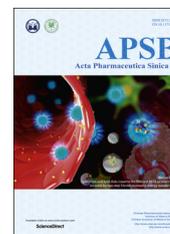




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ORIGINAL ARTICLE

Biosynthesis of antibiotic chuangxinmycin from *Actinoplanes tsinanensis*



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KEY WORDS

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Actinoplanes tsinanensis;
Biosynthesis gene cluster;
Heterologous expression;
Cytochrome P450;
Seco-chuangxinmycin;
C–S bond formation;
Sulfur incorporation

Abstract Chuangxinmycin is an antibiotic isolated from *Actinoplanes tsinanensis* CPCC 200056 in the 1970s with a novel indole-dihydrothiopyran heterocyclic skeleton. Chuangxinmycin showed *in vitro* antibacterial activity and *in vivo* efficacy in mouse infection models as well as preliminary clinical trials. But the biosynthetic pathway of chuangxinmycin has been obscure since its discovery. Herein, we report the identification of a stretch of DNA from the genome of *A. tsinanensis* CPCC 200056 that encodes genes for biosynthesis of chuangxinmycin by bioinformatics analysis. The designated *cxn* cluster was then confirmed to be responsible for chuangxinmycin biosynthesis by direct cloning and heterologous expressing in *Streptomyces coelicolor* M1146. The cytochrome P450 CxnD was verified to be involved in the dihydrothiopyran ring closure reaction by the identification of seco-chuangxinmycin in *S. coelicolor* M1146 harboring the *cxn* gene cluster with an inactivated *cxnD*. Based on these results, a plausible biosynthetic pathway for chuangxinmycin biosynthesis was proposed, by hijacking the primary sulfur transfer system for sulfur incorporation. The identification of the biosynthetic gene cluster of chuangxinmycin paves the way for elucidating the detail biochemical machinery for chuangxinmycin biosynthesis, and provides the basis for the generation of novel chuangxinmycin derivatives by means of combinatorial biosynthesis and synthetic biology.

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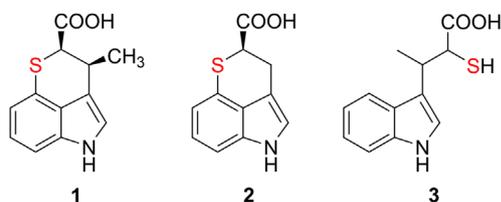


Figure 1 Chemical structures of chuangxinmycin (1), demethyl-chuangxinmycin (2) and seco-chuangxinmycin (3).

1. Introduction

Chuangxinmycin (1, Fig. 1), discovered in the 1970s by scientists from Institute of Antibiotics (now Institute of Medicinal Biotechnology), Chinese Academy of Medical Sciences, is a secondary metabolite containing a novel indole-dihydrothiopyran heterocyclic skeleton isolated from *Actinoplanes tsinanensis* CPCC 200056¹. It showed *in vitro* antibacterial activity against both Gram-negative and Gram-positive bacteria, and *in vivo* efficacy in mouse infection models against *Escherichia coli* and *Shigella dysenteriae*, as well as in preliminary clinical trials for septicemia, urinary and biliary infections caused by *E. coli*¹. Although chuangxinmycin did not come into clinical use, its unique antibacterial mechanism as a bacterial tryptophanyl tRNA synthetase (TrpRS) inhibitor² and the unusual heterocyclic skeleton with a sulfur have attracted the interests of pharmacologists and medicinal chemists, especially recently when the antibiotic-resistant bacteria such as ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) become a serious emerging problem.

Since the successful total organic synthesis of racemic chuangxinmycin in 1976³, many derivatives of chuangxinmycin were obtained, a few of which showed improved anti-microbial activities^{4–8}. However, biogenetic origins and synthetic mechanisms of chuangxinmycin have remained largely obscure for over 40 years. The preliminary investigation into the biosynthetic pathway for chuangxinmycin has started since its discovery. For example, Cao et al.⁹ identified a methyltransferase activity with specificity for 3-indolylpyruvate from *A. tsinanensis*, which might have played a role in chuangxinmycin biosynthesis. Xu et al.¹⁰ proposed that the sulfur atom in chuangxinmycin might come from cysteine by feeding [³⁵S]-cystine during the fermentation of *A. tsinanensis*. Zhou et al.¹¹ suggested vitamin B12 is indispensable for the biosynthesis of chuangxinmycin. Since then, no significant progress has been achieved on chuangxinmycin biosynthesis.

Structurally, chuangxinmycin bears some resemblance to indolmycin, another known inhibitor of TrpRS¹². It is also somehow similar to L-tryptophan, the substrate of TrpRS. Therefore, tryptophan is generally regarded as the precursor for chuangxinmycin biosynthesis. On the other hand, sulfur is essential and ubiquitous in living systems, thus the incorporation of sulfur atom is the most remarkably fascinating biosynthetic feature. Although sulfur atom appears in many primary and some secondary microbial metabolites, knowledge about the intriguing enzymatic machinery driving the incorporation of sulfur atom into microbial secondary metabolites remains limited. Recently, Hungwen Liu's group¹³ disclosed a strategy of secondary metabolite biosynthesis by hijacking the primary sulfur-delivery system including sulfur carrier protein (SCP), SCP-activating enzyme, etc. Wen Liu's

group¹⁴ established the metabolic coupling of two small-molecule thiols, mycothiol and ergothioneine, in the biosynthesis of lincomycin A. These findings provide valuable clues for analyzing the biosynthesis pathway of dihydrothiopyran ring which will facilitate the identification of biosynthetic gene cluster of chuangxinmycin in the genome of *A. tsinanensis* CPCC 200056.

The advent of next-generation sequencing technologies has revolutionized the search for novel antibiotic biosynthetic gene cluster which is of great importance in proposing a plausible biosynthetic pathway in its producing microorganism. In this work, we report the successful mapping of putative chuangxinmycin biosynthetic gene cluster (*cxn*) in the genome of *A. tsinanensis* CPCC 200056 by searching adjacent genes encoding: (1) TrpRS, which is supposed to be a self-resistant mechanism for chuangxinmycin producer; (2) aminotransferase and methyltransferase, which are responsible for the transformation of L-tryptophan; and (3) protein members of primary sulfur transfer system or low-molecular-mass thiol biotransformations, which might be involved in the incorporation of sulfur atom. We describe the identification and characterization of the *cxn* biosynthesis gene cluster and propose a rational and convincing chuangxinmycin biosynthetic pathway in *A. tsinanensis* CPCC 200056.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

All the strains and plasmids used in this study are listed in Table 1^{1,15–21}. The chuangxinmycin-producing strain *A. tsinanensis* CPCC 200056, obtained from China Pharmaceutical Culture Collection, was grown at 28 °C on solid ISP2²² medium for sporulation and fermentation. The medium 65 plates (4 g/L of glucose, 4 g/L of yeast extract, 10 g/L of malt extract, 2 g/L of CaCO₃, 12 g/L of agar, pH 7.2)²³ were used for conjugation between *A. tsinanensis* and *E. coli*. The heterologous expression host strain *Streptomyces coelicolor* M1146¹⁵ and its derivatives were cultured at 28 °C on Mannitol soya flour (MS) agar²⁴, which was also used for conjugation between *Streptomyces* and *E. coli*. Liquid phage medium²⁵ were used for isolation of genomic DNA. *E. coli* DH5α¹⁷ was used as a host for general cloning experiments. *E. coli* ET12567/pUZ8002¹⁸ were used for conjugal transfer according to the established protocol. All of the *E. coli* strains were incubated in Luria–Bertani medium (LB) at 37 °C. Yeast YPAD medium containing 1% yeast extract, 2% peptone and 2% dextrose supplemented with 0.01% adenine hemisulfate was used to grow *Saccharomyces cerevisiae* VL6-48 (*MATα*, *his3-Δ200*, *trp1-Δ1*, *ura3-52*, *lys2*, *ade2-101*, *met14*, *psi+cir⁰*)¹⁶ at 30 °C, which was used as the host for DNA assembler. Synthetic tryptophan dropout agar (SD-Trp agar, purchased from Clontech, California, USA) was used to select yeast transformants containing the assembled biochemical gene clusters of interest. Apramycin (Am, 50 μg/mL), kanamycin (Km, 50 μg/mL), chloramphenicol (Cm, 30 μg/mL), hygromycin B (Hyg, 200 μg/mL) and nalidixic acid (25 μg/mL) were used for selection of *E. coli* and *Streptomyces* recombinant strains.

