Precise cloning and tandem integration of large polyketide biosynthetic gene cluster using *Streptomyces* artificial chromosome system

Hee-Ju Nah, Min-Woo Woo, Si-Sun Choi and Eung-Soo Kim*

**Abstract**

**Background:** Direct cloning combined with heterologous expression of a secondary metabolite biosynthetic gene cluster has become a useful strategy for production improvement and pathway modification of potentially valuable natural products present at minute quantities in original isolates of actinomycetes. However, precise cloning and efficient overexpression of an entire biosynthetic gene cluster remains challenging due to the ineffectiveness of current genetic systems in manipulating large-sized gene clusters for heterologous as well as homologous expression.

**Results:** A versatile *Escherichia coli*-*Streptomyces* shuttle bacterial artificial chromosomal (BAC) conjugation vector, pSBAC, was used along with a cluster tandem integration approach to carry out homologous and heterologous overexpression of a large 80-kb polyketide biosynthetic pathway gene cluster of tautomycetin (TMC), which is a protein phosphatase PP1/PP2A inhibitor and T cell-specific immunosuppressant. Unique XbaI restriction sites were precisely inserted at both border regions of the TMC biosynthetic gene cluster within the chromosome of TMC-producing *Streptomyces* sp. CK4412, followed by site-specific recombination of pSBAC into the flanking region of the TMC gene cluster. The entire TMC gene cluster was then rescued as a single giant recombinant pSBAC by XbaI digestion of the chromosomal DNA as well as subsequent self-ligation. Next, the recombinant pSBAC construct containing the entire TMC cluster in *E. coli* was directly conjugated into model *Streptomyces* strains, resulting in rapid and enhanced TMC production. Moreover, introduction of the TMC cluster-containing pSBAC into wild-type *Streptomyces* sp. CK4412 as well as a recombinant *S. coelicolor* strain resulted in a chromosomal tandem repeat of the entire TMC cluster with 14-fold and 5.4-fold enhanced TMC productivities, respectively.

**Conclusions:** The 80-kb TMC biosynthetic gene cluster was isolated in a single integration vector, pSBAC. Introduction of TMC biosynthetic gene cluster in TMC non-producing strains has resulted in similar amount of TMC production yield. Moreover, over-expression of TMC biosynthetic gene cluster in original producing strain and recombinant *S. coelicolor* dramatically increased TMC production. Thus, this strategy can be employed to develop a custom overexpression scheme of entire metabolite pathway clusters present in actinomycetes.

**Keywords:** *Streptomyces* artificial chromosome, Pathway tandem integration, Polyketide biosynthetic gene cluster
within chromosomes, identification of the entire biosynthetic gene cluster is relatively straightforward. Unfortunately, some of these biosynthetic genes are derived from non-culturable or not amenable to genetic manipulation microorganisms and thus do not easily express the target compounds [3]. To bypass such intrinsic limitations and achieve functional expression of uncharacterized potentially-valuable natural product biosynthetic pathways, a relatively well-characterized heterologous host should be utilized [4, 5]. Recent genome mining approaches have also identified a significant number of metabolite biosynthetic gene clusters, some of which must be expressed in a heterologous host. Synthetic biology techniques have also made it possible to produce novel and/or improved natural products through reconstitution of biosynthetic gene clusters in an appropriate host system [3, 6–8].

Since the gene clusters responsible for the biosynthesis of microbial secondary metabolites are typically as large as 100 kb, appropriate vector systems capable of cloning an entire gene cluster as well as transferring these genetic segments between different hosts are necessary. Capturing an entire biosynthetic gene cluster in a single E. coli clone can facilitate the genetic manipulation of secondary metabolite biosynthetic pathways using the PCR-targeted gene replacement method [9–11]. Recently, a new E. coli-Streptomyces shuttle bacterial artificial chromosomal (BAC) vector system, pSBAC, which conveniently switches single-copy to high-copy replication in E. coli as well as utilizing the phage ΦBT1 attP-int site-specific integration system, was successfully used for the heterologous expression of a meridamycin (mer) biosynthetic gene cluster in S. lividans [12]. Specifically, to clone and express the entire mer cluster, a total genomic DNA library was first constructed through ligation of EcoRI-digested pSBAC and MfeI-digested total genomic DNA, followed by intergeneric conjugation of the mer cluster containing pSBAC into S. lividans [12]. Unique XbaI restriction sites were first inserted at both border regions of the TMC biosynthetic gene cluster within the chromosome of TMC-producing Streptomyces sp. CK4412, followed by site-specific recombination of pSBAC into the flanking region of the TMC gene cluster. Moreover, introduction of the TMC cluster-containing pSBAC into wild-type Streptomyces sp. CK4412 as well as a recombinant S. coelicolor strain resulted in a chromosomal tandem repeat of the entire TMC cluster with 40-fold enhanced TMC productivities. This strategy consisting of site-specific restriction site insertion, recombinant pSBAC plasmid rescue, intergeneric conjugation, and cluster tandem repeat introduction can be employed to develop a custom overexpression scheme of entire metabolite pathway clusters present in actinomycetes (Additional file 1: Table S1).

