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Construct of *Streptomyces Scabies* Knock-Out Mutant of TXT Biosynthetic Gene *txtB* Via Intergeneric Conjugation



Lin Lan* and Xu Xu-Dong

Department of Bioengineering, Southeast University, China

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*Corresponding author: Lin Lan, Department of Bioengineering, School of Medicine, Southeast University, Nanjing, 210009, P. R. China

Abstract

Production of thaxtomin A (TXT), a nitrated dipeptide which inhibits cellulose synthesis in expanding plant tissue, is regarded as the major pathogenicity determinant in *Streptomyces scabies*, the most widely studied pathogen causing scab disease in economically important crops such as potato. It is known that TXT biosynthetic genes *txtA*, *txtB*, *txtC* and their regulator *txtR* are clustered together on the pathogenicity island (PAI) of the genome of *S. scabies* and are conserved in TXT-producing *Streptomyces* spp. including *S. scabies*. Here we reported an efficient method to establish the knock-out mutant of *txtB* (*txtBA*), by using intergeneric conjugal transfer system constituting *Escherichia coli* ET12567 /pUZ8002, *S. scabies* and the vector pKC1139. Briefly, a *txtB* gene-specific deletion cassette was constructed by PCR and long multiple fusion (LMF), which was subcloned into the *Streptomyces-E. coli* shuttle vector pKC1139, generating pKC1139-x. By mixing the donor *E. coli* ET12567 /pUZ8002/ pKC1139-x and the recipient *S. scabies* at the suitable condition, the exconjugants were successfully obtained and verified by colony PCR. Subsequently, TXT was analyzed for the knock-out mutant (*txtBA*) in comparison to wild-type of *S. scabies*. In addition, the transcriptional levels of *txtB* were studied between the two genotypes. Altogether, *txtB* gene was successfully knocked out in the exconjugants obtained herein. This would lay a foundation for studying the TXT-biosynthesis associated genes and pathogenicity in scab-causing plant pathogen, facilitating the mechanistic elucidation of plant-pathogen interactions.

Keywords: *Streptomyces scabies*; Thaxtomin A (TXT) Biosynthesis; Conjugation; pKC1139

Abbreviations: TXT: Thaxtomin A; PAI: Pathogenicity Island; LMF: Long Multiple Fusion; OMB: Oatmeal Broth; RP: Reverse Phase; RT: Reverse Transcription; KO: Knockout

Introduction

Omnipresent in marine and terrestrial habitats, streptomycetes are prolific producers of diverse bioactive metabolites and thus of pharmaceutical and agricultural significance. In spite of a multitude of *Streptomyces* species characterized to date, only a handful of them are pathogenic to eukaryotic organisms including plants and animals. Among these pathogens, phytopathogens *Streptomyces scabies*, *S. acidiscabies*, *S. turgidiscabies*, draw much research interests because they are known causal agents of common scab diseases in potatoes and other tuber crops such as carrots. The production of phytotoxin thaxtomin A (TXT), an inhibitor of plant cellulose biosynthesis, is regarded to contribute to their pathogenicity [1-3]. TXT has been demonstrated to induce plant cell hypertrophy in expanding tissues. Globally distributed common scab pathogens are represented by *S. scabies*, the first documented species causing scabs on potatoes [4]. The TXT biosynthetic genes are highly conserved across plant-

pathogenic streptomycetes and are found on a pathogenicity island (PAI) that is mobilized among soil-borne streptomycetes, which is considered to be the predominant reason for the emergence of new scab-causing *Streptomyces* spp [1,5].

To explore and develop the feasible measures to control the rapid spreading of pathogenic streptomycetes in the ecosystem, the molecular mechanism underlying the TXT biosynthesis pathway and the regulation thereof need to be clarified. In this paper we presented an efficient method to establish the knock-out mutant of *txtB* (*txtBA*), by using intergeneric conjugal transfer system, consisting of *Escherichia coli* ET12567 /pUZ8002, *S. scabies* and the shuttle vector pKC1139. Our study would help deepen the understandings about the mechanism of *S. scabies* pathogenicity and virulence, thereby providing insight into the elucidation of plant-pathogen interactions.

