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# Functional Analysis of Type II Thioesterase of *Streptomyces lydicus* AS 4.2501

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## Abstract

Constructing a mutant strain of single gene disruption is the basis for the study of gene function and metabolomics. Systematic and complete genome sequencing is the basis of genetic manipulation. In the case of a little knowledge about the *Streptomyces lydicus* genome and the speculation that polyketide synthases (type I) might be responsible for the polyketide side chain biosynthesis of streptolydigin, a 588-bp fragment was amplified by polymerase chain reaction (PCR) according to the homology existing in the same functional genes among *Streptomyces*. A mutant strain of this gene was constructed by single crossover homologous recombination. The results of sequence analysis as well as the metabolite analysis of the mutant and the original strain by liquid chromatography/mass spectroscopy indicated that this fragment was part of type II thioesterase (TE) gene, which was required for streptolydigin biosynthesis like other type II TEs function in related antibiotics biosynthesis. Furthermore, targeted gene manipulation based on PCR was a powerful tool for studying gene function and metabolomics, especially when little was known about the genomic sequence of streptomyces.

**Index Entries:** Type II thioesterase; polymerase chain reaction; mutant strain construction; functional analysis; single crossover homologous recombination; *Streptomyces lydicus* AS 4.2501.

## Introduction

Streptolydigin ($C_{32}H_{44}N_2O_9$) produced by *Streptomyces lydicus* has activity against Gram-positive bacteria *(1)*. Its mechanism of inhibiting

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bacterial RNA polymerase has been defined *(2)*. Because it has limited or no cross resistance with other inhibitors of bacterial RNA polymerase *(3–6)*, streptolydigin is a potent antibiotic in a time of the emergence of multi-antibiotic-resistant bacterial strains.

Streptolydigin is an acyl tetramic acid antibiotic. There is substantial evidence that the acyl groups are derived from a polyketide, while the ring is derived from a $C_2$ unit and an amino acid *(7)*. All four $C_2$ units come from malonate, four propionate residues are incorporated into the acyl side chain of streptolydigin, and type I polyketide synthase (PKS) is speculated to participate in its synthesis *(8,9)*. However, these results are short of the demonstrations coming from molecular biological study.

Type I PKSs are large, multifunctional enzymes that are organized into repeated units (modules), and each module contains three domains—ketosynthases, acyltransferase, and acylcarrier protein— that are necessary for elongation of the $C_2$ unit. In addition, modules may contain one to three of the following enzymes: keto reductase, dehydratase, and enoyl reductase. The complete polyketide chain is released from the PKS through the action of a thioesterase (TE), which is probably also involved in cyclization of the final product during macrolide formation *(10)*. Moreover, within the cluster of antibiotic biosynthetic genes, many type I PKSs contain additional TE gene located adjacent to the PKS genes *(11)*. To distinguish them from the chain-terminating thioesterase (TE I), they are named TE II. Disruption of the TE II genes from several PKS clusters has shown that TE II plays an important role in maintaining normal levels of antibiotic production *(12,13)*. The behavior of tylosin TE II in vitro is consistent with its proposed role as an editing enzyme *(11)*. In view of the emergence of drug-fast bacteria, new kinds of antibiotics are needed. The library of secondary metabolites might be constructed by module recombination of type I PKSs to meet this demand. For example, moving the TE domain from the end of module 6 to the end of module 2 of erythromycin biosynthesis gene cluster results in the production of novel 6-member triketides, whereas moving the TE domain to the end of module 5 results in the production of a 12-member macrolide related to methymycin *(14,15)*. Thus, the TE domain has recently received much attention *(10,13)*.

Genetic manipulation requires information about the targeted gene sequence, which is usually acquired by construction of a gene library, targeted gene screening, cloning, and sequencing. According to the homology existing in the same functional genes from *Streptomyces*, polymerase chain reaction (PCR) could be used to amplify the targeted gene in order to study its function by disruption. With the development of analytic tools such as chromatography and spectroscopy, analysis of the genome at the metabolome level could provide further insight into the function of orphan genes.

