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토토마이세틴 생합성 조절 네트워크 규명

Dual Regulatory Systems of Tautomycetin Biosynthesis in *Streptomyces* sp. CK4412;
WblA<sub>inc</sub>-independent TmcN and WblA<sub>inc</sub>-dependent TmcT

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生物工學科

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主審：_________________________

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Abstract

Tautomycetin (TMC) is an unusual linear polyketide compound esterificated with a cyclic anhydride, which exhibits a novel activated T cell-specific immunosuppressant and anti-cancer activities. Previously, we isolated and characterized the entire TMC biosynthetic gene cluster including a TMC pathway-specific gene, tmcN from Streptomyces sp. CK4412, whose over-expression led to significantly increased TMC productivity. In addition, we also reported that wblA acted as a global down-regulator of antibiotic biosynthesis through pathway-specific regulator Streptomyces species. Here, we confirm that tmcT is another TMC pathway-specific regulatory gene present within the TMC biosynthetic cluster, because tmcT deletion resulted in the complete loss of TMC production and complementation by a tmcT-carrying integrative plasmid significantly restored TMC biosynthesis. We also identified a 0.39 kb wblA ortholog (named wblAtmc) via genomic DNA library screening from Streptomyces sp. CK4412, showing 96% amino acid identity compared to a previously known S. coelicolor wblA. A targeted gene disruption of wblAtmc from the Streptomyces sp. CK4412 exhibited approximately 2-fold higher TMC productivity than that from the wild-type strain. Moreover, transcript analyses of the TMC biosynthetic and regulatory genes revealed that the expression of tmcT is strongly down-regulated by wblAtmc. These results imply that TMC biosynthetic regulation network is controlled by two pathway-specific positive regulatory genes, wblAtmc-independent tmcN as well as wblAtmc-dependent in Streptomyces sp. CK4412.
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1. Introduction

1.1 *Streptomyces*

The bacterial genus *Streptomyces* is high G+C Gram-positive filamentous soil bacteria with a complex life cycle including morphological differentiation and spore formation (Del Sol *et al.*, 2007). They have long been appreciated as a rich source for the production of various secondary metabolites including many pharmaceutically valuable compounds such as antibiotics, anti-cancer agents, immunosuppressants and enzyme inhibitors (Hindra and Elliot, 2010; Hranueli *et al.*, 2005; Myles, 2003). Among them is a protein phosphatase PP1/PP2A inhibitor named tautomycetin (TMC), which is also a pharmacokinetically-superior T-cell-specific immunosuppressant produced by *Streptomyces* sp. CK4412 (Shim *et al.*, 2002; Chae *et al.*, 2004).

1.2 Antibiotics Biosynthesis Regulatory Systems in *Streptomyces*

It is now well documented that the biosynthesis of *Streptomyces* secondary metabolites is typically regulated via multiple regulatory pathways operating at several layers of complicated control systems (Lee *et al.*, 2005; Chen *et al.*, 2010). Some global regulatory systems affect both morphological differentiation and secondary metabolite production, suggesting that both processes share some common elements of genetic control in *Streptomyces* species (Chater and Bibb, 1997; Liu *et al.*, 2005). In addition, there is a different type of regulatory system which affects only the corresponding target metabolite biosynthesis in *Streptomyces* species, which is commonly-named ‘pathway-specific’ regulatory system (Lombó *et al.*, 1999; Retzlaff and Distler 1995; Tang *et al.*, 1996; Niraula *et al.*, 2010). Among the best-characterized pathway-specific regulatory protein is ActII-ORF4 for actinorhodin biosynthesis from *S. coelicolor*, which belongs
to the so-called *Streptomyces* Antibiotic Regulatory Protein (SARPs) family (Arias *et al*., 1999; Wietzorrek and Bibb, 1997). Another pathway-specific regulator called Large ATP-binding regulator of the LuxR (LAL) family also has been identified and characterized in several macrolide antibiotic pathways including PikD for pikromycin from *S. venezuelae* (Wilson *et al*., 2001). Surprisingly, however, the detailed mechanisms and networks how these global regulatory systems are connected to the most of the pathway-specific regulatory genes at the molecular level, still remain largely unknown (Kitani *et al*., 2009). Previously, *whiB*-like putative transcription regulatory gene A (named *wblA*) was identified as a pleiotropic down-regulator of antibiotic biosynthesis in *Streptomyces* species (Kang *et al*., 2007; Noh *et al*., 2010). In *wblA*-overexpressing *S. coelicolor*, the biosynthesis of three major antibiotics, actinorhodin (ACT), undecylprodigiosin (RED), and calcium-dependent antibiotic (CDA), were inhibited and the transcripts of pathway-specific activator genes of these antibiotics (i.e., *actII-ORF4* for ACT, *redD/Z* for RED, and *cdaR* for CDA) were significantly reduced (Kang *et al*., 2007). Gene disruption of *wbla* (*wblA* ortholog founded in *S. peucetius*) from the *S. peucetius* overproducing industrial mutant (OIM) resulted in an additional increase in the production of both doxorubicin and daunorubicin (Noh *et al*., 2010), implying that *wblA* acts broadly as a down-regulator of antibiotic biosynthesis.