2.2. Genomic DNA extraction and genome sequencing

Total DNAs were extracted from *A. tsinanensis* CPCC 200056 grown in liquid phage medium at 28 °C for 48 h. Mycelium was

Table 1 Strains and plasmids used in this study.

Strains/plasmid	Relevant characteristic	Ref.
Strain		
<i>A. tsinanensis</i> CPCC 200056	Wild-type strain (chuangxinmycin-producing strain)	1
<i>S. coelicolor</i> M1146	The heterologous expression host strain, Δact , Δred , Δcpk , Δcda	15
<i>S. coelicolor</i> M1146/pCAP-CM ($\Delta cxnD$)	A copy of <i>cxn</i> biosynthetic gene cluster* (<i>cxnD</i> inactivated) was integrated on the chromosome of <i>S. coelicolor</i> M1146, Am ^r	This study
<i>S. coelicolor</i> M1146/pCAP-CM	A copy of <i>cxn</i> biosynthetic gene cluster* (<i>cxnD</i> inactivated) and a complement coding region of <i>cxnD</i> were integrated on the chromosome of <i>S. coelicolor</i> M1146, Am ^r , Hyg ^r	This study
<i>S. coelicolor</i> M1146/pCAP-CM ($\Delta cxnA\sim F$)	The majority of genes (<i>cxnA\sim F</i>) in <i>cxn</i> cluster were deleted in <i>S. coelicolor</i> M1146, Am ^r	This study
<i>S. cerevisiae</i> VL6-48	The homologous recombination host for DNA assembly, MAT α , <i>his3-Δ200</i> , <i>trp1-Δ1</i> , <i>ura3-52</i> , <i>lys2</i> , <i>ade2-101</i> , <i>met14</i> , <i>psi+cir</i> ^o	16
<i>E. coli</i>		
DH5 α	General cloning host	17
ET12567/pUZ8002	Donor strain for intergeneric conjugation between <i>E. coli</i> and <i>S. coelicolor</i> and <i>A. tsinanensis</i> , Cm ^r , Km ^r	18
Plasmid		
pCAP01	Gene cluster capture vector; ARSH4/CEN6, pUC <i>ori</i> , <i>aph(3)III</i> , ϕ C31 <i>intlattP</i> , <i>oriT</i> (RP4)	16
pCAP-CM-LR	The <i>cxn</i> gene cluster specific capture vector with two homology arms corresponding to flanking regions of the target gene cluster into pCAP01, Km ^r	This study
pCAP01-CM	pCAP01 carrying the entire <i>cxn</i> biosynthetic gene cluster* (<i>cxnD</i> inactivated), Km ^r	This study
pCAP-CM($\Delta cxnD$)	A derivative of pCAP01-CM, Am ^r	This study
pCAP-CM($\Delta cxnA\sim F$)	pCAP-CM($\Delta cxnD$) derivative plasmid with deletion of the majority of <i>cxn</i> genes (<i>cxnA\sim F</i>), Am ^r	This study
pIJ10500	<i>Streptomyces</i> integrative vector containing ϕ BT1 integrase gene (<i>int</i>) and its attachment site (<i>attP</i>), Hyg ^r	19
pIJ-CxnD	pIJ10500 derivative plasmid containing complete coding region of <i>cxnD</i> downstream of <i>ermE</i> *p, Hyg ^r	This study
pSET152	<i>Streptomyces</i> integrative vector consisting of the ϕ C31 integrase gene (<i>int</i>) and its attachment site (<i>attP</i>), Am ^r	20
pL646	pSET152 derivative containing the constitutive promoter <i>ermE</i> *p, Am ^r	21
pL-CxnR	pL646 derivative plasmid containing 942 bp complete coding region of CxnR, Am ^r	This study

Am^r, apramycin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Hyg^r, hygromycin B resistance; *S. coelicolor*, *Streptomyces coelicolor*; *A. tsinanensis*, *Actinoplanes tsinanensis*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *E. coli*, *Escherichia coli*.

harvested and washed with STE buffer (0.3 mol/L sucrose, 25 mmol/L Tris-HCl, 10 mmol/L EDTA, pH 8.0), then was lysed by STE buffer plus 5 mg/mL lysozyme and 1% SDS. Phenol-chloroform solution was used to remove proteins. The total DNAs was digested with DNase-free RNase A to remove RNAs, purified by isopropanol precipitation. Genomic DNA was sequenced with Pacific Biosciences (PacBio) RSII SMRT technology and Illumina HiSeq. 4000 platform (commissioned to Beijing Genomics Institute, Shenzhen, China). The genome was directly assembled by SMRT Analysis v2.3.0²⁶ based on the subreads obtained from Pacbio RSII platform. SOAPsn²⁷, SOAPindel²⁸ and GATK²⁹ were used to correct the errors found in the assembled sequence based on the data obtained from Illumina HiSeq. 4000 platform. Putative protein-coding sequences (CDSs) were predicted using Glimmer 3.0³⁰. Gene functional annotation was performed using BLASTP with KEGG, COG, Swiss-Port, TrEMBL, NR and GO databases.

2.3. Intergenic conjugation between *E. coli* and *A. tsinanensis*

The vector pL646²¹, a pSET152²⁰-derived expression plasmid containing a ϕ C31 *attP-int* locus, was used for intergeneric conjugation between *E. coli* and *A. tsinanensis* (Table 1). A

942 bp DNA fragment containing the complete *cxnR*-coding region was amplified using pL-CxnR-F and pL-CxnR-R as PCR primers (Table S1 in Supplementary information), and cloned into the *Nde*I and *Bam*HI sites of pL646, under the control of a strong constitutive promoter *ermE**p, to give pL-CxnR. Then it was transferred into methylase-negative strain *E. coli* ET12567/pUZ8002 which was used as the *E. coli* donor strain. The *A. tsinanensis* recipient strain was cultivated in 100 mL of CRM medium²⁴ in a 500-mL baffled Erlenmeyer flask which was inoculated with one cultured square (about 1 cm \times 1 cm in size). The incubation was carried out at 28 °C for 5 days on a rotary shaker (Zhicheng, Shanghai, China) at 200 rpm. The culture was diluted 1 to 10 and further incubated in 50 mL of tryptic soy broth (TSB) medium (BD, Franklin Lakes, USA) for 16 h under the same conditions. Then, the mycelium was homogenized and diluted 1 to 5 (10 mL TSB plus 2.5 mL of mycelium), and incubation was carried out for from 1 h to a maximum of 5 h under the conditions described above. For the conjugation approach, the cells were pelleted by centrifugation (5000 \times g, 10 min) and resuspended in 2 mL of TSB. A total of 200 μ L of *A. tsinanensis* cells (approximately 10⁸ cells/mL) and 200 μ L of the *E. coli* donor strain (a washed overnight culture; approximately 10⁸ cells/mL) were mixed and then spread onto medium 65 plates containing 20 mmol/L MgCl₂. After 16–18 h, the plates were

