGCTGCTTC-3'). The lower-case type represents 40 nt homologous extensions to the DNA regions inside *tmcN*. This cassette was introduced into *E. coli* BW25113/pIJ790 containing pTMC2290. Gene replacement in *tmcN* was confirmed by restriction analysis of the mutated pTMC2290

Fig. 1. TMC biosynthetic pathway gene cluster.

Table 1. *Streptomyces* strains and plasmids used for this study

Strain or plasmid	Relevant characteristics	Source
<i>Streptomyces</i> sp.		
CK4412	Wild-type TMC-producing strain	Choi <i>et al.</i> (2007)
CK4412-002	<i>tmcN</i> -disruptant tautomycin non-producing mutant strain	This work
CK4412-002/ <i>tmcN</i>	<i>Streptomyces</i> sp. CK4412-002 (<i>tmcN</i> -disruptant) containing pSETNEO <i>tmcN</i>	This work
CK4412/ <i>tmcN</i>	Wild-type TMC-producing strain containing pSETNEO <i>tmcN</i>	This work
Plasmids		
pTMC2290	Cosmid vector including <i>tmcN</i>	Choi <i>et al.</i> (2007)
pTMC2290Δ <i>tmcN</i>	pTMC2290 mutant cosmid vector containing <i>tmcN</i> disrupted with <i>apr^R/oriT</i>	This work
pSETNEO <i>tmcN</i>	pSET152-based insertional vector including single copy of <i>tmcN</i> and additional selection marker neomycin-resistance gene	This work

(pTMC2290Δ*tmcN*). pTMC2290Δ*tmcN* 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 mg ml⁻¹ and nalidixic acid at a final concentration of 25 mg ml⁻¹. Incubation was continued at 28 °C until conjugants appeared. The double-crossover exconjugants were selected using a standard Apr^R/Kan^S method (Gust *et al.*, 2003), followed by the confirmation of both *Streptomyces* sp. CK4412 and *Streptomyces* sp. CK4412-002 (Δ*tmcN* mutant of *Streptomyces* sp. CK4412) genomic DNAs by both PCR and Southern blot hybridization. Three different PCR primers for the confirmation of the double-crossover recombinants were *tmcN* test primer F (5'-AAGCTTAGTCGTCGTCGGGTCGCCGGGCTGGACA-3'), *tmcN* test R (5'-AAAGCGTCATCCTGCTCAA-3') and *oriT* test primer F (5'-GAATTCAGCGTGACATCATTCTGTGG-3'), which is in the *apr^R/oriT* cassette. (Fig. 3a).

Construction of integrative plasmid for *tmcN* complementation. For complementation of the *tmcN*-disrupted mutant, a 3 kb DNA fragment including the entire *tmcN* gene was amplified by PCR using genomic DNA from the wild-type (wt) TMC-producing strain (*Streptomyces* sp. CK4412) as a template and the primer pair *PtmcN*-forward (5'-TCTGGAATTCCGAGGGTGAGCTCTTCACGACCATT-3') and *PtmcN*-reverse (5'-AAGCTTAGTCGTCGTCGGGTCGCCGGGCTGGACA-3'). The underlined sequences indicate *EcoRI* and *HindIII* restriction sites, respectively. PCR was performed in a final volume of 20 µl containing 0.4 µM each primer, 0.25 mM of each of the four dNTPs (Roche), 1 µl extracted DNA, 1 U Ex Taq polymerase with its recommended reaction buffer (TaKaRa) 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. The amplified PCR product was analysed by electrophoresis in 1% (w/v) agarose gel, purified via a DNA extraction kit (Qiagen) and then ligated into pMD18-T (TaKaRa); the sequence was confirmed by complete nucleotide sequencing (Genotech Korea). The PCR-amplified *tmcN* gene was cloned into *EcoRI*-digested pSET152 (*apr^R*, pUC18 replicon, ΦC31, *attP*); the neomycin-resistance gene originating from pFDNEO-S plasmid (Denis & Brzezinski, 1991) was cloned into the *EcoRV* restriction site in pSET152 (Bierman *et al.*, 1992). Finally, neomycin was used for selection of the recombinant *Streptomyces* sp. CK4412-002/*tmcN*⁻ strain, which was already resistant to apramycin due to *tmcN* gene disruption.