### 1.3 Tautomycetin

TMC has a structurally-unique ester bond linkage between a terminal cyclic C8 dialkylmaleic anhydride moiety and a linear polyketide chain bearing an unusual terminal alkene, and its chemical structure is identical to a previously reported antifungal compound produced by *S. griseochromogenes* (Fig. 1A, Kobayashi *et al*., 1989). More recently, TMC also has been
reported to possess anti-cancer activities against colorectal and thyroid cancer cells (Lee et al., 2006), implying that TMC could be a potentially-valuable immunosuppressive-&-anticancer drug lead compound. Recently, we isolated and characterized the entire TMC biosynthetic gene cluster from *Streptomyces* sp. CK4412, and demonstrated its identity by gene disruption analysis (Choi et al., 2007). The TMC biosynthetic gene cluster revealed two ORFs which encodes a typical modular polyketide synthetase (PKS) gene as well as 18 ORFs located at both flanking regions, the deduced functions of which were consistent with TMC biosynthesis (Fig. 1B). In addition, we identified and characterized *tmcN*, which located at the downstream of the TMC biosynthetic gene cluster, as a LAL-family pathway-specific transcriptional activator for TMC biosynthesis (Hur et al., 2008). By over-expressing *tmcN* gene from wild type *Streptomyces* sp. CK4412, TMC productivity increased approximately 5.5-fold higher than those produced from wild type (Hur et al., 2008).

Here, we verify that *tmcT* is another functional TMC pathway-specific regulatory gene through targeted gene-disruption and functional complementation, in addition to the previously-reported *tmcN*. We also isolate and characterize a global antibiotic down-regulatory gene, *wblA* ortholog (*wblA_{tmc}* ) from *Streptomyces* sp. CK4412 via genomic DNA library screening, suggesting that *wblA_{tmc}* plays a critical role in TMC biosynthesis. Moreover, transcript analyses of the TMC pathway regulatory genes via RT-PCR reveal the TMC regulatory network is arranged as a cascade system of *wblA*, *tmcT*, and *tmcN*. 
Figure 1. (A) TMC structure, and (B) TMC biosynthetic pathway gene cluster
2. Experimental section

2.1. Bacterial strains and culture conditions

*Streptomyces* sp. CK4412 was used as a TMC-producing strain (Choi *et al.*, 2007). For TMC production, *Streptomyces* sp. CK4412 cells were pre-cultured for 2 days in TSB and then cultured for 7 more days in R2YE or MS liquid medium (Choi *et al.*, 2007). *Escherichia coli* DH5α strain was used for DNA cloning and plasmid propagation. *E. coli* ET12567/pUZ8002 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).

2.2. Screening and sequence analysis of *wbla*<sub>mc</sub>

The cosmid library, which was previously generated (Choi *et al.*, 2007), was screened by polymerase chain reaction (PCR) using the degenerate primers. The PCR primer pair [forward primer 5’- TGC CGC ACT ACC GAT CCG GA -3’ and reverse primer 5’- GTA CTC CGT MCG CGC S(C/G)GT CT - 3’] was designed based on the *wbla* sequences from *S. coelicolor* A3(2) (Kang *et al.*, 2007). The amplified PCR product was analyzed by electrophoresis in 1% (w/v) agarose gel and purified via a DNA extraction kit [COSMO, Korea]. This was ligated into pMD18-T [TaKaRa, Japan], followed by complete nucleotide sequencing confirmation by Genotech Korea. DNA sequences between *wbla* from *S. coelicolor* and *wbla* ortholog (*wbla*<sub>mc</sub>) from *Streptomyces* sp. CK4412 were assembled using BLAST searches on the National Center for Biotechnology Information (NCBI) server.
2.3. Construction of Δwbla_{tmc} and Δtmc\text{\textit{T}} mutants via PCR-targeted gene disruption