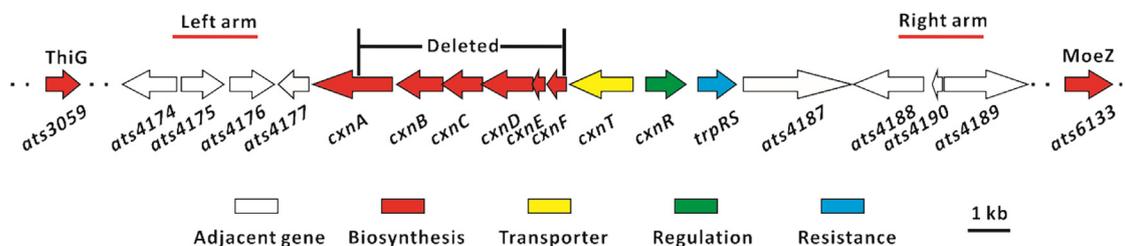


Figure 2 Organization of the chuangxinmycin biosynthetic gene cluster (*cxn*) of *A. tsinanensis*. Left arm and right arm represent the capture arms in the direct cloning of the cluster. “Deleted” represents the deletion fragment in pCAP-CM(Δ *cxnA*~*F*).

overlaid with 3 mL of H₂O containing nalidixic acid and apramycin (*A. tsinanensis* is intrinsically resistant to apramycin). After 5–7 days, the transconjugants were picked onto ISP2 agar containing apramycin at 28 °C for 7 days and then inoculated into liquid phage medium for isolation of the genomic DNA for PCR verification using pSET152 and attB_Streptomyces as primers (Table S1).

2.4. Chuangxinmycin biosynthetic gene cluster assembly using DNA assembler

The capture vector pCAP01¹⁶ has three elements which were the yeast element consisting of ARSH4/CEN6 (replication origin) and TRP1 auxotrophic marker, the *E. coli* element containing the pUC *ori* from SuperCos1 (Stratagene, La Jolla, USA) and the *Streptomyces* element consisting of the ϕ C31 integrase gene (*int*) and its attachment site (*attP*), origin of DNA transfer (*oriT*), and kanamycin resistance gene (*aph(3)III*), respectively. The target cluster-specific capture vector was constructed by introducing into the vector two short homology arms (~2 kb) corresponding to flanking regions of the target gene cluster. Two arms and gene cluster fragments were amplified from the genomic DNA of *A. tsinanensis* using primers whose sequences are listed in Table S1. The two PCR amplified capture arms were introduced into pCAP01 by restriction/ligation, yielding the target cluster-specific capture vector pCAP-CM-LR. Then the “circular” construct was digested with *EcoRI*, and dephosphorized with calf intestine alkaline phosphatase, generating linear capture vector flanked by 2 kb capture arms at both ends (Fig. 2). To ensure high efficiency of yeast homologous recombination, each fragment was built to generate a 100–800 bp overlap region with adjacent fragments. 500–600 ng of each individual cassette/fragment (~4.0 kb) and 2 μ g linear capture vector were mixed and precipitated with ethanol. The resulting DNA pellet was air-dried and resuspended in 4 μ L of Milli-Q double deionized water. The concentrated mixture of DNA was electroporated into *S. cerevisiae* using the protocol reported elsewhere³¹. Desired transformants were selected on synthetic tryptophan dropout agar (SD-Trp agar) at 30 °C for 2–3 days and firstly identified by colony PCR. The correct clones with the target cluster were further confirmed by restriction analysis. Cells were spheroplasted using Zymolyase-20T (MP Biomedicals, Irvine, USA) at 37 °C for 0.5–1 h and harvested by centrifugation at 2000 \times g for 10 min. Then the plasmid DNA was purified following the protocols of HiPure BAC DNA Kits (Magen, Guangzhou, China) and introduced into *E. coli* DH5 α by chemical transformation or electroporation. The plasmids were then purified from Km^r *E. coli* clones, and confirmed by restriction mapping/analysis with *SpeI*, *NdeI*, *EcoRI*, *KpnI* and *XhoI*. The yielded construct was designated as pCAP01-CM.

2.5. Heterologous expression in *S. coelicolor* M1146

To facilitate the following conjugation, the kanamycin resistance gene *aph(3)III* in pCAP01-CM was replaced by apramycin resistance gene *aac(3)IV* using PCR-targeting. Then the new plasmid was sequenced and a point mutation was present (the GAA codon of Glu₁₄₉ in CxnD was changed to termination codon TAA) which led to early termination of CxnD translation, so it was designated as pCAP-CM(Δ *cxnD*). When pCAP-CM(Δ *cxnD*) was digested by *EcoRI*, the 23 kb DNA fragment (with the deletion of *ats4178*~*4183*) was extracted from agarose gel and ligated by itself, produced the plasmid pCAP-CM(Δ *cxnA*~*F*) deleting the majority of biosynthetic genes. The pCAP-CM(Δ *cxnD*) and pCAP-CM(Δ *cxnA*~*F*) were introduced into *E. coli* ET12567/pUZ8002 and transferred to *S. coelicolor* M1146 by intergeneric conjugation according to a standard protocol²⁴. Am^r exconjugants were selected on MS agar containing nalidixic acid and apramycin at 28 °C and then inoculated into liquid phage medium for isolation of the genomic DNA. The correct exconjugants were verified by PCR (Fig. S2) and designated as *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*) and M1146/pCAP-CM(Δ *cxnA*~*F*), respectively. The early termination of CxnD may cause functional inactivation in *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*), so the complete coding region of *cxnD* was introduced into *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*) for functional complementation. The strong constitutive promoter *ermE**_p and coding region of *cxnD* were cloned to vector pIJ10500¹⁹ by Gibson assembly kit (NEB, Ipswich, USA), resulting the plasmid pIJ-CxnD. Then it was conjugated into *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*) to get heterologous expression strain *S. coelicolor* M1146/pCAP-CM.

2.6. Production and analysis of chuangxinmycin and its derivatives

A. tsinanensis CCCC 200056, *S. coelicolor* M1146 and their derivatives were fermented on ISP2 medium at 28 °C for 7 days. Every culture on ISP2 plate was cut into squares (about 1 cm \times 1 cm in size), and extracted once with 2-fold ethyl acetate overnight. The ethyl acetate was concentrated to the same volume (250–500 μ L). The point injection volume during the silica gel TLC analysis was 5 μ L. The developing solvent system includes ethyl acetate/*n*-hexane/dichloromethane/glacial acetic acid (9:7:6:0.2, v/v/v/v), observed at 254 and 365 nm respectively. The final ethyl acetate extract was filtered with organic membrane filter of 0.22 μ m and the filtrate was analyzed by HPLC. The HPLC conditions were as follows: XSelect CSHTM C18 column (150 mm \times 4.6 mm, 5 μ m, Waters, Ireland), or Eclipse Plus C18 column (150 mm \times 4.6 mm, 5 μ m, Agilent, USA), or CAPCELL PAK ADME column (250 mm \times 4.6 mm, 5 μ m, SHISEIDO, Japan), mobile phase MeCN–H₂O (water phase containing 0.1% glacial acetic acid), 15%–100%, 30 min, flow rate of 1.0 mL/min, detection

Table 2 Proposed chuangxinmycin biosynthetic gene cluster and gene functions.