Materials and Methods

Strains, Plasmids and Culture Conditions

Streptomyces scabies strain 49173 was grown in the TSB agar (Elite Biotech. Co., Shanghai, China) as previously described [6]. Spore suspensions of *Streptomyces* strains were prepared as described before [7]. Fresh cultures of *Streptomyces* cultures grown in TSB tryptone soya broth (tryptone 17g l⁻¹, soya peptone 3g l⁻¹, glucose 2.5g l⁻¹, NaCl 5g l⁻¹, K₂HPO₄ 2.5g l⁻¹) within 48h on a rotary shaker (200rpm, at 30 °C) were used to extract genomic DNA using a bacterial genomic DNA extraction kit (Sangon Biotech Co, Shanghai, China). *Streptomyces* spores were grown at 26±2 °C in International *Streptomyces* Project medium 4 (ISP-4) and shaking cultures were harvested for RNA extraction at 4 d. *Streptomyces* spores were inoculated in oatmeal broth (OMB) [8] for 8-9 d for thaxtomin enrichment and extraction. As for intergeneric conjugation, *E. coli* strain ET12567 harboring pUZ8002 (*E. coli* ET12567/pUZ8002) was used as the donor and pKC1139 (Huayueyang Biotech Co., Beijing, China) as conjugative vector. *E. coli* ET12567/pUZ8002 and *E. coli* 12567/pKC1139 were maintained in LB medium (Luria-Bertani broth, Sangon Biotech Co, Shanghai, China) supplemented with apramycin (Apra, 50µg ml⁻¹), kanamycin (Kana, 50µg ml⁻¹) and chloramphenicol (Cm, 25µg ml⁻¹).

Extraction of Genomic DNA of *S. Scabies*

The genomic DNA was prepared from 48h shaking culture of *S. scabies* by using EZ-10 spin column bacterial genomic DNA isolation kit (Sangon Biotech Co, Shanghai, China), with the addition of lysozyme solution (20mg ml⁻¹) which facilitated the removal of *Streptomyces* cell wall.

Deletion of the *txtB* Gene

Construction of Plasmids For Gene Deletion: A *txtB* deletion mutant was created using PCR-based homologous recombination method [9] by replacing the gene of interest with a *txtB* gene-specific deletion cassette. A thiostrepton resistance gene [*tsr* (Thio^R)] from the plasmid pIJ8600 was PCR-amplified using the primers P3 and P4 (Table 1), resulting in a 1058bp fragment. A 428bp fragment designated *txtB*_1, encompassing the nucleotides upstream of *txtB* gene CDS (coding sequence) and some of its CDS, was PCR-amplified from the *S. scabies* genomic DNA using the primers P1 and P2 (Table 1). A 531bp fragment designated *txtB*_2, encompassing the some of *txtB* gene CDS and the nucleotides downstream of this gene, was amplified from the *S. scabies* genomic DNA using the primers P5 and P6 (Table 1). The three fragments were ligated according to the LMF method described by Shevchuk et al. [10], yielding fragment *txtB*_1-*tsr*-*txtB*_2. A deletion cassette containing the upstream homologous arm (~420bp), and the downstream homologous arm (~530bp), between which is the *tsr* marker gene (Thio^R) was PCR-amplified with the *txtB* gene-specific primer pairs that carry BamHI, HindIII site sequences, respectively using the fragment *txtB*_1-*tsr*-*txtB*_2 as the template. The obtained deletion cassette was cloned into pMD19T Simple (Takara Biotech Co., Dalian, China), followed by BamHI and HindIII digestion, ligation into BamHI/

HindIII digested linearized pKC1139, generating the *txtB* deletion vector pKC1139-x.

Table 1: Primers used in PCR.

Primer	Sequence (5'→ 3')
P1	GGCTCTACGACGAGGTCTA
P2	ATGAAGTATTTCGCTTGATCAATCCAGTACTTTCGTCGGCC
P3	TGATCAAGGCGAATACTTCAT
P4	TCATCACTGACGAATCGAGGT
P5	ACCTCGATTTCGTCAGTGATGATCGTCGGCCGTATCGCGAAA
P6	TCCTGTCCGTGCCGGAACA