Inactivation of \( wbla_{tmc} \) or \( Tmc\text{\textit{T}} \) was performed using a PCR-targeted gene disruption system (Hur et al., 2008). A spectinomycin-resistance gene/or\textit{i}T cassette for the replacement of \( wbla_{tmc} \) was amplified using the pIJ778 as a template. The lower-case type represents 40-nt homologous extensions to the DNA regions inside \( wbla_{tmc} \). This cassette was introduced into \( E. \text{\textit{coli}} \) BW25113/pIJ790 containing pTMC2360. Gene replacement in \( wbla_{tmc} \) was confirmed by restriction analysis of the mutated pTMC2360, which \( wbla_{tmc} \) in pTMC2360 replaced to spectinomycin-resistance gene/or\textit{i}T. pTMC2360Δ\( wbla_{tmc} \) was introduced into \( Streptomyces \) sp. CK4412 by conjugation from \( E. \text{\textit{coli}} \) ET12567/pUZ8002 (Fig. 2A). After incubation at 28°C for 16h, each plate was overlaid with 1ml sterile water containing spectinomycin at a final concentration of 400\( \mu \)g ml\(^{-1}\) and nalidixic acid at a final concentration of 25\( \mu \)g ml\(^{-1}\). Incubation was continued at 28°C until conjugants appeared. Construction of the Δ\( wbla_{tmc} \) mutant generated by PCR-targeted disruption was confirmed by using both standard Spe\text{\texte{R}}/Kan\text{\texte{S}} method and PCR (Fig. 2B). Similarly, inactivation of \( tmc\text{\textit{T}} \) was also performed using a same PCR-targeted gene disruption system. An apramycin-resistance gene/or\textit{i}T cassette for the replacement of \( tmc\text{\textit{T}} \) was amplified using the pIJ773 as a template. Introduction of mutated pTMC2290, which \( tmc\text{\textit{T}} \) in pTMC2290 replaced to apramycin-resistance gene/or\textit{i}T, into \( Streptomyces \) sp. CK4412. Genetic confirmation strategy of Δ\( tmc\text{\textit{T}} \) mutant (named \( Streptomyces \) sp. CK4412-003) was almost identical as that of \( wbla_{tmc} \) mutant (named \( Streptomyces \) sp. CK4412-003), except for the use of apramycin instead of spectinomycin (Fig. 2C, 2D).
Figure 2. Gene replacement of the \textit{wbla}_{tmc} gene and \textit{tmcT} gene. (A) Schematic representation of PCR-targeted gene replacement disruption of \textit{wbla}_{tmc} and spectinomycin-resistance (\textit{spe}^R)/\textit{oriT}. (B) Confirmation of constructed \textit{Δwbla}_{tmc} mutant. Lanes: M, 1kb ladder; 1 and 3, \textit{Streptomyces} sp. CK4412 WT genomic DNA; 2 and 4, \textit{Streptomyces} sp. CK4412-001 genomic DNA. In 1 and 2, PCR was performed with \textit{oriT} and \textit{wbla-tmc} check F primers; in 3 and 4, PCR was performed with \textit{wbla-tmc} check F and R primers. (C) Schematic representation of PCR-targeted gene replacement disruption of \textit{tmcT} and apramycin-resistance (\textit{apr}^R)/\textit{oriT}. (D) Confirmation of constructed \textit{ΔtmcT} mutant. Lanes: M, 1kb ladder; 1 and 3, \textit{Streptomyces} sp. CK4412 WT genomic DNA; 2 and 4, \textit{Streptomyces} sp. CK4412-003 genomic DNA. In 1 and 2, PCR was performed with \textit{tmcT} check F and R primers; in 3 and 4, PCR was performed with \textit{oriT} and \textit{tmcT}
check F primers. Black arrow, \textit{wblA-tmc} check F and R primers; Grey arrow, \textit{tmcT} check F and R primers; Red arrow, \textit{oriT} check F primer.
2.4. Construction of integrative plasmid for complementation of ΔwblA<sub>mc</sub> or Δtmc<sub>T</sub>

For complementation of the ΔwblA<sub>mc</sub> mutant and Δtmc<sub>T</sub> mutant, a 700-bp of DNA fragment including entire the wblA<sub>mc</sub> and 1.3-kb of DNA fragment including the entire tmc<sub>T</sub> gene was amplified by PCR using genomic DNA from the TMC-producing Streptomyces sp. CK4412 wild-type strain as a template. The amplified PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel, purified via a DNA extraction kit and then ligated into pGEM®-T Easy vectors. The sequence was confirmed by complete nucleotide sequencing of each product (Macrogen, Korea).