**Results**

**PCR-targeting of unique restriction enzyme sites into borders of TMC gene cluster**

An E. coli-Streptomyces BAC conjugation vector, pSBAC (Fig. 1), has been successfully applied for heterologous phosphatase PP1/PP2A inhibitor and T-cell-specific immunosuppressant [16–18]. Unique XbaI restriction sites were first inserted at both border regions of the TMC biosynthetic gene cluster within the chromosome of TMC-producing Streptomyces sp. CK4412, followed by site-specific recombination of pSBAC into the flanking region of the TMC gene cluster. Moreover, introduction of the TMC cluster-containing pSBAC into wild-type Streptomyces sp. CK4412 as well as a recombinant S. coelicolor strain resulted in a chromosomal tandem repeat of the entire TMC cluster with 40-fold enhanced TMC productivities. This strategy consisting of site-specific restriction site insertion, recombinant pSBAC plasmid rescue, intergeneric conjugation, and cluster tandem repeat introduction can be employed to develop a custom overexpression scheme of entire metabolite pathway clusters present in actinomycetes (Additional file 1: Table S1).

**Fig. 1** Map of pSBAC. Essential components of the vector are indicated. Ori2 and oriV, replication origins; SopA-C, partitioning system; aac(III)IV, apramycin resistance gene; oriT, origin of transfer; ΦBT1 attP-int, integration system; Unique restriction enzyme recognition sites, BamHI, HindIII, and EcoRI
expression of the entire meridamycin (mer) biosynthetic gene cluster [12]. The entire mer gene cluster (~95 kb) could be captured in a single pSBAC clone by straightforward restriction enzyme digestion due to the presence of unique restriction enzyme MfeI sites in border regions of the mer biosynthetic gene cluster. In contrast, most secondary metabolite biosynthetic gene clusters such as the TMC gene cluster do not possess unique restriction sites in border regions (Fig. 2a). To apply the pSBAC cloning system to metabolite gene clusters lacking unique restriction enzyme sites in their border regions, we inserted unique XbaI restriction enzyme sites into border regions of the TMC biosynthetic gene cluster in the Streptomyces sp. CK4412 chromosome using PCR-targeted gene insertion. For this, two DNA fragments, each containing a selection marker, oriT, and XbaI restriction enzyme site, were synthesized and precisely inserted into TMC border-containing cosmids, pTM2982 and pTM2290, in E. coli. The modified cosmids were then conjugated into Streptomyces CK4412, followed by target sequence-specific recombination at the borders of the TMC gene cluster (Fig. 2b). The resulting ex-conjugants were isolated based on the selection markers and confirmed to possess the correct XbaI insertions by PCR analysis and sequencing (Additional file 2; Fig. S1).

Precise cloning of entire TMC biosynthetic gene cluster as a single giant recombinant pSBAC

The typical cloning method for large-sized DNA fragment isolation requires extra care so as to avoid unintended DNA fragmentation. Alternatively, the in vivo plasmid rescue method can be used to isolate a particular chromosomal locus through recovery of adjacent DNA sequences [13–15]. Here, we applied the plasmid rescue technique using pSBAC in order to clone a large DNA fragment containing the TMC biosynthetic gene cluster. A 3480-bp tmcI DNA fragment containing the gene at the left end of the cluster was first cloned into attP-int deleted pSBAC plasmid (named pSATNI), followed by conjugation into Streptomyces sp. CK4412. The presence of the tmcI fragment allowed pSATNI vector to integrate into the left site of the TMC biosynthetic gene cluster as a result of targeted homologous recombination (Fig. 2c). Genomic DNA from kanamycin-resistant conjugants was isolated and digested by XbaI restriction enzyme. XbaI-digested total chromosomal DNA fractions were self-ligated, followed by transformation into E. coli cells. DNA was then isolated from the transformants and analyzed by PCR, restriction enzyme digestion, and sequencing. Analysis revealed that the entire TMC biosynthetic gene cluster was successfully cloned as a single recombinant pSBAC vector (Additional file 3: Fig. S2). Finally, the DNA fragment containing ΦBT1 attP-int was re-introduced into the rescued recombinant pSBAC vector and named pMMBL101 (Fig. 2d).

