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Research Article Open Access

Introduction of Extra Copy of Oxytetracycline Resistance Gene *otrB* Enhances the Biosynthesis of Oxytetracycline in *Streptomyces rimosus*

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Abstract

The aromatic polyketide antibiotic oxytetracycline (OTC) is produced by *Streptomyces rimosus* as an important secondary metabolite. Enhancement of self-resistance is one effective way to improve antibiotic production in *Streptomyces* spp. In the present study, we aimed to improve the production of OTC by introducing extra copies of the OTC resistance genes, *otrA* and *otrB*, into the chromosome of the industrial strain of *S. rimosus* (SRI). First, *otrA* and *otrB* were cloned and ligated with pSET152 to generate the recombinant plasmids pSET152-*otrA/otrB*, the demethylated pSET152-*otrA/otrB* by *Escherichia coli* ET12567(pUZ8002) were then introduced into *SRI* to yield *otrA/otrB* knock-in mutants: *SRI-A* (*otrA*) and *SRI-B* (*otrB*). Ten selected mutants and the parent *SRI* strain were cultured in shake flasks. Production of OTC was increased by 67% in one *SRI-B* mutant compared with the parent strain, suggesting that the enhancement of resistance gene *otrB* in the antibiotic producer is an effective way to improve OTC biosynthesis. However, introduction of extra copy of *otrB* could retard growth of mutant cells.

Keywords: Antibiotic; Aromatic polyketide; Oxytetracycline (OTC) Biosynthesis; Self-Resistance

Introduction

Streptomyces spp. are filamentous Gram-positive aerobic soil-dwelling bacteria that belong to the family *Streptomycetaceae* and the order *Actinomycetales*. *Streptomyces* spp. and closely related genera have the ability to coordinate the production of a wide variety of secondary metabolites during morphological development [1]. Many of these secondary metabolites have antibiotic properties. Current methods employed to increase the antibiotic productivity of industrial microorganisms range from classical random mutagenesis studies performed in conjunction with the optimization of large-scale industrial fermentations. For more rational approaches, metabolic engineering is a common method used by researchers to regulate the production of many antibiotics. For example, genetic modifications of primary metabolic fluxes can lead to increases in the productivity of antibiotic synthesis [2,3], since the availability of biosynthetic precursors is a key factor that determines their production. To date, many studies have reported the improvement of antibiotic production by engineering the availability of certain precursors in the producer organisms [4-6]. Other methods, such as the optimization of fermentation conditions, are also available to regulate antibiotic production [7].

The aromatic polyketide antibiotic oxytetracycline (OTC) is produced *by Streptomyces rimosus* as an important secondary metabolite. There are three resistance genes in the OTC biosynthesis cluster, namely *otrA*, *otrB* and *otrC*, of which *otrA* and *otrB* are located at either end of this cluster. *otrA* changes the conformation of the 30S ribosome non-covalently and prevents the binding of OTC . Furthermore, *otrA* may be a substitute for the regulatory elongation factor [8], and *otrB* encodes a membrane transport protein that aids the transportation of OTC out of the cell. The *otrB* sequence shares great similarity with other transport genes, including *tetA* from Tn10 [9]. However, the function of *otrC* remains to be elucidated. A traditional mutation program has resulted in the improvement of OTC production from 2 $g \bullet l^{-1}$ to 80 $g \bullet l^{-1}$, and OTC production can also be improved by disrupting the zwf (coding glucose-6-phosphate dehydrogenase) gene [10]. Nevertheless, there is no information concerning the effects of *otrA*, *otrB* and *otrC* on OTC production.

Strains or plasmids	Function	Source
E. coli Top10	plasmid amplification	our laboratory
E. coli ET12567(pUZ8002)	plasmid demethylation	John Innes Center, UK
Industrial *S. rimosus* (SRI)	OTC producer	Shanxi Datong Antibiotic Company
pMD19T pMD19T-*otrA* pMD19T-*otrB*	gene amplification gene amplification gene amplification	TARAKA, Japan this study this study
pSET152	gene integration	
pSET152-*otrA* pSET152-*otrB*	gene integration gene integration	this study this study

Table 1: Strains and plasmids used in this present study.

In this present study, we aimed to investigate the influence of *otrA* and *otrB* on OTC production by introducing extra copies of these resistance genes into the genome of the industrial strain of *S. rimosus* (*SRI*). Herein, it is shown that production of OTC was increased in the *otrB* mutant, though an extra copy of *otrA* had no effect on OTC production.