The PCR-amplified wblA<sub>mc</sub> or tmc<sub>T</sub> gene was cloned into pSET152 (Choi et al., 2007) followed by hygromycin resistance gene cloning to the BamHI and XbaI restriction site of pSET152 (Bierman et al., 1992). Finally, MS medium containing 50μg ml<sup>-1</sup> of hygromycin was used for selection of the recombinant Streptomyces sp. CK4412-001/wblA<sub>mc</sub> or CK4412-003/tmc<sub>T</sub> strain, which is already resistant to spectinomycin or apramycin due to the wblA<sub>mc</sub> or tmc<sub>T</sub> targeted gene disruption (Table 1). Each of the final true exconjugant was selected and confirmed by PCR analysis.
Table 1. *Streptomyces* strains and plasmid used for this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptomyces</strong> sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK4412</td>
<td>Wild-type TMC-producing strain</td>
<td>Choi et al. (2007)</td>
</tr>
<tr>
<td>CK4412-001</td>
<td><em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt;-disrupted strain (replaced with <em>spe&lt;sup&gt;R&lt;/sup&gt;</em>/<em>oriT</em>)</td>
<td>This work</td>
</tr>
<tr>
<td>CK4412-001/<em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt;</td>
<td><em>Streptomyces</em> sp. CK4412-001 (<em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt;-disruptant) containing pSETHYG&lt;sub&gt;wbla&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>CK4412/<em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt;</td>
<td>Wild-type TMC-producing strain containing pSETAP&lt;sub&gt;wbla&lt;/sub&gt;T</td>
<td>This work</td>
</tr>
<tr>
<td>CK4412-002</td>
<td><em>tmcN</em> disrupted strain (replaced with <em>apr&lt;sup&gt;R&lt;/sup&gt;</em>/<em>oriT</em>)</td>
<td>Hur et al. (2008)</td>
</tr>
<tr>
<td>CK4412-003</td>
<td><em>tmcT</em> disrupted strain (replaced with <em>apr&lt;sup&gt;R&lt;/sup&gt;</em>/<em>oriT</em>)</td>
<td>This work</td>
</tr>
<tr>
<td>CK4412-003/<em>tmcT</em></td>
<td><em>Streptomyces</em> sp. CK4412-003 (<em>tmcT</em>-disruptant) containing pSETHYG&lt;sub&gt;tmcT&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>CK4412/<em>tmcT</em></td>
<td>Wild-type TMC-producing strain containing pSETHYG&lt;sub&gt;tmcT&lt;/sub&gt;</td>
<td>This work</td>
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<tr>
<td><strong>Plasmid</strong></td>
<td></td>
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<tr>
<td>pTMC2360</td>
<td>Cosmid vector including <em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt;</td>
<td>This work</td>
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<tr>
<td>pTMC2360&lt;sub&gt;ΔwbLA&lt;/sub&gt;</td>
<td>pTMC2360 mutant cosmid vector containing <em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt; disrupted with <em>spe&lt;sup&gt;R&lt;/sup&gt;</em>/<em>oriT</em></td>
<td>This work</td>
</tr>
<tr>
<td>pTMC2290</td>
<td>Cosmid vector including <em>tmcT</em></td>
<td>Choi et al. (2007)</td>
</tr>
<tr>
<td>pSETHYG&lt;sub&gt;wbla&lt;/sub&gt;</td>
<td>pSET152-based insertional vector including single copy of <em>wbLA</em>&lt;sub&gt;mc&lt;/sub&gt; and additional selection marker hygromycin-resistance gene</td>
<td>This work</td>
</tr>
<tr>
<td>pSETHYG&lt;sub&gt;tmcT&lt;/sub&gt;</td>
<td>pSET152-based insertional vector including single copy of <em>tmcT</em> and additional selection marker hygromycin-resistance gene</td>
<td>This work</td>
</tr>
</tbody>
</table>
2.5. HPLC quantification and antifungal bioassay for TMC

For HPLC analysis of TMC production, \textit{Streptomyces} sp. CK4412, the \textit{Streptomyces} sp. CK4412-001 or \textit{Streptomyces} sp. CK4412-003, those complemented strains and over-expressed strains were cultivated at 28°C in MS liquid media for 7 days after sub-cultivation in MS liquid medium for 2 days and CK4412 and \textit{wbla-tmc}-related mutants were also cultivated on MS agar medium for 9 days at 28°C. The culture broth supernatant was extracted with an equal volume of ethyl acetate. The extracts were dried using a rotor-evaporator and then resuspended in methanol. Extracts were fractionated by HPLC using binary gradient conditions with methanol and distilled water (both included 0.2% formic acid) on a Genesis C18 4 μm column with UV detection at 273 nm. TMC production was also evaluated by a biological assay against \textit{Aspergillus niger} as an indicator using a paper disc containing the same culture broth extract used in the HPLC assay. The paper disc was placed on top of \textit{A. niger} that had been incubated on ME medium (0.05% malt extract, 0.05% glucose, 0.001% peptone in 1L ddH₂O) for 6 h at 30°C, followed by measurement of the inhibition zone after overnight incubation at 30°C.