Gene	<i>cxn</i> name	Size (aa)	Protein homologue	Identity/Similarity (100%)	Proposed function
<i>ats3059</i>		264	ThiG, thiazole synthase (WP_055570175.1), <i>Streptomyces atriruber</i>	94/96	Thiazole synthase
<i>ats4175</i>		331	Hypothetical protein (WP_047121450.1), <i>Streptomyces leeuwenhoekii</i>	55/63	HTH_XRE family transcriptional regulator
<i>ats4176</i>		346	Peptidoglycan-binding protein (WP_044378158.1), <i>Streptomyces cyaneogriseus</i>	72/83	Peptidoglycan-binding protein
<i>ats4177</i>		240	Hypothetical protein (WP_086718244.1), <i>Streptomyces angustmyceticus</i>	81/85	Associated to methylation
<i>ats4178</i>	<i>cxnA</i>	623	Maturation radical SAM protein 1 (WP_063754376.1 RiPP), <i>Streptomyces</i> sp. NRRL S-1813	93/96	Radical SAM protein, methylation
<i>ats4179</i>	<i>cxnB</i>	362	ThnJ, aminotransferase (AMR44310.1), <i>Streptomyces</i> sp. FXJ1.172	52/63	Aminotransferase
<i>ats4180</i>	<i>cxnC</i>	318	2-Dehydropantoate 2-reductase (WP_030988196.1), <i>Streptomyces</i> sp. NRRL S-1813	87/90	Reductase
<i>ats4182</i>	<i>cxnD</i>	404	ThnC, P450 (AMR44303.1), <i>Streptomyces</i> sp. FXJ1.172	37/48	Cytochrome P450
<i>ats4181</i>	<i>cxnE</i>	100	MoaD family protein (KRT67113.1), <i>Candidatus</i> Dadabacteria CSP1-2	43/71	Sulfur-carrier protein
<i>ats4183</i>	<i>cxnF</i>	238	ThnF, hypothetical protein (AMR44306.1), <i>Streptomyces</i> sp. FXJ1.172	44/61	JAMM (JAB1/MPN/Mov34) metalloprotease
<i>ats4184</i>	<i>cxnT</i>	496	MFS transporter (WP_051818417.1), <i>Streptomyces</i> sp. NRRL S-1813	86/90	Transporter
<i>ats4185</i>	<i>cxnR</i>	313	LysR family transcriptional regulator (AJK58337.1), <i>Amycolatopsis lurida</i> NRRL 2430	37/50	Pathway-specific regulator
<i>ats4186</i>	<i>trpRS</i>	300	Ind0, tryptophanyl-tRNA synthetase (AJT38681.1), <i>Streptomyces griseus</i> subsp. <i>griseus</i>	73/83	Tryptophan-tRNA synthetase
<i>ats4187</i>		846	Membrane protein (ANZ15560.1), <i>Streptomyces noursei</i> ATCC 11455	39/52	Membrane protein
<i>ats4188</i>		548	Sodium/proline symporter (WP_037838132.1), <i>Streptomyces</i> sp. NRRL S-337	68/81	Transporter
<i>ats4190</i>		79	No homologous protein		Unknown
<i>ats6133</i>		392	Adenylyltransferase/sulfurtransferase MoeZ (WP_067275253.1), <i>Streptomyces jeddahensis</i>	98/98	SCP-activating enzyme

wavelength of 254 nm and column temperature of 25 °C. LC–MS analysis was monitored by HPLC as described above (except the flow rate decreased to 0.8 mL/min) coupled to an ESI mass spectrometer (1290 (LC)-1956 single quadrupole MS (Agilent, USA) or 1100 (LC)-6300 MSD Trap MS (Agilent, USA)). Electrospray ionization (negative ionization) in Ultra Scan mode with a capillary voltage of 3.5 kV and a heated capillary temperature of 325 °C was used for LC–MS analysis. The compound **3** (Fig. 1) was prepared by HPLC chromatography as described above from the ISP2 culture of *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*). The HR-ESI-MS and HR-ESI-MS/MS analysis of the standard compound **1** (chemically synthesized³) and **3** was achieved by a QSTARTM Elite LC–MS/MS system (Applied Biosystems/MSD Sciex, Singapore).

3. Results and discussion

3.1. Bioinformatic identification of the chuangxinmycin biosynthesis gene cluster

The genome sequence of *A. tsinanensis* CPCC 200056 was obtained using a third generation sequencing platform Pacbio

RSII, together with a second generation sequencing platform Illumina Hiseq. 4000, and yielded approximately 7.7 Mb of contiguous sequence plus one 13.5 kb circular plasmid with a GC content of 70.3% and 69.0%, respectively. The genome of CPCC 200056 totally contains 7060 protein coding genes, accounting for 87.03% of the genome.

Chuangxinmycin is a potent and selective inhibitor of bacterial tryptophanyl tRNA synthetase (*TrpRS*)², thus we speculated that a tryptophanyl-tRNA synthetase gene (*trpRS*) might colocalize with the chuangxinmycin biosynthetic genes, as resistant and biosynthetic genes for a specific secondary metabolite are usually clustered in bacteria. Manual BLASTP searching of the *A. tsinanensis* CPCC 200056 genomic data uncovered two putative *trpRS* genes throughout the genome, and *ats4186* drew our attention. Sequence analysis of its neighboring genes showed the presence of an aminotransferase gene (*ats4179*) with significant similarity with the tryptophan aminotransferase (*ind8* and *orf2651*) involved in the indolmycin biosynthetic pathway³². The biosynthetic gene cluster of indolmycin, another known inhibitor of *TrpRS*, has recently been identified, suggesting the involvement of a tryptophan aminotransferase (*ind8* and *orf2651*) as the first step

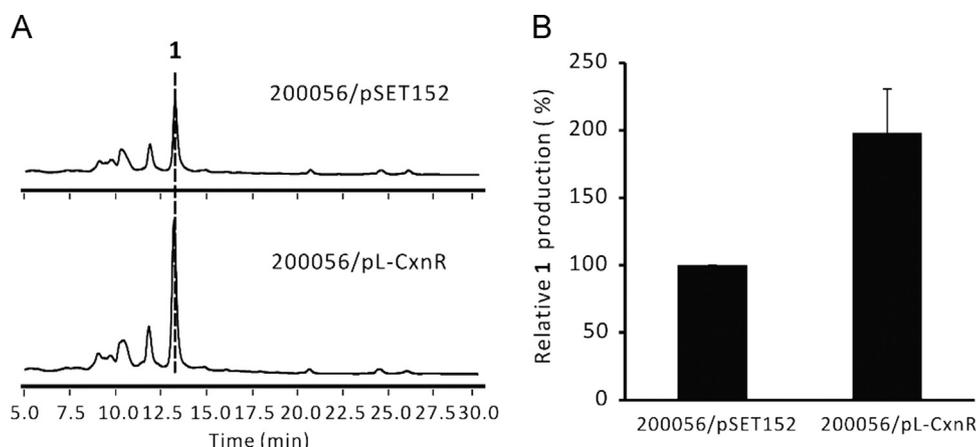


Figure 3 Comparison of chuangxinmycin production between overexpression strain 200056/pL-CxnR and control strain 200056/pSET152. (A) The HPLC profile of representative conjugant of 200056/pL-CxnR and 200056/pSET152; (B) Quantitative comparison of **1** production (statistical results of 4 conjugants of 200056/pSET152 and 8 conjugants of 200056/pL-CxnR, respectively).