Materials and Methods

Bacterial strains, media, and plasmids

The strains and plasmids used in this present study are listed in

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Primer	Sequence	Digestion site
P1(*otrA*-F)	5' CGCCATATGATGAACAAGCTGAATCTGGG 3'	*NdeI*
P2(*otrA*-R)	5' GGAAGCTTTCTAGATCACACGCGCTTGAGC 3'	*XbaI*
P3(*otrB*-F)	5' CGCCATATGGTGTCATCCGCAAATCCG 3'	*NdeI*
P4(*otrB*-R)	5' CCAAGCTTGCTCTAGATCAGGCGTCCGACGC 3'	*XbaI*
P5(*attB*-F)	5' GTTCACCAACAGCTGGAGGC 3'	
P6(*attB*-R)	5' CGTCATGCCCGCAGTGACC 3'	

Table 2: Primers used in this present study.

Table 1. Organisms were grown at 37˚C in Luria-Bertani medium (1% tryptone, 1% NaCl, 0.5% yeast extract), and standard procedures were used for transformations [11]. *Escherichia coli* transformants were selected with ampicillin (100 mg•ml^{-1}), apramycin (50 mg•ml^{-1}), kanamycin (10 mg•ml^{-1}) or chloramphenicol (25 mg•ml^{-1}). *SRI* (S. rimosus M4018) was grown and manipulated as described previously [12].

Construction of recombinant plasmids and *SRI* mutants

Primers: All the primers used are listed in Table 2.

Construction of recombinant plasmids: The *otrA* (2.1 kb) and *otrB* (1.7 kb) gene fragments were amplified using primers P1 and P2 or P3 and P4. Genomic DNA of *SRI* was used as the template. The fragments were cloned into the pMD19T vector to yield pMD19T-*otrA*/*otrB*, and then transformed into competent cells of *E. coli* Top10. Recombinant clones were screened by white-blue plaque selection and recombinant plasmids were analyzed by both single and double restriction enzyme digestion. pMD19T-*otrA*/*otrB* were digested with *Nde*I and *Xba*I, and then cloned into pSET152, which was digested with the same restriction enzymes to give pSET152-*otrA*/*otrB*. Then, the plasmids were transferred into *E. coli* ET12567 for demethylation and stored at -20˚C until later use.

Gene enhancement and identification of mutants: Demethylated pSET152-*otrA*/*otrB* were electroporated into *SRI* competent cells at 2 kV, 25 μF and 400Ω. Exconjugants were selected on tryptone soy agar plates containing apramycin 500 μg•mL^{-1} and incubated at 30˚C for 4–6 days. Mutants were confirmed by polymerase chain reactions (PCR) using *aprF* (P5) and *aprR* (P6) as primers.

Fermentation experiments

A spore suspension was inoculated into 30 ml of seed medium containing glucose (10 g•l^{-1}), yeast extract (0.5 g•l^{-1}), tryptone (15 g•l^{-1}), sucrose (2.8 g•l^{-1}) and calcium carbonate (0.1 g•l^{-1}). The first seed cultures were grown for 3 days at 260 rpm and 30˚C. Then, 2 ml of the first seed culture was inoculated into 50 ml of fermentation medium [12] in a 500-ml shaking flask with a spring. The second cultures were grown for 8 days at 260 rpm and 30˚C.

For determination of dry cell weight, 5-ml samples of each culture were collected every 24 h and dried at 105˚C to constant weight. OTC production *in vivo* and *in vitro* was analyzed by high performance liquid chromatography according to reference 9.

Results

Validation of the introduction of extra *otrA* and *otrB* genes

The recombinant plasmids pSET152-*otrA* and pSET152-*otrB* were verified by *Xba*I and *Nde*I digestion (Figure 1A). As indicated in Figure 1B, the positive clones of *otrA* and *otrB* transformants showed strong signals at 750 bp (apramycin resistance gene), while the wild-type pSET152 and pKC1139 showed no bands. After site-specific integration, the entire recombinant plasmid should be inserted into the genome of *SRI* as shown in Figure 1C. Then, positive clones were identified by cross-over sites (Figure 2) using the primers *otrA*-F and *attB*-R or *attB*-F and *apr*-R.