Isolation of total RNA and gene expression analysis by RT-PCR. *Streptomyces* sp. CK4412 and *Streptomyces* sp. CK4412-002 were grown for 72 h in R2YE medium, and the cultures were washed twice with 1 vol. sterile water. The mycelia were subsequently harvested by centrifugation and immediately frozen by immersion in liquid

nitrogen. The frozen mycelia were then broken by shearing in a mortar, and the frozen lysate was added to buffer RLT (Qiagen) in the presence of 1% β-mercaptoethanol. RNeasy mini spin columns were used for RNA isolation according to the manufacturer's instructions. RNA preparations were treated with DNase I (Qiagen) to eliminate possible chromosomal DNA contamination.

DNase I-treated RNA (7 µg) was used as a template for reverse transcription at 50 °C with an AVM Reverse Transcriptase XL (TaKaRa) and random hexamers. The conditions for cDNA synthesis were as follows: 30 °C for 10 min, 50 °C for 1 h, 99 °C for 2 min, 5 °C for 5 min. The resulting cDNA was used for PCR amplification under the following conditions: 25 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 68 °C for 35 s. Each primer pair of TMC biosynthetic genes was carefully designed to generate a PCR product of approximately 150 to 200 bp using a genscript site (<http://www.genscript.com/ssl-bin/app/primer>). The complete RT-PCR primer pair sequences are listed in Supplementary Table S1; the RNA samples that had not been subjected to RT reaction were used for PCR as negative controls.

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 and with UV detection at 273 nm. TMC production was also evaluated by a biological assay against *Aspergillus niger*, using a paper disc containing the same culture broth extract as used in the HPLC assay. The paper disc was placed on top of *A. niger* that had been incubated on ME medium (0.05% malt extract, 0.05% glucose, 0.001% peptone in 1 l double-distilled H₂O) for 6 h at 30 °C, followed by measurement of the inhibition zone after overnight incubation at 30 °C.

RESULTS AND DISCUSSION

In silico sequence analysis of *tmcN*

Previously, we reported that a 3 kb ORF (*tmcN*) located downstream of *tmcB* resembled a pathway-specific regulatory gene due to its chromosomal location within *tmc*, as well as its homology to LuxR-family regulatory genes (Choi *et al.*, 2007), often found in secondary metabolite gene clusters of Gram-positive bacteria (Haydock *et al.*, 2005). Further characterization of the *tmcN* gene product via database-

assisted *in silico* analysis revealed that it encodes a protein of 1 029 aa and shows 33 % identity at the amino acid level to ThcG, a regulator of the LuxR family with an ATP-binding site in *Rhodococcus erythropolis* (De Schrijver & De Mot, 1999), 35 % identity to a LuxR regulatory protein from *Frankia* sp. EAN1pec (Copeland *et al.*, unpublished data) and 30 % identity to a putative transcriptional regulator from *Streptomyces ambifaciens* (Choulet *et al.*, 2006).

Notably, the predicted amino acid sequence of TmcN shows two highly conserved domains: a putative HTH motif in the C-terminal region typically found in various bacterial DNA-binding proteins (Pabo & Sauer, 1992) and Walker A and B nucleoside triphosphate binding motifs at the N-terminal region (Walker *et al.*, 1982; Fig. 2). The latter have also been found in polyketide biosynthetic regulatory proteins, including PikD for pikromycin from *S. venezuelae* (Wilson *et al.*, 2001), RapH for rapamycin from *S. hygroscopicus* (Aparicio *et al.*, 1996; Molnár *et al.*, 1996) and NysRI/RIII for nystatin from *S. nouresii* (Brautaset *et al.*, 2000). Since all these proteins were previously assigned to the LAL-family (Demain & Fang, 1995), *tmcN* is also presumed to encode a LAL-family pathway-specific regulatory protein involved in TMC biosynthesis from *Streptomyces* sp. CK4412. Interestingly, however, TmcN exhibited relatively low amino acid identities to the following *Streptomyces* LAL-type

regulators: 17 % with PikD, 16 % with RapH and 17 % with NysRI/RIII. In addition, *tmcN* contains two rare TTA leucine codons (codon 81, codon 375), which indicates its dependence on *bldA*, the structural gene for tRNA^{UUA}. This may be a further indication of a regulatory role of TmcN in TMC biosynthesis, since most of the known TTA-containing genes specify regulatory or resistance proteins associated with antibiotic biosynthesis gene clusters (Leskiw *et al.*, 1991; Li *et al.*, 2007).