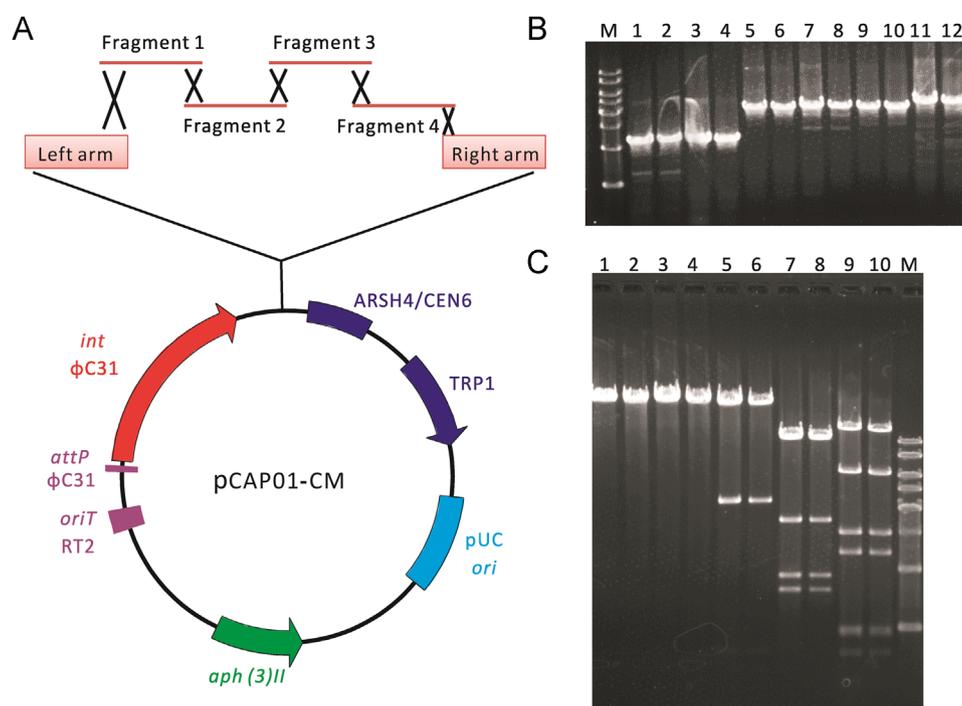


Figure 4 Direct cloning of the *cxn* cluster by DNA assembler. (A) Schematic representation for the construction of pCAP01-CM; (B) PCR verification of pCAP01-CM isolated from *E. coli* DH5 α . Lane M: 1 kb plus DNA ladder (10,000, 8000, 6000, 5000, 4000, 3000, 2000, 1500, 1000, 800, 500, 300 bp); Lane 1~2: amplification of the left arm (2265 bp); Lane 3~4: amplification of the right arm (2225 bp); Lane 5~6: amplification of the fragment 1 (4003 bp); Lane 7~8: amplification of the fragment 2 (4101 bp); Lane 9~10: amplification of the fragment 3 (4049 bp); Lane 11~12: amplification of the fragment 4 (4388 bp); (C) Restricted enzymatic analysis of pCAP01-CM isolated from *E. coli* DH5 α . Lane 1~2: *Spe*I (28,242 bp); Lane 3~4: *Nde*I (28,242 bp); Lane 5~6: *Eco*RI (23,132, 4336 bp); Lane 7~8: *Kpn*I (10,834, 10,483, 3340, 1884, 1601 bp); Lane 9~10: *Xho*I (12,553, 6319, 3031, 2415, 1002, 951, 714, 444, 399, 237, 141 bp); Lane M: 1 kb DNA ladder (10,000, 8000, 6000, 5000, 4000, 3000, 2000, 1000 bp).

in its biosynthetic pathway to convert tryptophan to indolepyruvate. More intriguing, an SCP gene (*ats4181*) was present in the neighborhood, suggesting its possible involvement in the sulfur incorporation into the chuangxinmycin molecule. Thus, we hypothesized that this gene cluster, named the *cxn* cluster, is potentially involved in the chuangxinmycin biosynthetic pathway.

The organization of *cxn* cluster is shown in Fig. 2 and the proposed function of each ORF is given in Table 2. Sequence

analysis of this gene cluster predicts that *cxnA* (*ats4178*) encodes a cobalamin (vitamin B12)-dependent radical *S*-adenosylmethionine (SAM) methyltransferase. *cxnB* (*ats4179*) encodes a pyridoxal 5'-phosphate (PLP)-dependent aminotransferase. *cxnC* (*ats4180*) is homologous to dehydropantoate reductase. *cxnD* (*ats4182*) encodes a cytochrome P450. *cxnE* (*ats4181*) encodes an SCP gene. *cxnF* (*ats4183*) encodes a protein with a MPN domain, belonging to JAMM (JAB1/MPN/Mov34) metalloprotease family³³. *cxnT* (*ats4184*) encodes a transporter of major facilitator

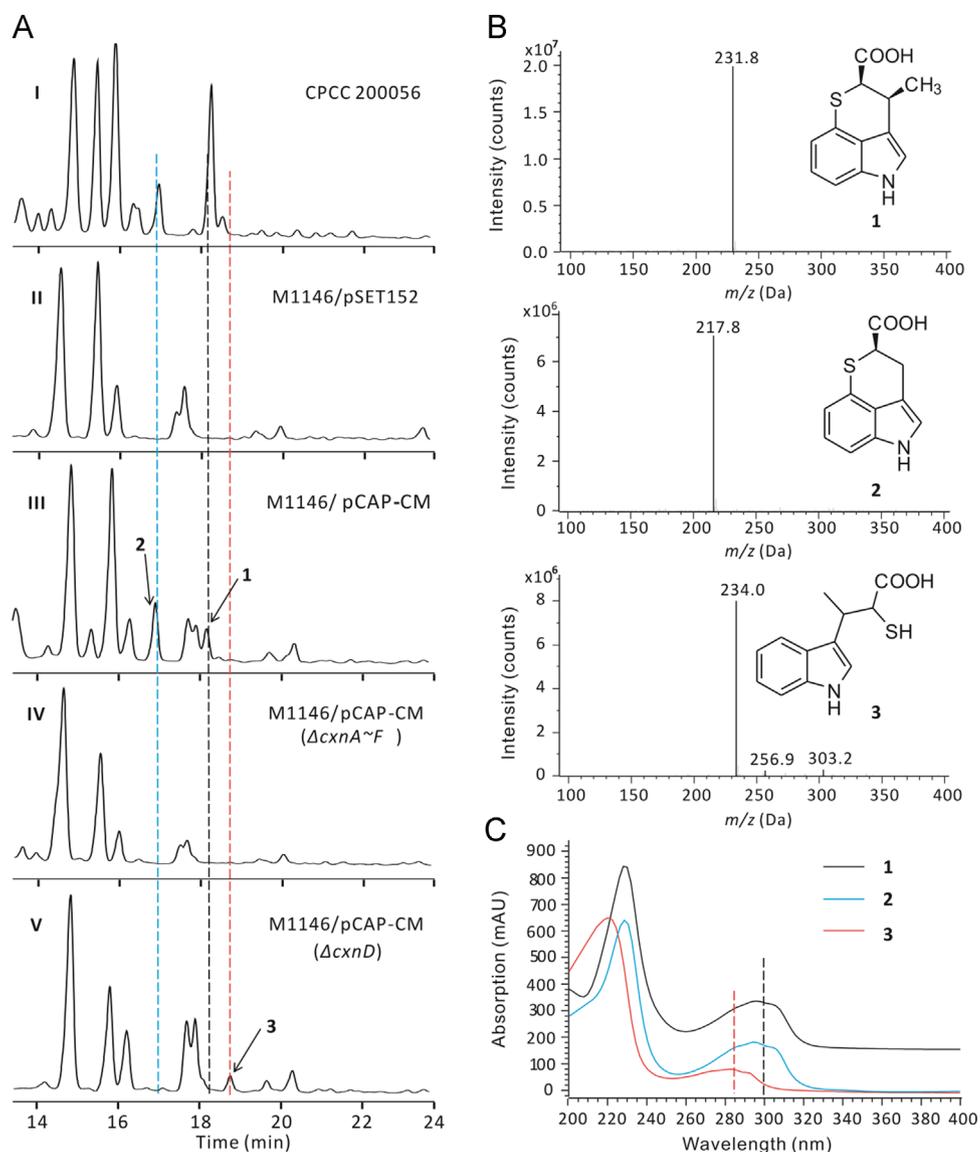


Figure 5 Heterologous expression of *cxn* cluster in *S. coelicolor* M1146. (A) HPLC analysis of different strains; (B) Molecular ion peaks of **1**, **2** and **3** in A; (C) UV absorption spectra of **1**, **2** and **3**.

superfamily. *cxnR* (*ats4185*) encodes an LysR family transcriptional regulator and *trpRS* (*ats4186*) encodes a putative resistant gene. Genes flanking this putative *cxn* cluster were also analyzed. *ats4177* encodes a hypothetical protein of unknown function. *ats4176* is homologous to a peptidoglycan-binding protein. *ats4175* encodes an HTH_XRE family transcriptional regulator. *ats4187* encodes a membrane protein, *ats4188* encodes an ion-coupled transporter, and *ats4190* encodes a protein of unknown function (Table 2).