Screening of the high-productivity mutants

Eight *SRI-A* mutants (containing an extra copy of *otrA*) and 12 *SRI-B* mutants (containing an extra copy of *otrB*) were chosen for the shake flask experiments. After 8 days of fermentation, there were no obvious differences in the levels of OTC in the *SRI* parent strain, the *SRI-A* mutants and most of the *SRI-B* mutants (Figure 3). However,

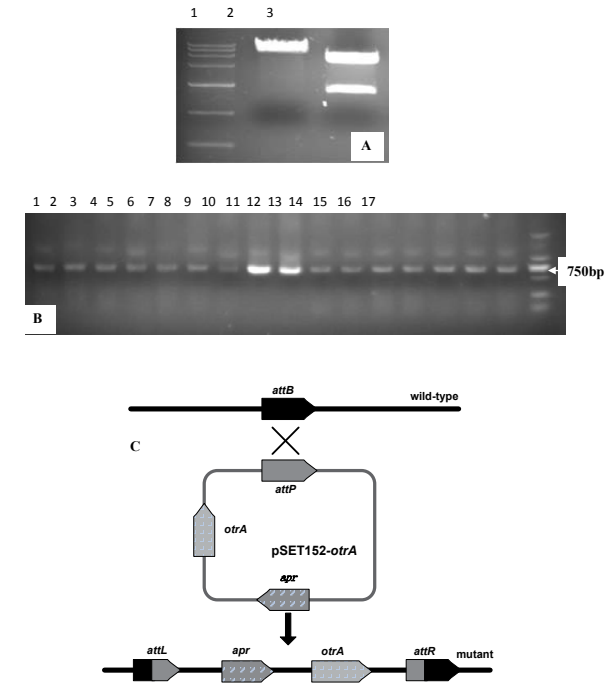

Figure 1: Generation and validation of the introduction of extra *otrA* and *otrB* genes.
A.Digestion of pSET152-*otrB*. Lane 1: marker DL15000; lane 2: pSET152-*otrB* digested with *NdeI*; lane 3: pSET152-*otrB* digested with *Xba*I and *Nde*I.
B. PCR identification of *SRI* mutants. Lanes 1–7: *SRI*-A mutants; lane 8–9: positive control(750bp for apramycin resistance gene); lane 10–16: *SRI*-B mutants; lane 17: marker III.
C. Site-specific integration of *otrA* in the SRI genome.

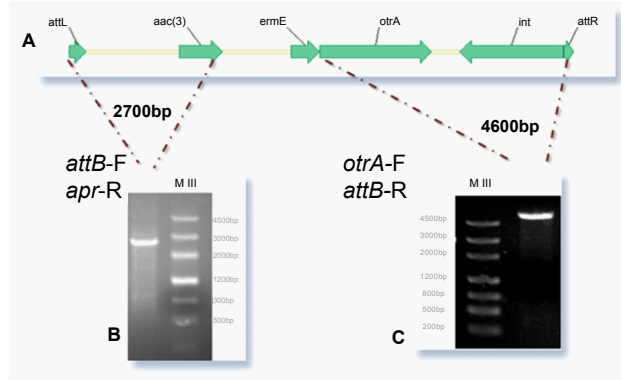

Figure 2: PCR identification of site-specific integration.

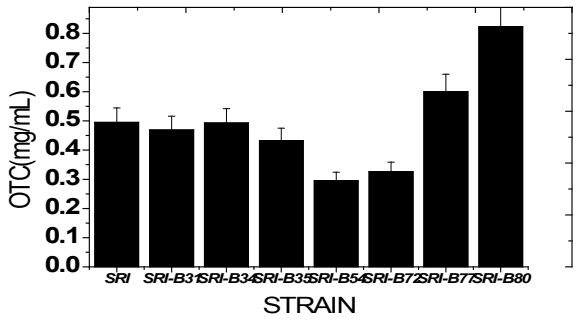

Figure 3: OTC levels in *SRI*, and *SRI-A* and *SRI-B* mutants.

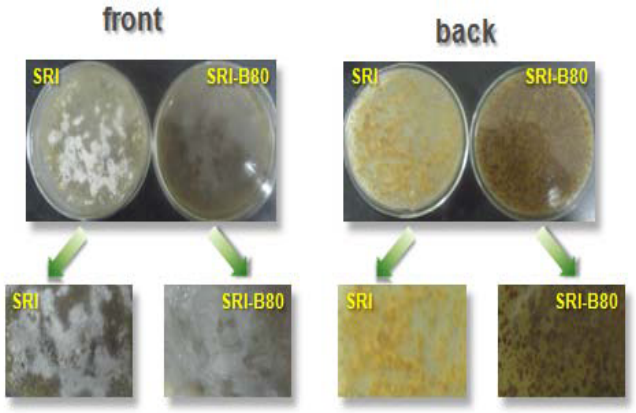

Figure 4: Pigment and spore formation by *SRI-B*80 and *SRI*.