2.6. Isolation of total RNA and gene expression analysis by RT-PCR

For RT-PCR, \textit{Streptomyces} sp. CK4412, CK4412-001, CK4412-002 and CK4412-003 were grown for 7 days in R2YE medium and the samples were taken at 72 hr time-point. The mycelia were harvested by centrifugation and immediately stored in -40°C deep freezer. The frozen mycelia were broken by shearing in a mortar, and the frozen lysate was added to RLT buffer [Qiagen, Germany] in the presence of 1.0% 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, Germany] to eliminate possible chromosomal DNA contamination.

DNase I-treated RNA (7 μg) was used as a template for reverse transcription (RT) at 50 °C with an AVM Reverse Transcriptase XL [TaKaRa, Japan] and random hexamers. The conditions for cDNA synthesis were as follows: 30 °C for 10 min, 50°C for 1 hr, 99 °C for 2 min, 5 °C for 5 min. The resulting cDNA was used for PCR amplification under the following conditions: denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 68 °C for 35 sec for 30 cycles. Each primer pair of the TMC biosynthetic genes was carefully designed to generate a PCR product of approximately 100 to 200 bp using a genscript site (http://www.genscript.com/ssl-bin/app/primer). The complete primer sequences pairs are listed in Table 2.
Table 2. Used PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Description</th>
</tr>
</thead>
</table>
| PTwblA-F1 | ATCCGGATGAACTCGTTCGTTCAAGGAGCAGCGCA GAACCAATTCCGGGACGAGCTGACC                   | Forward primer for wblA
| PTwblA-R1 | CTCGTACTCCGAACGCTTCGCTTCCAGGAGGCCTGC GCCAGGAGGTGTGATGGCACTGTCGCC              | Reverse primer for wblA
| PTtmcT-F1 | AAGATACATGACTGAGAAGGAGATAGAGTCAAGTT ACTCAATTCCGGGATCCGCTGACC                   | Forward primer for tmcT
| PTtmcT-R1 | TCGTGTGCCTTACAGCGTGAGACCTGAGTTCAT GGAATGAGCAGCTGACGCTGCTC                     | Reverse primer for tmcT
| CPwblA-F2 | GGATCCTCCGAATCGCCTCGTGTGTT                                                    | Forward primer for wblA
| CPwblA-R2 | TCTAGAACTCGCGAGATCGCGCA                                                        | Reverse primer for wblA
| CPtmcT-F2 | CGGGATCCGGTGAATGCCGGCAGTGATAGA                                                 | Forward primer for tmcT
| CPtmcT-R2 | GCTCTAGAGCCTCACGAGCCAGCTCCGCTGCT                                               | Reverse primer for tmcT
| CHwblA-F3 | AGCCTCGATTCCGAGAGGAGGA                                                          | Forward primer for wblA
| CHwblA-R3 | ATGTAGTTTCTCGTACACCT                                                           | Reverse primer for wblA
| CHtmcT-F3 | TTTACCCGCATGTAACCCAT                                                           | Forward primer for tmcT
| CHtmcT-R3 | CGTCGGGACATGCAACGGTT                                                          | Reverse primer for tmcT
| RTtmcB-F4 | TCCGGTGTTGTCGAACTTGA                                                           | Forward primer for tmcB
| RTtmcB-R4 | GCACTCGGTGACTGGTCC                                                           | Reverse primer for tmcB
| RTtmcC-F4 | GTGCTGGTGTCGACTGGCAGGGTCACTT                                                   | Forward primer for tmcC
| RTtmcC-R4 | ATCTGGTGCAGGCGAGGCA                                                           | Reverse primer for tmcC
<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTtmcJ-F&lt;sup&gt;4&lt;/sup&gt;</td>
<td>CGAGACCCATCTCGTGCTGA</td>
<td>Forward primer for tmcJ</td>
</tr>
<tr>
<td>RTtmcJ-R&lt;sup&gt;4&lt;/sup&gt;</td>
<td>CGAGCGTCTTCATGGTGAG</td>
<td>Reverse primer for tmcJ</td>
</tr>
<tr>
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<td>TGACGCAATGTCCTGACGTG</td>
<td>Forward primer for tmcN</td>
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<tr>
<td>RTtmcN-R&lt;sup&gt;4&lt;/sup&gt;</td>
<td>GGACGAGACCCGGAGGAGTT</td>
<td>Reverse primer for tmcN</td>
</tr>
<tr>
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<tr>
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<tr>
<td>RTwblA-F&lt;sup&gt;4&lt;/sup&gt;</td>
<td>CCGTGGAGAGTTCGGCGTGT</td>
<td>Forward primer for wblA&lt;sub&gt;enc&lt;/sub&gt;</td>
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<tr>
<td>RTwblA-R&lt;sup&gt;4&lt;/sup&gt;</td>
<td>CCGTGGAGAGTTCGGCGTGT</td>
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<tr>
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<td>GCGGTGGAGAGGGTGACTA</td>
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<td>CACAGTTGGAGGTGCTGGA</td>
<td>Reverse primer for hrdB</td>
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1. Used for PCR targeting system; 2. Used for complementation and over-expression experiments; 3. Used for disruption check; 4. Used for Real-Time PCR.
3. Results and discussion