3.2. Overexpression of cluster-situated regulator *CxnR*

There is a possible pathway-specific regulator *CxnR* situated in the predicted *cxn* cluster. It is homologous to LysR family transcriptional regulator in *Amycolatopsis lurida* NRRL 2430 (37% identity) and FkbR1 (35% identity) which was a positive regulator for ascomycin production in *S. hygroscopicus* var. *ascomyticus* ATCC 14891³⁴. The effect of overexpression of the regulator

CxnR in wild type strain on the production of chuangxinmycin was detected to determine whether the gene cluster was responsible for the biosynthesis of chuangxinmycin.

The overexpression plasmid pL-*CxnR* was introduced into the wild type strain *A. tsinanensis* CPC 200056 by conjugation to give the overexpression strain 200056/pL-*CxnR*, with pSET152 transferred strain 200056/pSET152 as a control. Then these strains were cultured simultaneously on ISP2 agar with the same inoculum size and the fermentation product was extracted with ethyl acetate and analyzed by TLC and HPLC. The result of TLC displayed that the **1** production in 200056/pL-*CxnR* was significantly improved than that in 200056/pSET152 (Fig. S1 in Supplementary information). Furthermore, relative quantitative analysis was carried out by HPLC and showed that the production level of **1** in 200056/pL-*CxnR* was increased to 198% compared with that in 200056/pSET152 (Fig. 3). These results suggest that *CxnR* is the pathway-specific positive regulator for chuangxinmycin biosynthesis and support that the predicted cluster is responsible for the production of chuangxinmycin.

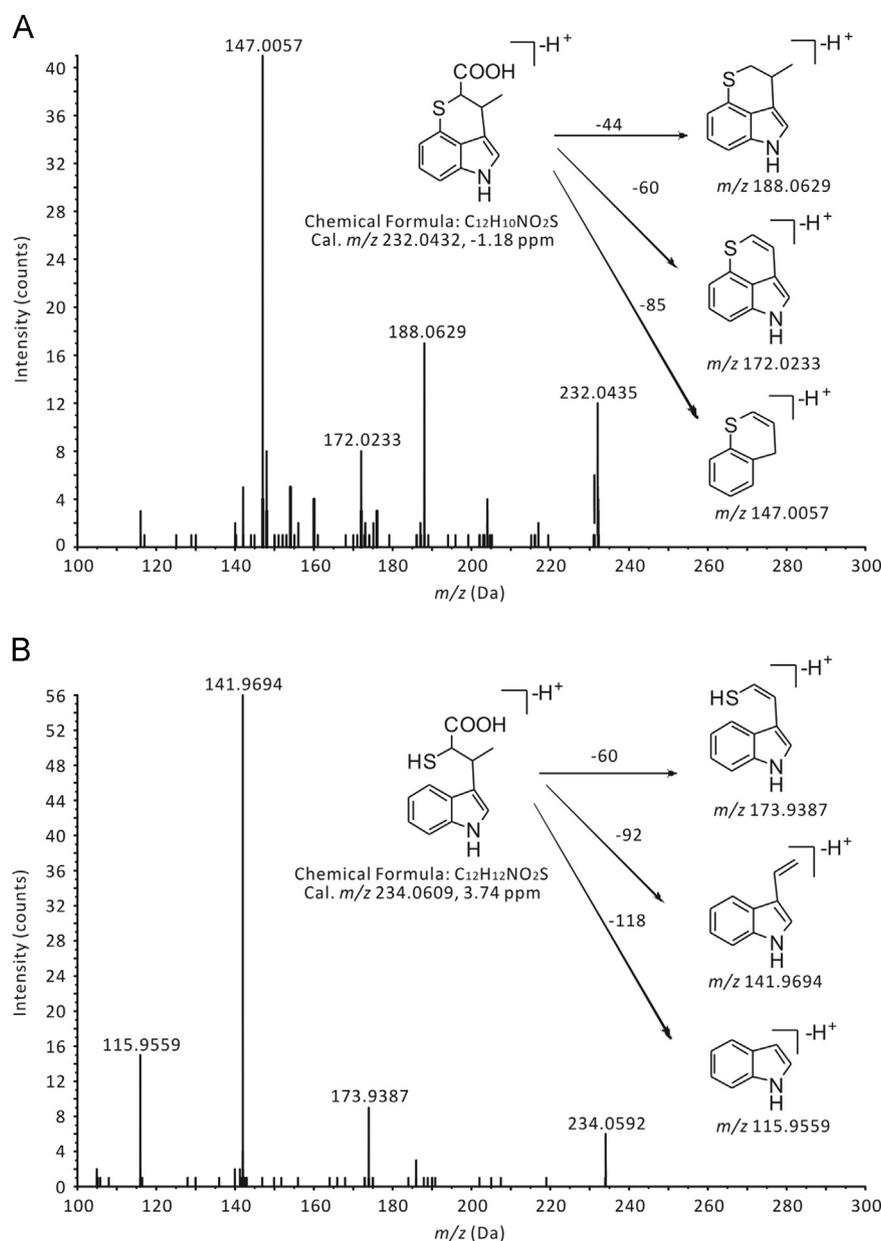


Figure 6 HR-MS/MS analysis of **1** (A) and **3** (B).

3.3. Direct cloning and heterologous expression of *cxn* cluster

To heterologously express the *cxn* cluster in *S. coelicolor* M1146, DNA assembler³¹ was used to directly clone the proposed *cxn* cluster by the *in vivo* homologous recombination mechanism in *S. cerevisiae*. Two capture arms (Fig. 2) were amplified and cloned into pCAP01¹⁶ to get the *cxn* cluster capture vector pCAP-CM-LR. Four fragments of the gene cluster were obtained using PCR and electroporated into *S. cerevisiae* together with linear pCAP-CM-LR, resulting in the construct pCAP01-CM carrying the entire gene cluster (*ats4175~4190*, Fig. 4A). The plasmid pCAP01-CM was isolated from *S. cerevisiae*, transferred into *E. coli* DH5 α and then verified by PCR and restricted enzymatic analysis (Fig. 4B). To facilitate the following conjugation, the kanamycin resistance gene in pCAP01-CM was replaced with apramycin resistance gene by PCR-targeting³⁵. However, sequencing of the assembled *cxn* cluster in this plasmid showed that there is a point mutation in *cxnD* which led to the early

translation termination of CxnD (148 vs. 404 amino acids), thus this plasmid was named pCAP-CM(Δ *cxnD*). The further deletion plasmid pCAP-CM(Δ *cxnA~F*) with disruption of the majority of the *cxn* cluster was constructed by digestion of pCAP-CM(Δ *cxnD*) with *EcoRI* (there are 4 *EcoRI* sites situated within *cxnA~F* in this plasmid) and then re-ligation of the large fragment. These two plasmids were introduced into *S. coelicolor* M1146¹⁵ by conjugation, resulting in two disruption strains *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*) and *S. coelicolor* M1146/pCAP-CM(Δ *cxnA~F*). To fulfill the CxnD function in the disruption strain *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*), the entire *cxnD* ORF was cloned into pIJ10500¹⁹, a plasmid containing a ϕ BT1 *attP-int* locus. The resulting plasmid pIJ-CxnD, with *cxnD* under the control of promoter *ermE**_p, was transferred into *S. coelicolor* M1146/pCAP-CM(Δ *cxnD*) to give a complete *cxn* heterologous expression strain *S. coelicolor* M1146/pCAP-CM. The PCR primers used for verification of all the recombinant strains are shown in Table S1.