Construction of a *tmcN* disruption mutant, *Streptomyces* sp. CK4412-002

Although the organization and *in silico* sequence analysis of *tmcN* in the TMC cluster from *Streptomyces* sp. CK4412 is consistent with a regulatory role in TMC biosynthesis, we sought to confirm the *in vivo* function of *tmcN* using a gene disruption approach. Inactivation of *tmcN* was performed using a PCR-targeted gene disruption system (see Methods). pTMC2290 was replaced by an apramycin-resistance/*oriT* cassette, generating pTMC2290Δ*tmcN*, which was introduced into *Streptomyces* sp. CK4412 by conjugative gene transfer (Fig. 3a). Construction of the *tmcN* mutant (named *Streptomyces* sp. CK4412-002) generated by PCR-targeted disruption was confirmed by both PCR and Southern hybridization analysis. The

Fig. 2. Domain structure and amino acid sequence alignments of parts of the TmcN protein. Since a RapH Walker A motif (GXXXXTP) is different from a classic Walker A motif (GXXXXKS), RapH is probably not functional as a Walker A-like ATPase domain due to the lack of the lysine and serine.

Fig. 3. Gene replacement of the *tmcN* gene. (a) Schematic representation of PCR-targeted gene replacement disruption of *tmcN* and apramycin-resistance (*apr^R*)/*oriT*. (b) Confirmation of constructed *tmcN* mutant by PCR. Lanes: M, 100 bp ladder; 1 and 3, *Streptomyces* sp. CK4412 wt genomic DNA; 2 and 4, *Streptomyces* sp. CK4412-002 genomic DNA. In 1 and 2, PCR was performed with *tmcN* F and R primers; in 3 and 4, PCR was performed with *oriT* and *tmcN* R primers. (c) Confirmation of constructed *tmcN* mutant by Southern hybridization. A diagrammatic representation of the digest and probe binding sites is shown to the left of the blot; B, *Bam*HI restriction sites. Lanes: 1, *Streptomyces* sp. CK4412 wt genomic DNA; 2, *Streptomyces* sp. CK4412-002 genomic DNA; 3, undigested pGEM-T vector containing a 409 bp PCR-amplified DNA fragment probe.

expected 1.43 kb PCR-amplified band was observed in genomic DNA samples isolated from *Streptomyces* sp. CK4412, and a band of the expected size (1.53 kb) was observed in genomic DNA samples isolated from *Streptomyces* sp. CK4412-002 (Fig. 3b). A PCR-amplified fragment of the expected size (0.47 kb), amplified using an alternative PCR primer pair designed to detect an apramycin resistance gene/*oriT* cassette, was observed only in the *Streptomyces* sp. CK4412-002 (Fig. 3b). Moreover, two different theoretically calculated *Bam*HI digestion patterns were observed in Southern hybridization with *Streptomyces* sp. CK4412 and *Streptomyces* sp. CK4412-002 (Fig. 3c), implying that *tmcN* was specifically disrupted as expected.

Loss of TMC production by *tmcN* disruption and rescue by *tmcN* complementation

Fermentation broths of both *Streptomyces* sp. CK4412 and *Streptomyces* sp. CK4412-002 grown under conditions

optimal for TMC production were extracted with chloroform, followed by an antifungal bioassay and HPLC quantification for the presence of TMC. Although very weak antifungal activity was detected, presumably due to either residual TMC or some other unconfirmed metabolites produced by *Streptomyces* sp. CK4412, the significantly reduced antifungal activity against *A. niger*, as well as the absence of TMC in extracts of *Streptomyces* sp. CK4412-002 under the same culture conditions, provide strong evidence that *tmcN* plays an essential regulatory role in TMC biosynthesis (Fig. 4).

To prove further that the inactivation of *tmcN* was indeed responsible for TMC production, we performed genetic complementation of *Streptomyces* sp. CK4412-002 by expressing *tmcN* under the control of its own promoter. For this purpose, we used the integrating conjugative vector pSET152 (Bierman *et al.*, 1992), into which the coding region of *tmcN* and 144 bp of the DNA region upstream of the putative translational start point was

Fig. 4. (a) TMC volumetric productivities measured by quantitative HPLC analyses from the chloroform-extracted broths were 3.42 mg l⁻¹ from the wt strain (CK4412), 1.02 mg l⁻¹ from the empty vector conjugant (CK4412/pSET152), non-detectable from the *tmcN* gene disruptant (CK4412-002), 1.52 mg l⁻¹ from the *tmcN* complementation strain (CK4412-002/*tmcN*) and 18.86 mg l⁻¹ from the *tmcN* overexpression strain (CK4412/*tmcN*). Right top; control authentic TMC sample kindly provided by the ForHumanTech Company in Korea. (b) Comparison of antifungal activity of TMC against *A. niger*.