3.1. In silico analysis of pathway-specific regulatory gene, tmcT from Streptomyces sp. CK4412

Previously, we reported that a 1.3kb ORF (tmcT) located at the right-most flanking region of the cluster could be another 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 (Antón et al., 2004; Wilson et al., 2001). Notably, the predicted amino acid sequence of tmcT shows a putative Helix-turn-helix motif in the C-terminal region typically found in various bacterial DNA-binding proteins (Fig. 3A). Further characterization of the tmcT gene product via database-assisted in silico analysis revealed that tmcT encodes a protein that comprises 434 a.a (and shows 36 % identity at the amino acid level to StaR, a regulator of the LuxR family with C-terminal DNA binding domain in Streptomyces sp. TP-A0274 (Hiroyasu et al., 2002). In addition, tmcT contains two rare UUA leucine codons, which indicates its dependence on bldA, the structural gene for tRNA_{UUA}. This may also be further indication of a regulatory role of TmcT in TMC biosynthesis, since most of the known TTA-containing genes specify regulatory or resistance proteins associated with biosynthetic gene clusters for antibiotics (Chater and Chandra, 2008).

3.2. Cloning and sequence analysis of wblA ortholog from Streptomyces sp. CK4412

To isolate the wblA ortholog gene from Streptomyces sp. CK4412, a previously-constructed total genomic DNA library from the Streptomyces sp. CK4412 (Choi et al., 2007) was screened using PCR degenerate primers based on the highly conserved regions present in
both \textit{wblA} sequences from \textit{S. coelicolor} A3(2) and \textit{S. avermitilis} ATCC31780. After screening approximately 300 cosmids, one positive candidate, named pTMC2360, containing the entire \textit{wblA} ortholog gene (\textit{wblA}_{tmc}) was successfully selected. The complete sequence analysis of 390 bp-containing \textit{wblA}_{tmc} revealed a sequence encoding a 130 aa protein that showed a high degree of amino acid identity with the translated products of \textit{wblA} genes from \textit{S. coelicolor} (96%), \textit{S. avermitilis} MA-4680 (93%), \textit{S. griseus} NBRC 13350 (89%), and \textit{S. clavuligerus} ATCC 27064 (93%), all of which contain four conserved cysteines and HTH (Helix-Turn-Helix) motif (Fig. 3B).
Figure 3. (A) C-terminal sequence alignment of LuxR family with TmcT. (B) Amino acid sequence alignments of *Streptomyces* WhiB-family transcriptional regulators. Conserved (asterisk) and homologous (colon) amino acids were marked underneath. Grey box; cysteine conserved region.
3.3. TMC productivity stimulation via \textit{wblA}_{\text{tmc}} disruption from \textit{Streptomyces sp. CK4412} \\

Although \textit{in silico} sequence analyses of \textit{wblA}_{\text{tmc}} and \textit{tmcT} from \textit{Streptomyces sp. CK4412} are consistent with their regulatory roles in TMC biosynthesis, we sought to confirm the \textit{in vivo} function of \textit{wblA}_{\text{tmc}} and \textit{tmcT} using a gene disruption approach. Inactivation of \textit{wblA}_{\text{tmc}} or \textit{tmcT} was performed using a PCR-targeted gene disruption system (see Methods). pTMC2360 and pTMC2290 were replaced by an apramycin resistance/\textit{oriT} cassette generating pTMC2360\textDelta \textit{wblA} and pTMC2290\textDelta \textit{tmcT}, respectively, which were introduced into \textit{Streptomyces sp. CK4412} by conjugative gene transfer (Table 1, Fig. 2A, C). Construction of the \textit{wblA} and \textit{tmcN} mutants (named \textit{Streptomyces} sp. CK4412-001 and CK4412-003, respectively) generated by PCR-targeted disruption was confirmed by PCR analysis. The expected size of 0.36 kb and 1.7 kb for the PCR-amplified bands was observed in genomic DNA samples isolated from \textit{Streptomyces sp. CK4412}, while a band of the expected size (1.64 kb and 1.6 kb) was observed in genomic DNA samples isolated from \textit{Streptomyces sp. CK4412-001} and CK4412-003 (Fig. 2B, 2D). The expected size (0.6 kb and 0.7 kb) PCR-amplified fragment using an alternative PCR primer pair designed to detect an antibiotic resistance gene/\textit{oriT} cassette was observed only in the \textit{Streptomyces} sp. CK4412-001 and CK4412-003 (Fig. 2B, 2D), implying that the \textit{wblA} and \textit{tmcT} were specifically disrupted as expected.