Intergeneric Conjugation and Identification of Exconjugants: The donor cells of *E. coli* ET12567 /pUZ8002 containing pKC1139-1 were grown to an OD600 of 0.4–0.6 with the appropriate antibiotics. MS medium (mannitol 20g L⁻¹, soya flour 20g L⁻¹) containing 10mM MgCl₂ and TSB medium were used for plating of *E. coli*/*Streptomyces scabies* in intergeneric conjugation and the efficiency of conjugation was evaluated between MS and TSB. The conjugation plates were incubated at 28 °C for 16-20h, and subsequently overlaid with 1.5mL of sterile water containing 500µg mL⁻¹ nalidixic acid and 1000µg mL⁻¹ thiostrepton, which were incubated further for 10-12d, until the exconjugants emerged. Apra^R (100µg mL⁻¹) exconjugants were screened for Cm^S (25µg mL⁻¹), indicating a double-crossover allelic exchange in *S. scabies*, and were subsequently verified by colony PCR.

TXT Extraction and Assay: *S. scabies* cultures of wildtype and mutant were grown for 8 d and cells were pelleted by centrifugation (10000×g). The supernatants were extracted with ethyl acetate, followed by evaporation, and the residues were dissolved in methanol, and analyzed for the presence of TXT using an Agilent 1200 HPLC (Agilent Technologies, CA, USA) on a C-18 reversed-phase(RP) column eluting with a gradient series of 20% to 100% methanol in water as the mobile phase.

Transcriptional Analysis of *txtB* With RT-PCR: RNA was extracted from 3d mycelia grown on ISP-4 at 28 °C using EZ-10 total RNA Mini-Preps Kit (Bio Basic Canada Inc. Ontario, Canada). Genomic DNA was removed from total RNA using DNase I (Bio Basic Canada Inc. Ontario, Canada). DNase-treated RNA (1µg) was reverse transcribed using the SuperscriptTM III 1st stand synthesis system kit and random hexamer primers (Invitrogen, USA) according to the manufacturer's protocol. Gene expression was verified by PCR amplifying the cDNA using gene-specific primers. The *gyrA* gene (NZ_LRTN01000850.1:272-2866, http://www.sanger.ac.uk/Projects/S_scabies/), which encodes DNA gyrase subunit A, was used to normalize the amount of RNA in the samples. Each measurement was performed in triplicate.

Results and Discussion

Construction of the Plasmid pKC1139-x for Gene Knock-Out

We aimed to construct a pKC1139-based vector, designated pKC1139-x, which carried gene-specific homologous upstream and downstream arms for knock-out purpose (Figure 1). By PCR

amplification, the *txtB* gene-specific upstream and downstream fragments [homologous arms, (Figure 2a)] and the *tsr*, a thiostrepton resistance marker (Figure 2b), were obtained respectively. The individual band was purified using DNA gel extraction kit for further use.

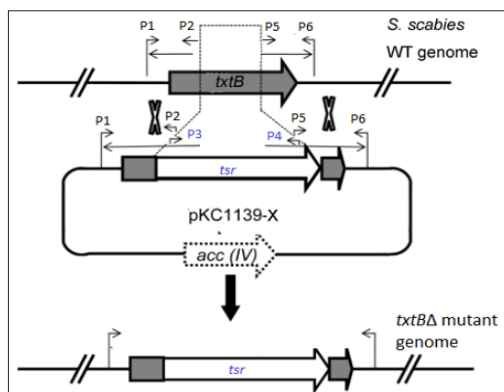


Figure 1: Schematic diagram showing the construct of gene (*txtB*) inactivation mutants via the homologous recombination.

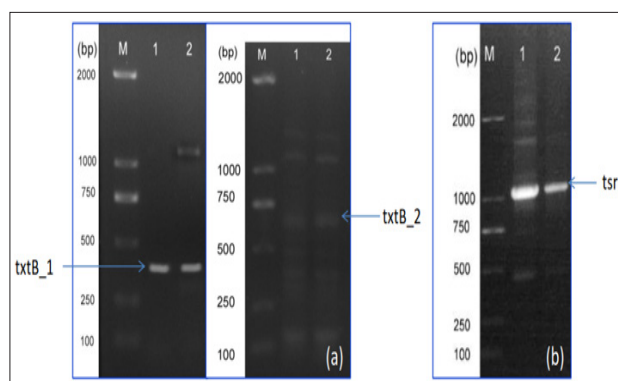


Figure 2: Electrophoretic gel analysis.