cloned, resulting in pSETNEO*tmcN*. Also, a neomycin-resistance gene, originated from plasmid pFDNEO-S (Denis & Brzezinski, 1991), was inserted into the multiple cloning site (MCS) of parental pSET152 to select the recombinant strains bearing complementation plasmids. Both HPLC and the bioassay confirmed that TMC productivity and antifungal activity were restored to significant levels in the *Streptomyces* sp. CK4412-002 mutant strain carrying pSETNEO*tmcN* (Fig. 4), implying that the absence of TMC productivity from *Streptomyces* sp. CK4412-002 strains was due to a lack of a TMC-specific positive regulatory gene, *tmcN*. Moreover, the wild-type *Streptomyces* sp. CK4412 strain containing an extra copy of *tmcN* via chromosomal integration of pSETNEO*tmcN* led to an approximately 5.5-fold increase in TMC biosynthesis (Fig. 4a).

Transcriptional control of TMC biosynthetic pathway genes

Total RNA samples were prepared from the *Streptomyces* sp. CK4412 wild-type and the *Streptomyces* sp. CK4412-002 mutant after 72 h of growth and used as a template for gene expression analysis by RT-PCR. Primers for RT-PCR were specific to sequences within *tmc* genes (Table 2) and were designed to produce cDNAs of approximately 200 bp. A primer pair designed to amplify a cDNA of the rRNA gene was used as an internal control. Transcripts were analysed from the 14 genes located within the *tmc* cluster, including *tmcN*, after 25 PCR cycles. This analysis was carried out at least three times for each primer pair. In the RT-PCR analysis, the transcripts of all 14 genes were detected in the *Streptomyces* sp. CK4412 wt, while the

Fig. 5. Gene expression analysis of the TMC gene cluster by RT-PCR. Analysis was carried out on *Streptomyces* sp. CK4412 wild-type (+) and *Streptomyces* sp. CK4412-002 (-) strains as indicated in Methods. Transcription of the rRNA gene was also assessed as an internal control. The diagram indicates the organization of the genes within the TMC cluster and their putative transcripts.

transcription pattern in the *Streptomyces* sp. CK4412-002 was significantly reduced for most of the *tmc* genes within the cluster, except for *tmcA* and *tmcB* (Fig. 5). Interestingly, the transcripts of the putative PKS operon containing the translationally coupled genes *tmcA* and *tmcB* were apparently much less affected by the absence of the *tmcN* gene in the *Streptomyces* sp. CK4412-002 (Fig. 5). The absence of the *tmcM* transcript is believed to be a polar effect derived from *tmcN* disruption in the *Streptomyces* sp. CK4412-002 due to translational coupling of these two genes, even though the PCR-targeting method is designed to limit this effect. Real-time RT-PCR analysis also confirmed that the absence of *tmcN* reduced transcripts of the PKS operon less significantly (only 33 % reduction), while both flanking operons were severely affected by the absence of *tmcN* (88 % reduction for *tmcC* operon and 63 % reduction for *tmcJ* operon, respectively) (see Supplementary Fig. S1, available with the online version of this paper). These results strongly suggest that TmcN might activate all three putative TMC operons at different levels, either directly or indirectly. Taken together, these data demonstrate that *tmcN* encodes a LAL-family pathway-specific activator of the TMC biosynthetic pathway in *Streptomyces* sp. CK4412.

Concluding remarks

Regulation of natural product biosynthesis in *Streptomyces* spp. continues to represent an understudied aspect of the field. Despite the power of genome sequencing and transcriptomics, understanding the details of regulation of secondary metabolic systems demands genetic and biochemical dissection of specific regulators to elucidate their genetic targets and effects. The LAL family of regulators has been recognized in several systems (Wilson *et al.*, 2001; Aparicio *et al.*, 1996; Molnár *et al.*, 1996; Brautaset *et al.*, 2000), and the study described in this report has revealed that TmcN exerts its effects at different levels on individual operons in this large biosynthetic gene cluster. Gene disruption and complementation studies have confirmed its role as a positive regulator, and the ability to increase production of this important metabolite by adding an additional copy of *tmcN* into the chromosome demonstrates the value of this strategy for strain improvement. Currently, one of the key factors for further development of TMC as a

medicinal agent is the limited amount of this natural product available through fermentation methods. Thus, this initial study of TmcN paves the way for further analysis of this growing class of regulatory factors and offers a new approach to improving access to TMC for detailed biological studies, target identification and analogue development.

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