Culture broths of \textit{Streptomyces} sp. CK4412, CK4412-001, CK4412-001/\textit{wblA}_{\text{tmc}} and CK4412/\textit{wblA}_{\text{tmc}} grown in MS media were extracted with ethyl acetate, followed by an antifungal bioassay and HPLC quantification for the presence of TMC. Although no noticeable differences in cell growths and phenotypes were observed in these strains cultured in both plate
and liquid cultures, *Streptomycyes* CK4412-001 produced approximately 2-fold more TMC compared with the *Streptomycyes* CK4412 (Fig. 4A). The significantly enhanced antifungal activity against *A. niger* in extracts of the *Streptomycyes* sp. CK4412-001 under the same culture condition was also observed (Fig. 5A). The *Streptomycyes* sp. CK4412-001 shows bald type (Fig. 5C). WblA_{tmc} is supposed to essentially role of sporulation in *Streptomycyes* sp. CK4412 which was likely the function of WhiB (Kim *et al*., 2007; Horinouchi, 2007). We conducted scanning electron microscope (SEM) analysis to examine morphological differentiation result of wild type and CK4412-001. Briefly, samples of each strain were prepared as described above, and the strains are incubated on MS agar for 6 days were fixed 2 hours at room temperature in 2% gluteraldehyde, 0.1 M phosphate buffer (pH7.4).

Moreover, an integrating conjugative vector pSET152, into which the coding region of *wblA_{tmc}* and 673 bp of its own upstream promoter region were cloned, was constructed to result in pSETHYG_{wblA}. Both HPLC and the bioassay confirmed that TMC productivity and antifungal activity were reduced back to the WT level in the *Streptomycyes* sp. CK4412-001 mutant strain carrying pSETHYG_{wblA} (Fig. 4A), implying that *wblA_{tmc}* plays a global antibiotics down-regulatory role in TMC biosynthesis.

### 3.4. Loss of TMC production via *tmcT* disruption from *Streptomycyes* sp. *CK4412*

Culture broths of both *Streptomycyes* sp. CK4412 and *Streptomycyes* sp. CK4412-003 grown under conditions optimal for TMC production were also extracted with chloroform, followed by an antifungal bioassay and HPLC quantification for the presence of TMC. The significantly reduced antifungal activity against *A. niger* as well as the absence of TMC in extracts of the *Streptomycyes* sp. CK4412-003 strain under the same culture conditions provide
strong evidence that \textit{tmcT} plays an essential regulatory role in TMC biosynthesis (Fig. 4C, 5B). To further prove that the inactivation of \textit{tmcT} was indeed responsible for TMC production, we complemented \textit{Streptomyces} sp. CK4412-002 by expressing \textit{tmcT} under the control of its own promoter. Both HPLC and the bioassay confirmed that TMC productivity and antifungal activity were significantly restored in the \textit{Streptomyces} sp. CK4412-003 mutant strain carrying pSETHYG\textit{tmcT} (Fig. 4C), implying that the absence of TMC productivity from \textit{Streptomyces} sp. CK4412-003 strain was due to a lack of a TMC-specific positive regulatory gene, \textit{tmcT}. Moreover, the wild type \textit{Streptomyces} sp. CK4412 strain containing an extra copy of \textit{tmcT} via chromosomal integration of pSETHYG\textit{tmcT} led to an approximately 20-fold increase in TMC biosynthesis (Fig. 4C).
Figure 4. (A) Control TMC standard. (B) TMC volumetric productivities measured by quantitative HPLC analyses from the ethyl acetate-extracted broths. (B) TMC volumetric productivities measured by quantitative HPLC analyses from the ethyl acetate-extracted broths. a and e, Streptomyces sp. CK4412; b, Streptomyces sp. CK4412-001; c, Streptomyces sp. CK4412-001/wblA-tmc; d, Streptomyces sp. CK4412/wblA-tmc; f, Streptomyces sp. CK4412-003; g, Streptomyces sp. CK4412-003/tmcT; h, Streptomyces sp. CK4412/tmcT.
Figure 5. (A) Comparison of antifungal activities between wild type and CK4412-001. a and b, day3 culture extracts; c and d, day 5 culture extracts; a and c, wild type; b and d, CK4412-001. (B) Comparison of antifungal activities between wild type and CK4412-003. e and f, day3 culture extracts; g and h, day5 culture extracts; i and j; day7 culture extracts; e, g and i; wild type; f, h and j, CK4412-003. (C) The effect of wblAtmc disruption on morphological differentiaction grown for 6 days. k, grown CK4412 (left) and CK4412-001 (right) on MS agar at 30°C; l, scanning electron microscope (SEM) analysis to examine morphological differentiation result of CK4412 (left) and CK4412-001 (right).
3.5. TMC biosynthetic regulatory cascade of global regulatory $wblA_{tmc}$ and two pathway-specific regulatory $tmcN$ and $tmcT$