Heterologous expression of TMC biosynthetic gene cluster in Streptomyces strains

The newly formed pMMBL101 vector was conjugated into Streptomyces strains, including S. coelicolor M145 and S. lividans TK21. Both S. lividans and S. coelicolor have been successfully used for the heterologous expression of various Streptomyces secondary metabolite biosynthetic gene clusters. pMMBL101 was first transferred into S. lividans TK21 via conjugation, and the resulting transformant strain containing the tmc gene cluster was named S. lividans TMC002 (Fig. 3a). pMMBL101 was also introduced into S. coelicolor M145 by PEG-mediated transformation, resulting in S. coelicolor TMC003. These two recombinant strains along with wild-type strain were cultured in R5 media for 5 days. Although TMC was not detected in the 3-day wild-type culture, both S. lividans TMC002 and S. coelicolor TMC003 showed TMC production by day 3 (Fig. 3b). After 5 days of culture, TMC production levels in TMC002 and TMC003 were about 1.3-fold (4.05 mg/L) and 1.26-fold (3.91 mg/L) higher than that in wild-type (3.1 mg/L), respectively (Fig. 3b). These results reveal that the pSBAC-driven heterologous expression of an entire TMC biosynthetic gene cluster resulted in rapid and enhanced TMC production.

Homologous or heterologous tandem integration of entire TMC cluster

To further stimulate TMC productivity, an additional copy of the TMC cluster was introduced into the TMC single copy-containing wild-type Streptomyces sp. CK4412 and S. coelicolor TMC003 strains (Fig. 4). pMMBL101 was first introduced into Streptomyces sp. CK4412 by conjugation. Among the resulting ex-conjugants, four were randomly selected for further analysis by PCR amplification of attP-int-amplifying primer sets. PCR analysis showed that pMMBL101 integrated adjacent to the original tmc cluster in three of the four selected strains (named CK4412-TMC001), whereas pMMBL101 inserted into the attB site of the Streptomyces sp. CK4412 chromosome in only one strain. Chromosomal integration of pMMBL101 was confirmed by rapid draft genome sequencing. Total length of complete genome sequence was 9,803,578 bp. G + C content was determined to be 71.27 %. From the gene prediction results, 9141 CDSs were identified. The contig arrangements show that the pSBAC was inserted between two TMC biosynthetic gene clusters (Additional file 4: Fig. S3). Streptomyces sp. CK4412-TMC001 cultured in R5 media for 5 days showed a 14-fold increase in TMC production (34.47 mg/L) compared to the parental strain.
Fig. 2. Schematic description of pMMBL101 construction. TmcA and tmcB, type I polyketide synthase; tmcC-I and tmcP-Q, dialylic maleic anhydride moiety processing; tmcJ and tmcK, decarboxylase; tmcL, crotonyl-CoA reductase; tmcM and tmcT, pathway-specific regulator; tmcO, thioesterase; tmcR, cytochrome P450; tmcS, transporter. a Tautomycin structure and its biosynthetic gene cluster organization in Streptomyces sp. CK4412. b Insertion of XbaI recognition sequences into both flanking regions of tmc cluster via PCR-targeting system. c Modification of pSBAC and introduction of modified pSBAC (pSATNI) into Streptomyces sp. CK4412 chromosome. d XbaI digestion of CK4412 chromosome and self-ligation of digested chromosomal DNA to generate pTMC. After construction of pTMC, attP-int gene was inserted into AvrII recognition site of pTMC to generate pMMBL101.
Comparative qRT-PCR results also confirmed that transcription of three biosynthetic genes (tmcB, tmcC, and tmcJ) as well as two pathway-specific regulatory genes (tmcN and tmcT) was significantly stimulated in CK4412-TMC001 (Fig. 6). This implies that the presence of an additional copy of the entire TMC biosynthetic gene cluster was responsible for the increased transcription of TMC biosynthetic genes. To further verify introduction of a tandem repeat of the TMC cluster into a heterologous host, an apramycin-resistant gene of pMMBL101 was replaced by a spectinomycin/streptomycin-resistant gene to generate pMMBL102, followed by introduction into S. coelicolor TMC003 (named S. coelicolor TMC004). Similar to CK4412-TMC001 containing a tandem repeat of the TMC cluster, S. coelicolor TMC004 cultured in R5 media for 5 days also showed a 5.4-fold increase in TMC production (13.31 mg/L) compared to the original TMC-producing strain, S. sp. CK4412.
(2.47 mg/L) (Fig. 5), suggesting that pSBAC-driven introduction of a cluster tandem repeat was equally effective in both homologous and heterologous host systems.