OTC production was 67% higher in strain *SRI-B*80 compared with the control, indicating that *SRI-B*80 had high production of OTC.

Knowing that *Streptomyces* spp. spore formation is important for growth and the production of secondary metabolites [13], spore formation was compared between the *SRI* and *SRI-B*80 strains. After 6 days of incubation, mature spores were found on the surface of the *SRI* colony, but the *SRI-B*80 spores were malformed and immature. The onset of morphological differentiation generally coincides with the production of secondary metabolites and resistance genes are often co-regulated with these metabolites [14]. Indeed, spore formation is very closely related with the expression of resistance genes. *otrB* may be a negative regulator of spore formation and morphological differentiation. Moreover, the level of pigments produced by some *Streptomyces* spp. can reflect the magnitude of antibiotic production. Visual inspection of agar plates revealed that *SRI-B*80 produced more pigment than *SRI*, and this coincided with OTC production (Figure 4).

Growth characteristics of *SRI* and *SRI-B*80b

The physiological characteristics of *SRI-B*80 were further investigated and compared with the *SRI* parent. The growth curves of *SRI* and *SRI-B*80 are given in Figure 5A. The final biomass of *SRI-B*80 was markedly lower than *SRI*, indicating that the extra copy of *otrB* had a negative effect on growth. Furthermore, *SRI-B*80 had a slower growth rate, even during exponential growth phase. There are two possible reasons for this. First, the extra copy of *otrB* may increase the physiological burden of the cells. Second, re-distribution of cellular resources may occurred so that more materials could be directed into OTC biosynthesis in the *SRI-B*80 strain because the specific OTC productivity of *SRI-B*80 was twice that of *SRI* after 5 days of fermentation (Figure 5B).

OTC efflux mediated by the extra copy of *otrB* in *SRI-B*80

The intracellular and extracellular concentrations of OTC were determined after fermentation for the *SRI* and *SRI-B*80 strains. As expected, *SRI-B*80 showed enhanced efflux of OTC to some extent. After 8 days of fermentation, the introcellular OTC concentration in *SRI-B*80 was 13.6% lower than found in *SRI* (Figure 6A), which may reduce the risk of cell suicide due to high concentrations of OTC. Concomitantly, OTC concentration outside of the cells was 21.2% greater for *SRI-B*80 than detected for *SRI*. This shows that the extra copy of *otrB* played an important role in transporting OTC out of the cells. The extra *otrB* gene in *SRI-B*80 enabled greater transport of OTC out of the cells and this ensured that OTC in the cells was maintained at sufficiently low concentrations to cause any harm (Figure 6B).

Discussion

In this present study, extra copies of *otrA* and *otrB* were introduced into the genome of *SRI*. The extra copy of *otrA* did not exert any effects on OTC production; however, the extra copy of *otrB* enhanced OTC production markedly. Nevertheless, the *SRI-B*80 mutant had a slower growth rate, and spore formation and morphological differentiation were also affected, which suggests that *otrB* may be a multi-functional regulator in *SRI*. Through comparison of OTC concentrations *in vivo* and *in vitro* for *SRI* and *SRI-B*80, the extra *otrB* gene was shown to facilitate the intracellular efflux of OTC. Thus, the lower concentrations of OTC *in vivo* decrease the toxicity of the excess OTC, which may in turn stimulate the cells to produce more of this metabolite.