In the present work, according to the homology of TEs from different *Streptomyces*, a 588-bp fragment was amplified by PCR and sequenced from *Streptomyces lydicus* AS 4.2501, which was isolated by our group and could produce streptolydigin. A mutant strain of inactivating the target gene was

constructed. For determining the gene and its function in streptolydigin biosynthesis, the motif, active site, and homology of the sequence as well as the secondary metabolites of the mutant and the original strain were analyzed.

## Materials and Methods

### *Strains and Plasmids*

*S. lydicus* AS 4.2501 was isolated by our group and stored at our laboratory and the China General Microbiological Culture Collection Center. *Escherichia coli* $DH_{5\alpha}$ was used for cloning. *Bacillus subtilis* was used as bioactive indicator. *E. coli-Streptomyces* shuttle vector pKC1139, used for gene disruption, was kindly provided by Prof. Huarong Tan (Institute of Microbiology, Chinese Academy of Science). It has a temperature-sensitive replicon function only at less than 34°C. The pMD-18T vector was obtained from Takara Biotechnology and used for cloning the amplified fragment and the recombinant plasmid.

### *Medium and Culture Conditions*

*S. lydicus* AS 4.2501 was maintained on solid medium containing 20 g/L of soluble starch, 5 g/L of glucose, 2 g/L of peptone, 1 g/L of $K_2HPO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 0.5 g/L of NaCl, 2 g/L of corn steep liquor, and 20 g/L of agar. Spores were developed at 28°C for 7 d and stored at 4°C for later use.

Seed medium for *S. lydicus* AS 4.2501 and disruption strain contained 30 g/L of soluble starch, 5 g/L of glucose, 4 g/L of peptone, 2 g/L of yeast extract, 1.5 g/L of $K_2HPO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, and 0.5 g/L of NaCl. A loop of spores and mycelia was transferred into 250-mL conical flasks containing 50 mL of seed medium and incubated at 28°C for 48 h in an orbital incubator at 220 rpm.

Fermentation medium for *S. lydicus* AS 4.2501 and disruption strain contained 40 g/L of soluble starch, 5 g/L of glucose, 2 g/L of peptone, 1.0 g/L of $K_2HPO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, and 0.5 g/L of NaCl. Seed culture (5 mL) was transferred into a 250-mL baffled flask containing 50 mL of fermentation medium and incubated at 28°C for 96 h in an orbital incubator at 220 rpm.

*E. coli* strain was maintained on Luria-Bertani (LB) *(16)* agar plates and cultured in LB broth at 37°C with agitation. Ampicillin was supplemented at a concentration of 80 μg/mL when necessary.

$R_2$YE medium was used for protoplast regeneration *(17)*, and 50 mg/mL of apramycin was used for drug resistance selection.

### *DNA Manipulation Techniques*

The manipulations, including plasmid preparations, genomic DNA isolation, restriction endonuclease digests, DNA recovery, ligation, and

transformation into *E. coli* and protoplast of *S. lydicus* AS 4.2501, were performed according to established techniques *(16–18)* or standard protocols provided by manufacturers.

### Primers and PCR Conditions

Oligonucleotide primers P1 (forward primer: 5N-CGGCTGGTG TGCTTCCCGCACGC-3N) and P2 (reverse primer: 5N-AGGTAGAAG TGCCCGCCGGGGAAG-3N) were designed according to the homology of gene sequences of TE in PKS from *S. avermitilis* (AB070949), *S. fradiae* (U08223), *S. erythraeus* (X60379), and *S. griseus* (M93058) and synthesized by Shanghai Sangon Biological Engineering Technology and Services. The program of ClustalW Updated in www.ebi.ac.uk/services/ was used to compare the sequences' homology. PCR solution contained 5 mL of 10X buffer, 2 m*M* $MgCl_2$, 500 µ*M* dNTP, 400 ng of DNA template, 1 m*M* each primer, 2.5 U of *Taq* polymerase, and 7.5 mL of dimethyl sulfoxide with the volume brought to 50 mL with sterilized water. The reaction was conducted at 95°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (35 cycles), and 72°C for 10 min.