Discussion

Isolation and characterization of a secondary metabolite pathway gene cluster in *Streptomyces* species can elucidate its molecular biosynthetic and regulatory mechanisms. Here, a secondary metabolite produced by *Streptomyces* sp. CK4412, originally isolated from Jeju Island, Korea, was identified as an activated T cell-specific immunosuppressive compound with novel pharmacological activities in both in vivo and in vitro studies, and its chemical structure was shown to be identical to that of tautomycetin (TMC), an antifungal compound with a structurally-unique ester bond linkage between its terminal cyclic anhydride moiety and linear polyketide chain [19]. TMC is believed to specifically block tyrosine phosphorylation of intracellular signal mediators downstream of Src tyrosine kinases in a T cell-specific manner via selective inhibition of protein phosphatase 1 (PP1) and PP2A [18, 20, 21]. However, the Src homology-2 domain containing protein tyrosine phosphatase-2 (SHP2) was also recently shown to be a putative target for the immunosuppressive activity of TMC [22]. Moreover,
were inserted into the genome flanking an S. coelicolor RsA and RsB as well as site-specific relaxase gene zouA [23]. In this case, the 35-kb actinorhodin biosynthetic gene cluster in S. coelicolor was engineered to catalyze tandem amplification of a secondary metabolite gene cluster in Streptomyces. Specifically, a site-specific recombination system was engineered to catalyze tandem amplification of a 35-kb actinorhodin biosynthetic gene cluster in S. coelicolor [23]. In this case, the oriT-like recombination sites RsA and RsB as well as site-specific relaxase gene zouA were inserted into the S. coelicolor genome flanking an actinorhodin biosynthetic gene cluster. Recombination between RsA and RsB was followed by zouA-dependent DNA amplification, resulting in an average of nine tandem repeats of the actinorhodin gene cluster per genome as well as a 20-fold increase in actinorhodin production [23]. Unfortunately, the actinorhodin cluster in a 110-kb AUD (amplifiable units of DNA) failed to overproduce actinorhodin, implying this approach may not be applicable to overexpression of a large-sized gene cluster in Streptomyces. Another study directly cloned a gene cluster via transformation-associated recombination (TAR) in yeast as an alternative approach [24]. Further, a 67-kb cryptic non-ribosomal peptide synthase biosynthetic gene cluster identified by genome mining of the marine actinomycete Saccharomonospora sp. CNQ-490 was cloned and expressed in S. coelicolor, resulting in production of taromycin A as a new antibiotic related to daptoycin [24]. Although a TAR-based system might be suitable for cloning and expression of a large cryptic gene cluster screened from actinomycetes genome mining, TAR-based cloning must be performed in yeast before intergeneric conjugation into Streptomyces. In addition, a tandem repeat of the target gene cluster in a heterologous host is not applicable in a TAR-based expression system. pSBAC was previously applied for cloning a 95-kb Streptomyces meridamycin biosynthetic gene cluster into a heterologous S. coelicolor host [12]. Although unique restriction enzyme MfeI sites were absent inside the mer cluster, they were present just adjacent to the mer cluster in the chromosome. However, most Streptomyces secondary metabolite gene clusters, including the tmc cluster, have no unique restriction enzyme sites in their border regions, which make the pSBAC system less attractive for heterologous Streptomyces expression. To overcome the limited applicability of the pSBAC-based heterologous expression system, we developed a general method for the site-specific introduction of unique restriction enzyme XbaI sites into border regions of the tmc biosynthetic gene cluster within the Streptomyces sp. CK4412 chromosome, as illustrated in Fig. 2. Using this approach, we demonstrated that any restriction enzyme site could be introduced at any specific location of the chromosome. Cloning of a large-sized DNA fragment is challenging due to its in vitro physical instability. An attractive alternative to conventional in vitro cloning is in vivo plasmid rescue. Instead of cloning the entire tmc gene cluster into the pSBAC vector in vitro, a small DNA fragment containing the flanking region of the tmc gene cluster, tmcI, was cloned into pSBAC and underwent in vivo homologous recombination. Simple XbaI digestion of total chromosomal DNA followed by self-ligation, E. coli transformation, and apramycin selection were performed to recover the giant recombinant pSBAC vector containing the entire tmc biosynthetic gene cluster. Finally,
**Conclusions**