Note:

- PCR products of *txtB_1*, and *txtB_2*, sized 428bp and 531bp, respectively.
- PCR amplification of *tsr* gene, a thiostrepton resistance marker, using pIJ8600 as the template. M= D2000 DNA ladder (Tiangen Biotech. Co., Beijing, China)

The Construct of Long Fragment Used for Homologous Recombination

By adopting the long multiple fusion (LMF) method as described in Shevchuk et al. [10], the three fragments (*txtB_1*, *tsr*, *txtB_2*) were ligated together to generate a long fragment (~2kb) (Figure 3). This long fragment (sized ~2kb) was used for gene inactivation in *S. scabies* based on homologous recombination.

TXT Analysis by RP-HPLC

As demonstrated by previous studies, OMB was the suitable medium for *S. scabies* to biosynthesize TXT [8]. Following culturing

in OMB for 8-9d, the culture supernatants of *S. scabies txtBΔ* mutant and WT were subjected to organic-solvent extraction. Subsequently, TXT levels of both genotypes were determined against TXT standard by reverse phase(RP)-HPLC. As seen from the chromatographs, no TXT was detected in *txtBΔ* (Figure 4a), suggesting that the production of TXT was abrogated for *txtBΔ* as compared to WT which produced normal level of TXT (Figures 4b & 4c).

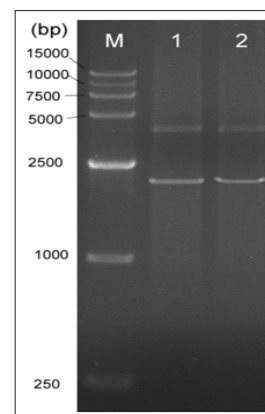


Figure 3: Electrophoretic gel of the fused long fragment (sized 2017bp) using LMF method. M: D15000 DNA ladder (Tiangen Biotech. Co., Beijing, China). Lane 1 and 2 indicates the sample 1 and 2, respectively.

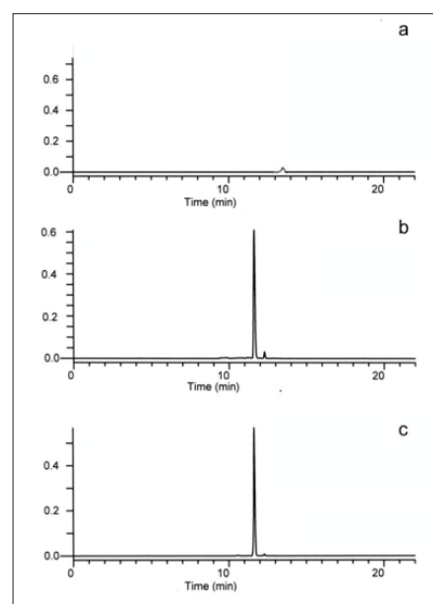


Figure 4: Reverse-phase(RP)-HPLC chromatographs of TXT extracted from OMB broth.

Note:

- txtBΔ* mutant;
- WT of *S. scabies*, and
- TXT standard.

Reverse Transcription (RT) -PCR of *txtB* Transcripts

In view of that *txtB* deletion generates *txtB* knockout (KO) mutant, it seemed reasonable to question whether KO abolished transcription of *txtB*. In this study, the transcriptional level of *txtB* was monitored by reverse transcription (RT) -PCR with the *txtB* gene-specific primers. As shown in Figure 5, transcription of *txtB* was diminished in KO mutant while WT displayed abundant transcripts of *txtB*. In summary, an efficient intergeneric DNA transfer system was successfully developed between *E. coli* and *Streptomyces* sp. This method would lay a basis for DNA transformation and mutational studies of economically important strain *S. scabies* and other *Streptomyces* species as well.

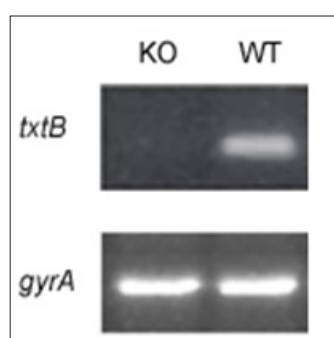


Figure 5: Relative abundance of *txtB* transcripts as assayed by RT-PCR from *S. scabies* WT versus *txtB*Δ (knockout, KO) mutant. The *gyrA* gene, was used as an internal control to normalize the amount of cDNA template in RT-PCR analysis.

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