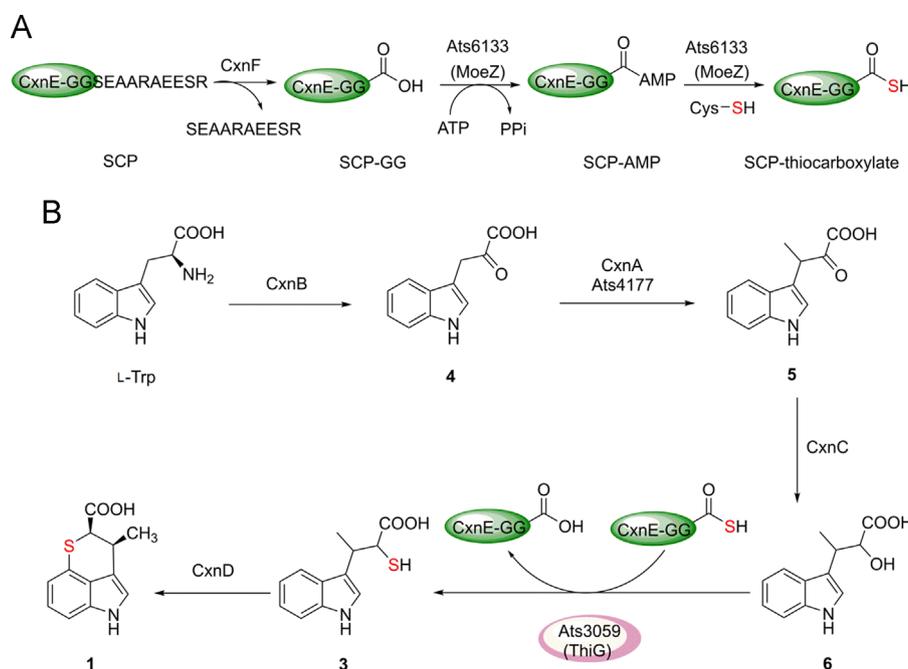


Figure 7 The proposed biosynthetic pathway of **1**. (A) Activation of *cxn* cluster-situated SCP CxnE. (B) The proposed biosynthetic pathway of **1**.

The three recombinant strains *S. coelicolor* M1146/pCAP-CM, M1146/pCAP-CM(Δ *cxnD*) and M1146/pCAP-CM(Δ *cxnA~F*), together with control strain M1146/pSET152 and chuangxinmycin producing wild type strain *A. tsinanensis* CPCC 200056, were cultured on ISP2 agar plates at 28 °C for 7 days. The culture was extracted with ethyl acetate and concentrated, and then analyzed by liquid chromatograph–mass spectrometer (LC–MS). Compared with that of control strain M1146/pSET152, two new HPLC peaks appeared at 18.1 and 16.9 min in the products of strain M1146/pCAP-CM containing complete *cxn* cluster (Fig. 5), with the maximum absorption wavelength (λ_{\max}) at the expected 230 and 300 nm, which were the same as those of chuangxinmycin (**1**) and demethyl-chuangxinmycin (**2**, Fig. 1), previously characterized in the wild type strain *A. tsinanensis* CPCC 200056²². In the LC–MS analysis, the two peaks revealed molecular ions at m/z 232 and 218 ($[M-H]^-$) respectively, identical to those of **1** and **2**. In the disruption strain M1146/pCAP-CM(Δ *cxnA~F*), these two peaks were not detected. These results demonstrated that the predicted *cxn* cluster was responsible for the biosynthesis of chuangxinmycin.

LC–MS analysis of extracts from the recombinant strain M1146/pCAP-CM(Δ *cxnD*) showed loss of **1** and **2** production, and the appearance of a new UV-absorbing peak (**3**) at a retention time of 18.8 min, displayed a molecular ion of m/z 234 $[M-H]^-$, 2 Da more than that of **1**. The UV absorption profile of **3** is similar to **1** but with a blue shift of 10–20 nm (λ_{\max} at 220 and 280 nm), suggesting the decreased electron density of the indole aromatic group in **3**. Thus the compound **3** was deduced as seco-chuangxinmycin (3-(1*H*-indol-3-yl)-2-thiolbutanoic acid) with an opened dihydrothiopyran ring compared with **1**, as a biosynthetic intermediate in **1** biosynthesis. Further investigation of this metabolite using LC–ESI–HR–MS identified its m/z as 234.0601 for $[M-H]^-$ (molecular formula deduced as $C_{12}H_{12}NO_2S$, Fig. S4, while Fig. S3 is for **1**), corresponding to that of seco-chuangxinmycin (calculated for 234.0609 for $M-1$). Additional MS/MS analysis of **3** (Fig. 6B) revealed its fragmentation pattern to be

analogous to that of **1** (Fig. 6A) to some extent. The fragment ions of m/z 173.9387 and 172.0233 in **3** and **1**, respectively, were possibly derived by lost the carboxyl group and 3-methyl group of **3** and **1**, respectively. The production of **3** in the recombinant strain M1146/pCAP-CM(Δ *cxnD*) verified that CxnD is involved in the second C–S bond formation (*i.e.*, the closure of the dihydrothiopyran ring) in chuangxinmycin biosynthesis.

3.4. Proposed pathway for chuangxinmycin biosynthesis

Chuangxinmycin bears a unique tricyclic indole-*S*-hetero scaffold as indolethiopyran that encompasses two C–S bonds. Thus, the most intriguing part of the biosynthesis is the mechanism of the incorporation of sulfur. Although the feeding of [³⁵S]-cysteine provided preliminary evidence that the sulfur atom is derived from cysteine¹⁰, the central metabolite of sulfur biochemistry in prokaryotic cells, the detailed mechanism of sulfur incorporation is still obscure. Fortunately, there is an SCP homologue (CxnE) in the *cxn* cluster we have identified, which strongly suggests that the first C–S bond is formed by hijacking the potential sulfur-transfer enzymes, including common SCP-activating enzymes, cysteine desulfurases, and rhodanese-like proteins from primary metabolism³⁶. In the synthesis of thiamine, molybdopterin, thionucleosides, etc., the C–S bonds were formed using SCPs (ThiS, MoaD, etc.) activated by SCP-activating enzyme (ThiF, MoeB, etc.). Some secondary metabolites, such as thioquinolobactin³⁷ and BE-7585A¹³, were reported to hijack the sulfur transfer systems from primary metabolism for incorporation of the sulfur into the secondary metabolite molecules. Recently, the mechanism of sulfur incorporation into the thiosugar of BE-7585A was elegantly described¹³. A ThiG (thiazole synthase for thiamin) homologue BexX is present in the BE-7585A biosynthetic cluster, and elsewhere in the genome a unique MoeZ (with a ThiF-like domain at N-terminus and a rhodanese homology domain at C-terminus) and several SCPs as well as cysteine desulfurases are present. The sulfur from L-cysteine charges the rhodanese domain of MoeZ

mediated by a cysteine desulfurase, and then catalyzes thiolation of the adenylated SCP activated by ThiF-like domain of MoeZ. The resulting SCP-thiocarboxylate is the ready-to-use form to transfer the sulfur atom into the thiosugar of BE-7585A¹³.

The enzymatic machinery for sulfur incorporation in primary metabolism was also present in the genome of *A. tsinanensis* CPCC 200056. There are a unique ThiG homologous protein Ats3059 within the thiamin biosynthesis cluster and a unique MoeZ homologous protein Ats6133. Several cysteine desulfurases (Ats6445, Ats3108, Ats2758 and Ats3097) and SCP homologues (Ats3085, Ats3815, Ats4181, Ats4502 and Ats4708) are present, and among them, *ats4181* is situated within the identified *cxn* cluster (Fig. 2). Thus, we envisaged that **1** utilized its own SCP (CxnE) and the primary sulfur transfer system, in a similar mechanism as used in the thiosugar of BE-7585A.

