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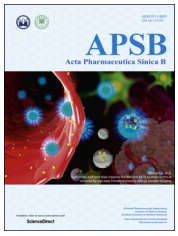

ORIGINAL ARTICLE

Biosynthesis of antibiotic chuangxinmycin from *Actinoplanes tsinanensis*

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KEY WORDS

Chuangxinmycin;
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Biosynthesis gene cluster;
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Cytochrome P450;
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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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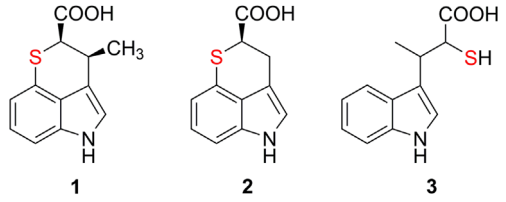

Figure 1 Chemical structures of chuangxinmycin (**1**), demethyl-chuangxinmycin (**2**) and seco-chuangxinmycin (**3**).

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 200056[1]. 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 septicaemia, 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) inhibitor[2] 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 1976[3], 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 [^{35}S]-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 TrpRS[12]. 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 group[13] 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 group[14] 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 ISP2[22] 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 $CaCO_3$, 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* M1146[15] and its derivatives were cultured at 28 °C on Mannitol soya flour (MS) agar[24], which was also used for conjugation between *Streptomyces* and *E. coli*. Liquid phage medium[25] were used for isolation of genomic DNA. *E. coli* DH5α[17] was used as a host for general cloning experiments. *E. coli* ET12567/pUZ8002[18] 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 (*MAT*α, *his3-Δ200*, *trp1-Δ1*, *ura3-52*, *lys2*, *ade2-101*, *met14*, *psi+cir^0*)[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