Many microbes can synthesize potentially toxic secondary metabolites (mainly antibiotics), and so there must exist pathways or mech-

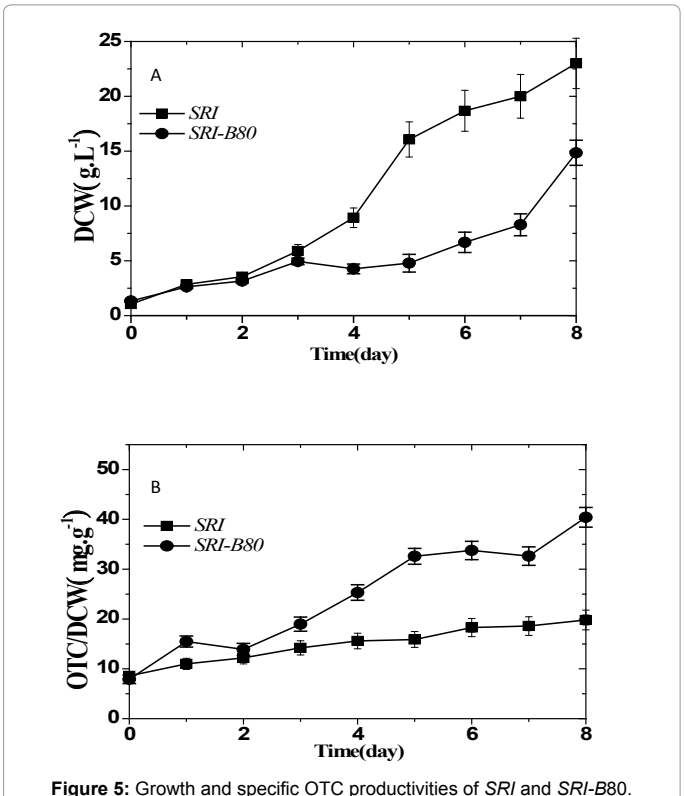

Figure 5: Growth and specific OTC productivities of *SRI* and *SRI-B*80.

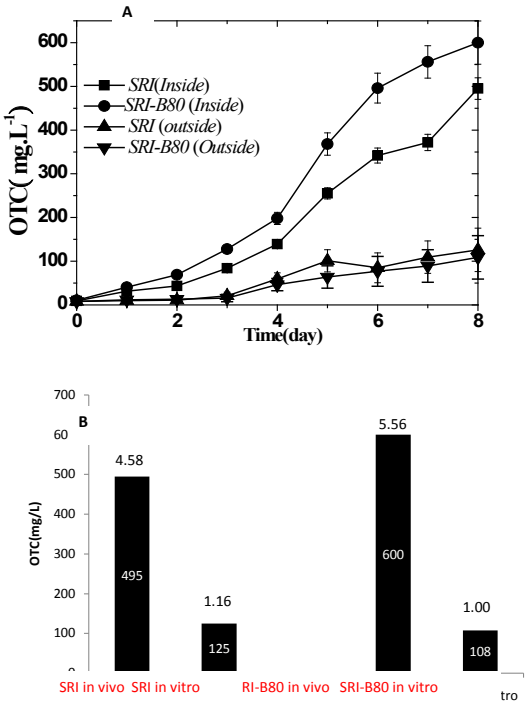

Figure 6: Intracellular and extracellular concentrations of OTC in *SRI* and *SRI-B*80.

anisms that protect the producer. Until now, various self-protection mechanisms have been reported [15], and by enhancing these self-protection mechanisms, the production of many antibiotics has been increased in various microorganisms. For example, Malla et al. [14] overexpressed the resistance genes *drrA*, *drrB* and *drrC* in *Streptomyces peucetius* ATCC 27952, such that the recombinant strains produced more doxorubicin (DXR) than the parental strain. Indeed, there was a 2.2-fold increase in DXR in the *drrAB* mutant, a 5.1-fold increase in the *drrC* mutant and a 2.4-fold increase in the *drrABC* mutant. Similarly, Olano et al. [16] demonstrated that improvements in self-resistance could increase the production of bioactive secondary metabolites in the actinomycetes. Thus, the introduction of extra resistance genes is an effective approach to enhance antibiotic production.

Although an extra copy of *otrB* improved OTC production, the additional copy of *otrA* had little or no effect on this parameter. This may be because *otrA* in *SRI* produces sufficient enzyme to modify the ribosome and provide a safe environment. Therefore, *otrA* may be saturating, but this assumption requires further investigation. Moreover, the observation that *SRI-B*80 has a slow rate of sporulation is consistent with the findings of Davies [17] and Den Hengst et al [18]. Specific growth states, such as biofilm formation, anaerobiosis and sporulation, can differentially impact on cell susceptibility to antimicrobials. It can be concluded that a series of genes that regulate *otrB* may have altered expression in *SRI-B*80, and these changes may have knock-on effects on growth and sporulation in these mutants. Since the regulation network in these cells is very complicated, further experiments are needed to understand the relations between all of these affected genes.

Acknowledgements

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