The C-terminus of the known SCPs such as ThiS and MoeZ has a highly conserved -GG-COOH motif that functions as a flexible linker and activation site for thiolation. Comparison of CxnE with known SCPs showed that the GG motif is followed by a decapeptide SEAARAEEESR in CxnE, *i.e.*, it might present as a precursor of SCP. A close investigation of the ORFs in *cxn* clusters showed that CxnF contained an MPN domain and was supposed to be a member of JAMM metalloproteinase family. Furthermore, it has the conserved JAMM motif (EX_nHS/THX₇SX₂D) (where X is any amino acid residue)³⁸ for isopeptidase activity. In the biosynthesis of a sulfur-containing siderophore thioquinolobactin produced in *Pseudomonas fluorescens*³³, the cluster situated SCP QbsE also has two more amino acids (CF) after the diglycine motif (GG). QbsD, a JAMM metalloproteinase, may digest CF of QbsE to expose GG as the new C-terminal for further adenylation and thiolation.

A summarized SCP-thiocarboxylate formation for **1** biosynthesis is proposed as in Fig. 7A. The C-terminal decapeptide SEAARAEEESR of CxnE (an SCP) is first hydrolytically cleaved off by CxnF (a JAMM family metalloproteinase), which exposes GG as C-terminal dipeptides for the following activation. The adenylation of CxnE is achieved by the ThiF-like domain of MoeZ (Ats6133). The conserved cysteine residue in rhodanese domain of MoeZ is converted to a persulfide group through the action of a cysteine desulfurase using L-cysteine as the sulfur source. Then the adenylated CxnE is thiolated by persulfide group of rhodanese domain of MoeZ to produce SCP-thiocarboxylate.

The chemical structure of **1** suggests the utilization of L-Trp as the starting material in a biosynthetic pathway, which is the case in another TrpRS inhibitor indolmycin³². Indolmycenic acid (**6**) has been reported as an intermediate in the biosynthesis of indolmycin³² and we predicted a similar mechanism in the biosynthesis of **1**. The aminotransferase (Ind8 and Orf2651), C-methyltransferase (Ind1) and NADH-dependent dehydrogenase (Ind2) are responsible for the biosynthesis of **6**. In the *cxn* cluster, CxnB shows high homology to a pyridoxal-5'-phosphate (PLP)-dependent aminotransferase Ind8 and Orf2651 as well as ThnJ in thienodolin biosynthesis³⁹, both responsible for the deamination of L-Trp to form indole-3-pyruvic acid (**4**). CxnA shows significant homology with C-methyltransferase of radical SAM superfamily enzyme GenK⁴⁰, TsrM⁴¹ and ThnK⁴² in the biosynthesis of different secondary metabolites. CxnA is thus supposed to catalyze methyl transfer from SAM to the β carbon of **4** to produce **5**. CxnC is a homologue of ketopantoate reductase PanE⁴³ or ApbA⁴⁴,

catalyzing the NAD(P)H-dependent reduction of ketopantoate to pantoate in the pantothenate (vitamin B5) biosynthetic pathway. CxnC might be a functional equivalent of Ind2 to fulfill the reduction of ketone in **5** to hydroxyl in **6**. Thus **6** is supposed to be an intermediate in **1** biosynthesis before the insertion of sulfur atom. Then the first C-S bond might be ready to be formed by the ThiG homologue (Ats3059) together with the CxnE-thiocarboxylate in a similar mechanism used by thiosugar of BE-7585A¹³.

Although CxnA shows low homology with C-methyltransferase Ind1, we speculate that they may catalyze the same reaction, as CxnA is a homologue of a vitamin B12-dependent SAM radical enzyme, catalyzing methylation of unreactive C or P atoms⁴⁵. An indolepyruvate C-methyltransferase transforming indolepyruvic acid (**4**) to **5** was partially purified and characterized from *A. tsinanensis* CPCC 200056 in 1989⁹, thus we speculated that the substrate of CxnA is **4**. However, we could not exclude the possibility that demethyl-chuangxinmycin (**2**) or other intermediates might be the substrate of this methyltransferase, and the substrate specificity of CxnA warrant further investigations. Zhou et al.¹¹ found that vitamin B12 could restore the production of **1** in 4 null mutants, and suggested it played an important role at late stage of **1** biosynthesis. This is consistent with the deduction that the CxnA is a vitamin B12-dependent C-methyltransferase, as well as that **2**, probably produced by the null mutants, losing its antibacterial activity²². Interestingly, Ats4177 exhibits significant 3D structure homology with chaperone PqqD³⁷ by the program HHpred⁴⁶ although it shows no homology to any known protein by BLASTP. PqqD is a structurally conserved peptide chaperone protein that forms a ternary complex with PqqE, a radical SAM enzyme in the biosynthesis of pyrroloquinoline quinone. So Ats4177 might be associated with the C-methylation activity by forming protein complex with CxnA.

A cytochrome P450-mediated C-S bond formation has been elucidated in several microbial secondary metabolite biosynthesis, such as SgvP in griseoviridin biosynthesis⁴⁷ and TlmF in five-membered thiolactone ring formation⁴⁸. In *thn* cluster, a P450 (ThnC) was speculated to catalyze the second C-S bond formation in a similar manner in indolethiophene scaffold of thienodolin³⁹. The P450 encoded by *cxnD* shows high homology with the above three P450s. The identification of the intermediate **3** in the *S. coelicolor* M1146 expressing *cxnD*-disrupted *cxn* cluster unambiguously verified that CxnD catalyzes the second C-S bond formation from **3**. Our study provides a substantial basis for the elucidation of two different mechanisms in the formation of two C-S bonds in chuangxinmycin biosynthesis.

On the basis of these bioinformatic analysis and genetic experiments, a complete biosynthetic pathway for **1** can now be proposed (Fig. 7B): L-tryptophan is deaminated by CxnB (a tryptophan aminotransferase) to generate indole-3-pyruvic acid (**4**), which is then methylated by CxnA, a radical SAM superfamily protein as an indolepyruvate C-methyltransferase. The resulted 3-methyl-indolepyruvic acid (**5**) was reduced to indolmycenic acid (**6**) by CxnC (NAD(P)H-dependent reductase). The sulfur transfer system from primary metabolism is hijacked, together with the cluster-situated sulfur carrier protein CxnE as thiol group donor (processed by CxnF, activated and thiolated by MoeZ), to catalyze sulfur incorporation into **6** to form the first C-S bond in **3** (3-(1*H*-indol-3-yl)-2-thiolbutanoic acid). Finally, **3** is further cyclized by cytochrome P450 CxnD to form the second C-S bond in **1**.

4. Conclusions

Bioinformatic analysis of the genome sequence of *A. tsinanensis* CPCC 200056 identified a contiguous stretch of DNA that contained some of the genes required for chuangxinmycin biosynthesis. We have unequivocally identified, cloned, genetically manipulated, and heterologously expressed the chuangxinmycin biosynthesis gene cluster (*cxn*) from *A. tsinanensis*. A plausible pathway was proposed mainly based on the *in silico* functional analysis of genes in *cxn* cluster, which provided new insights into chuangxinmycin biosynthesis by hijacking sulfur delivery system from primary metabolism. Furthermore, the involvement of the cytochrome P450 CxnD in the second C–S bond formation in chuangxinmycin biosynthesis was corroborated by the identification of an intermediate seco-chuangxinmycin in the *cxnD*-disrupted *cxn* heterologous expression strain. The feasibility of genetic manipulation of *cxn* cluster in *S. coelicolor* sets the basis for the future metabolic engineering and the direct production of novel chuangxinmycin derivatives.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.apsb.2017.07.005>.

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