Although actinomycetes continue to be a rich source of valuable secondary metabolites, their wild-type production levels are usually too low to be useful in the field of drug development. Here, we demonstrated an attractive genetic system for the efficient homologous and heterologous overexpression of a target cluster in *Streptomyces* species. Site-specific chromosomal integration of unique restriction sites as well as in vivo plasmid rescue of a *Streptomyces* bacterial artificial chromosome vector, pSBAC, containing a large biosynthetic gene cluster were performed for precise cloning and expression of the target cluster in a surrogate *Streptomyces* host. Re-introduction of a giant recombinant pSBAC vector into the chromosome of the *Streptomyces* host containing a single copy cluster resulted in a chromosomal tandem repeat of the entire TMC cluster with significantly enhanced TMC productivities. This approach demonstrates a platform technology for the precise cloning and functional overexpression of the entire biosynthetic gene cluster of any potentially-valuable low-titer metabolite in actinomycetes.

**Methods**

**Bacterial strains and culture media**

Various strains and plasmids used in this study are summarized in Table 1. *E. coli* strains were cultured at 37 °C in Luria–Bertani (LB) broth or on Luria–Bertani agar.

| Strain/plasmid | Relevant characteristics | Source/reference |
|----------------|--------------------------|------------------|
| **Plasmid** | | |
| pSBAC | aac III (IV), oriT, attP-int, backbone of pCC1BAC | [12] |
| pSATNI | Modified pSBAC which deleted attP-int and inserted Kan and tmcI fragment | This work |
| pTMC | pSATNI with 85 kb DNA insert containing whole tmc gene cluster | This work |
| pMMBL101 | pTMC with attP-int | This work |
| pMMBL102 | pMMBL101 which replaced AprR into SpeR | This work |
| **E. coli** | | |
| EPI300 | F- mcrA-D(xmr-hsdRMS-mcrBC)-trfA host for cloning and amplification of various BAC vectors and constructs | Epicenter |
| S17-1 | *E. coli* host for transferring various plasmids into *Streptomyces* via conjugation | |
| ET12567/ pUZ8002 | *E. coli* host for transferring various plasmids into *Streptomyces* via conjugation | |
| **Streptomyces sp.** | | |
| CK4412 | Original TMC-producing strain | [17] |
| CK4412-2XB | CK4412 with pSATNI and XbaI recognition sequences in both flanking region of TMC biosynthetic gene cluster | This work |
| TMC001 | pMMBL101-containing CK4412 | This work |
| **Streptomyces lividans** | | |
| TK21 | Non TMC-producing strain | This work |
| TMC002 | TK21 with pMMBL101 | This work |
| **Streptomyces coelicolor** | | |
| M145 | Non TMC-producing strain | This work |
| TMC003 | M145 with pMMBL101 | This work |
| TMC004 | pMMBL102-containing S. coelicolor TMC003 | This work |
supplemented with appropriate antibiotics [28]. *Streptomyces* sp. CK4412 was used as an original TMC-producing strain [17]. For production of TMC, all *Streptomyces* strains were grown at 28 °C in TSB media for 2 days and then cultured for 7 days in R5 media [17]. Modified ISP4 medium was used for conjugation while R2YE medium was used for PEG-mediated transformation.