To increase our understanding about the TMC biosynthetic regulatory network, RT-PCR transcript analyses of global regulatory $wblA_{tmc}$ and two pathway-specific regulatory $tmcN$ and $tmcT$ were performed. Total RNAs were prepared from the *Streptomyces* sp. CK4412, CK4412-001, CK4412-002, and CK4412-003 mutants after growth for 72 h and used as a template for gene expression analysis by RT-PCR. Primers for RT-PCR were specific to sequences within $tmc$ genes and were designed to produce cDNAs of approximately 200bp. A primer pair designed to amplify a cDNA of the $hrdB$ gene was used as an internal control. Transcripts from three biosynthetic genes such as $tmcB$, $tmcC$, $tmcJ$ and three regulatory genes such as $wblA_{tmc}$, $tmcN$ and $tmcT$ were analyzed after 30 PCR cycles. In the RT-PCR analysis, the transcripts of $tmcB$, $tmcC$ and $tmcJ$ genes, which located in three different putative operons within the cluster, were detected in the *Streptomyces* sp. CK4412, while the transcription patterns in the $tmcN$-deleted *Streptomyces* sp. CK4412-002 and $tmcT$-deleted *Streptomyces* sp. CK4412-003 mutants were not detected for all three genes. Moreover, the transcription pattern for the $wblA_{tmc}$-deleted CK4412-001 was significantly enhanced for all three biosynthetic genes (Fig. 5A). These results strongly suggest that TmcN and TmcT should act as pathway-specific positive regulators, and WblA$_{tmc}$ functions as a down-regulator in TMC biosynthesis. Although the transcript levels of $tmcN$ were comparable in all strains including the *Streptomyces* sp. CK4412-001, the $tmcT$ transcript was noticeably overexpressed only in $wblA_{tmc}$-deleted the *Streptomyces* sp. CK4412-001(Fig. 5A). These results suggest that the expression of $tmcT$ is strongly down-regulated by $wblA_{tmc}$ and the $tmcN$ is constitutively transcribed even in the
absence of \textit{wblA}_{\text{tmc}}$, implying that TMC biosynthetic regulation network is controlled by two pathway-specific positive regulatory genes, \textit{wblA}_{\text{tmc}}-independent \textit{tmcN} as well as \textit{wblA}_{\text{tmc}}-dependent in \textit{Streptomyces} sp. CK4412.(Fig. 5B)
Figure 6. (A) Gene expression analysis of the TMC biosynthetic gene cluster by RT-PCR. Analysis was carried out on *Streptomyces* sp. CK4412 (W), CK4412-001 (***), CK4412-002 (*) and CK4412-003 (**) as indicated in Experiments section. Transcription of the *hrdB* gene was also assessed as an internal control. The diagram indicates the organization of the genes within the TMC cluster and their putative transcripts. (B) Proposed regulatory network in TMC biosynthesis. Solid line, repression; dotted line, no repression; arrow, activation.
4. Conclusion

Despite the rapid progresses of genome sequencing and omics-driven analyses, regulatory network of secondary metabolite biosynthesis in *Streptomyces* species continue to demands genetic and biochemical dissection of specific regulators to elucidate their genetic targets and affects. The *wblA* ortholog (*wblA_{tmc}* in TMC-producing *Streptomyces* sp. CK4412 described here revealed that biological significance of *wblA_{tmc}* is quite similar to the previously-reported *wblAs* from various *Streptomyces* species, a key global antibiotic down-regulatory gene. The *in vivo* function of *wblA_{tmc}* using gene disruption and complementation approaches have proved that TMC production was significantly stimulated by the reduced transcription level of *wblA_{tmc}* in the *Streptomyces* sp. CK4412. Along with the previously-identified *tmcN*, another pathway-specific regulatory gene, *tmcT* described in this report is also confirmed via gene disruption and complementation analyses. The ability to increase titer of this important TMC compound by adding an additional copy of positive-regulatory *tmcT* or deletion of negative-regulatory *wblA_{tmc}* in the chromosome demonstrates the value of this strategy for *Streptomyces* strain improvement. Moreover, transcript analyses of the TMC biosynthetic and regulatory genes revealed that TMC biosynthetic regulation network is controlled by both *wblA_{tmc}*-dependent *tmcT* as well as *wblA_{tmc}*-independent *tmcN* in *Streptomyces* sp. CK4412. Thus, identification and characterization of *wblA* and *tmcT* in TMC biosynthesis from *Streptomyces* sp. CK4412 may lead to 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 analog development.
5. References


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