**Insertion of unique XbaI recognition sequences in both flanking regions of tautomycin biosynthetic gene cluster**

To isolate the TMC biosynthetic gene cluster, unique XbaI recognition sequences were inserted into both flanking regions of the TMC biosynthetic gene cluster using a PCR-targeted gene disruption system [29]. Briefly, an apramycin resistance gene (aac(3)Iv)/oriT cassette and spectinomycin resistance gene (aadA)/oriT cassette were used to insert XbaI recognition sequences into both flanking regions. These cassettes were amplified from pIJ773 and pIJ778 using XbaI recognition sequence-containing primers and then introduced into *E. coli* BW25113/pIJ790 containing pTMC2982 or pTMC2290, resulting in pTMC2982::aac(3)Iv/oriT and pTMC2290::aadA/oriT, respectively. Insertion of XbaI recognition sequences was confirmed by PCR applied to mutated pTMC2982 and pTMC2290. The mutated cosmids pTMC2982::aac(3)Iv/oriT and pTMC2290::aadA/oriT were then introduced into *Streptomyces* sp. CK4412 by conjugation with *E. coli* ET12567/pUZ8002. Conjugation experiments were performed as described previously [30]. Conjugation was repeated using the isolated *Streptomyces* sp. CK4412::aac(3)Iv strains in order to insert the XbaI recognition sequence into the opposite flanking region. The desired double cross-over mutants, selected by their apramycin-resistant (or spectinomycin-resistant) and kanamycin-sensitive phenotypes, were isolated. Their genotypes were verified using PCR.

**Isolation of entire tautomycin biosynthetic gene cluster into pSBAC**

To isolate the entire TMC biosynthetic gene cluster from the chromosome by XbaI digestion and ligation, attP-int containing XbaI recognition sequence was removed from pSBAC by *AvrII* digestion and ligation. To select the right colony, a kanamycin resistance gene was ligated into *BamHI*-EcoRI-digested pSBAC. To select the modified pSBAC into the desired location by homologous recombination, a 3480-bp DNA fragment including a part of tmcl (tmcl1) was amplified by PCR using the pTMC2982 cosmid as a template. The amplified PCR products were then ligated into a RBC T&A cloning vector. The ligated vector was completely sequenced in order to ensure its integrity (Macrogen, Korea). The tmcl1 fragment, digested using *BamHI* and *HindIII*, was cloned into modified pSBAC to generate pSATNI. Conjugation was performed to integrate pSATNI into the chromosome by homologous recombination. The desired mutant (named CK4412-2XB) was selected on kanamycin-included MS agar medium, and its genotypes were verified using PCR.

CK4412-2XB strain was cultured at 28 °C in TSB media for 2 days, and preparation of genomic DNA of CK4412-2XB was carried out using a Wizard® genomic DNA purification kit (Promega). Genomic DNA was digested by restriction enzyme XbaI, purified, and concentrated by ethanol precipitation before self-ligation using T4 ligase (TaKaRa). After desalting, the ligation mixture was used for electroporation of *E. coli* EPI300. Recombinant colonies were selected onto apramycin- and kanamycin-containing LB medium. Plasmids were isolated by alkali denaturation and screened by PCR using randomly selected primers within the tmc cluster to identify pTMC.

A 2-kb DNA fragment containing the attP-int of ФВТ1 was amplified by PCR using pSBAC as a template and ligated into RBC T&A cloning vector. The ligated vector was completely sequenced to ensure integrity. The attP-int, digested using *AvrII*, was cloned into pTMC to generate pMMBL101.

**HPLC quantification and antifungal bioassay for TMC**

Extraction of TMC and HPLC analysis were carried out according to previously reported methods [30]. Briefly, culture broth was extracted twice using an equal volume of ethyl acetate, followed by concentration using a rotary evaporator. The final extracts were dissolved in methanol. Analytical HPLC was carried out on a Grace C18 4-μm column at a flow rate of 1 ml/min with UV detection at 273 nm.

Antifungal bio assay was performed for qualitative analysis of TMC production yield of tmcl-containing heterologous hosts. Evaluation of TMC production was carried out by the paper disc diffusion method. *Aspergillus niger* stock (1 ml) was inoculated onto ME agar medium, after which extract-soaked discs were placed onto the prepared medium. The plates were incubated at 30 °C for 2 days.

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

For RNA preparation, CK4412, CK4412-TMC001, *S. lividans* TMC002, *S. coelicolor* TMC003, *S. coelicolor* TMC004 were grown for 5 days in R5 medium, after which samples were taken at 120 h. Mycelia were harvested by centrifugation and stored in a −40 °C deep freezer after washing twice with distilled water. RNA preparation and RT-PCR were carried out according to previously reported methods [25]. Briefly, after the frozen mycelia were broken by shearing in a mortar, total RNA was isolated by using RNeasy mini kit...
[Qiagen, Germany]. DNase-I treated RNA, AVM Reverse Transcriptase XL [TaKaRa, Japan] and random hexamers was used for cDNA synthesis. HrdB gene was used as internal control. Transcripts from three biosynthetic genes such as tmcB, tmcC, and tmcJ were analyzed after 30 PCR cycles. Primers used for RT-PCR were previously reported [25].

Replacement of apramycin-resistant gene by spectinomycin-resistant gene and introduction into tmc-containing Strains

To introduce the tmc cluster into S. coelicolor TMC003, the apramycin-resistant gene of pMMBL101 was replaced by a spectinomycin-resistant gene (named pMMBL102) using a Quick & Easy BAC modification kit (GeneBridges). The Red/ET plasmid was introduced into pMMBL101-containing E. coli EPI300, after which BAC modification was performed according to the manufacturer’s guide using PCR to amplify the spectinomycin-resistant gene in the aprR-homologous region. Transformants were selected on spectinomycin-containing LB medium by PCR and confirmed. After pMMBL102 was transformed into E. coli S17-1, it was introduced into S. coelicolor TMC003 by conjugation.

Rapid genome sequencing of Streptomyces sp.

CK4412-TMC001

Genomic DNA of Streptomyces sp. CK4412-TMC001 was prepared from 3-days culture with a Wizard® Genome DNA Purification Kit (Promega). The genomic DNA was fragmented by dsDNA fragmentase to make proper size for library construction. Resulting DNA fragments was processed to Illumine Nextera DNA sample preparation kit (Illumina, Inc., USA) following manufacturer’s instruction. Final library was quantified by Bioanalyzer 2100 (Agilent, USA) and average library size was 300 bp.

The genomic library was sequenced by Illumina MiSeq (Illumina, Inc., USA). Generated paired-end sequencing reads (23,891,700 method reads) were assembled using CLC genomics workbench 6.0 (CLC bio, Denmark) and resulted in 253 contigs. The contigs and PCR-based long reads were combined through manual curation by using CodonCode Aligner 3.7.1 (CodonCode Corp., Dedham, MA, USA). The final plasmid sequence was corrected by rearranging with raw reads to check errors and dubious regions.

The coding sequences (CDS) were predicted by Glimmer 3.02 [31]. tRNA were identified by tRNA-SCAN-SE [32], and rRNA were searched using HMMER with EzTaxon-e rRNA profiles [33, 34]. The predicted CDSs were compared to catalytic families (catFam) and NCBI COG by rpsBLAST and NCBI reference sequences (Ref-Seq) and SEED databases by BLASTP for functional annotation [35–38].

Additional files

Additional file 1: Table S1. Primers used in this study.

Additional file 2: Figure S1. Confirmation of XbaI insertion in both flanking region of TMC biosynthetic gene cluster (A) Diagram of XbaI inserted both flanking region of tmc cluster (B) PCR analysis of constructed strain. Left, confirmation of XbaI insertion in head of tmc cluster; Right, confirmation of XbaI insertion in tail of tmc cluster; 1 and 2, PCR products from CK4412-T2DNA; 3 and 4, PCR products from pMMBL101 inserted CK4412-T2DNA; 2 and 4, XbaI-digested PCR products.

Additional file 3: Figure S2. Confirmation of pMMBL101 (A) PCR analysis using randomly selected tmc gene primers (B) Enzyme mapping using various restriction enzyme, C, uncut pMMBL101; 1, EcoRI; 2, EcoRV; 3, NdeI, 4, HindIII, 5, XbaI-digested pMMBL101; M, λ-HindIII DNA ladder.

Additional file 4: Figure S3. Sequenced contig organization compared with predicted tandem repeated CK4412-TMC001.

Authors’ contributions

H-J Nah carried out experiments, analyzed the primary data and drafted the manuscript. M-W Woo participated in the production analysis in heterologous hosts. S-S Choi participated in the data analysis. E-S Kim supervised the whole research work and revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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