### DNA Sequencing and Analysis

The target fragment was cloned in pMD-18T vector (TaKaRa) according to the supplier's instructions and sequenced by Shanghai Sangon Biological Engineering Technology and Services. It was translated into amino acid sequence using a translation tool provided by www.expasy.org. Protein homolog analysis was performed with program NCBI-Blast2 Protein Database Query, and the deduced TE from *S. lydicus* AS 4.2501 and TEs from other *Streptomyces* strains were compared using ClustalW Updated and the program GeneDoc.

### Construction of Recombinant Plasmid

The fragment amplified by PCR was connected with pMD-18T and cloned in $DH_{5\alpha}$. The recombinant plasmid (named pMDTE) was extracted by alkaline decomposition and digested with *Eco*RI and *Hin*dIII to verify the positive clones. pMDTE was digested with *Eco*RI and *Hind*III. Then the target fragment was extracted from agarose gel using an Ultra-Sep Gel Extraction Kit (Omiga) and ligated into pKC1139 digested with the same two enzymes to construct the recombinant plasmid (named pKCTE). pKCTE was transformed into *E. coli* $DH_{5\alpha}$, and the positive clone was screened by apramycin resistance and α-complement and further verified by plasmid digestion with *Eco*RI and *Hind*III.

### Construction of Mutant Strain

Protoplast preparation, transformation, and regeneration were carried out according to the commonly used method *(17)*. TE gene was disrupted by inserting a fragment of pKC1139 through homologous recombination

(single crossover) *(19)*. The transformant was isolated by apramycin resistance and temperature sensitivity and further confirmed by apramycin-resistance gene amplification by PCR.

### *Detection of Secondary Metabolite*

After fermentation, the mycelium and broth were separated by centrifugation at 2000*g* for 10 min. Thirty milliliters of supernatant was extracted with an equal volume of ethyl acetate and repeated three times. The ethyl acetate extract was evaporated to dryness, and the residue was dissolved in methanol. For liquid chromatography-mass spectroscopy (LC-MS) analysis, the extract was eluted with acetonitrile and water (containing 100 μL of formic acid/L) in a gradient mode at 1.0 mL/min through a μBondapak C18 column (300 × 3.9 μm, 5–μm particle size; Waters), and the eluate was monitored using electrospray ionization (ESI). Acetonitrile concentration varied from 5 to 45% during 0–35 min, from 45 to 85% during 35–50 min, and from 85 to 100% during 50–80 min. All experiments were performed on an LCQ Advantage MAX instrument (Thermo Electron Corporation, San Jose, CA). Parameters were as follows: the temperature of the heated capillary was 300°C, the electrospray capillary voltage was 5 kV, and the detection of negative ions was 200–900 or 1200 u. Xcalubur1.3 software was used for data processing.

### *Nucleotide Sequence Accession Number*

The nucleotide sequence has been submitted to the GenBank database, and the assigned accession no. is DQ115803.

## Results

### *DNA Cloning and Sequencing Analysis*

A 588-bp DNA fragment was amplified from genomic DNA of *S. lydicus* AS 4.2501 by PCR (Fig. 1) and sequenced (accession no. DQ115803). The total percentage of G+C content of this fragment was 73.97%, which is typical in *Streptomyces (18)*.

Restriction analysis by DNAMAN software showed that 29 kinds of restriction endonucleases had one or more cut sites, and others, including *Bam*HI, *Eco*RI, and *Hind*III, had none among the 117 kinds of selected enzymes whose cutters are $6 bp.

The amino acid sequence deduced from this sequence (195 amino acids) showed 60% identity and 74% positives with putative TE II of *S. parvullus* and *S. rochei* (BAC76544) and 59% identity and 71% positives with TE II of *S. avermitilis* (BAC68663), and the percentage of G+C content at the third code was up to 92.3%, in accordance with severely biased use of *Streptomyces* codon *(20)*. Comparison of the deduced amino acid sequence with TE IIs from other microorganisms producing natural products showed that there were two important motifs (GHSMG and GGH) and an active-

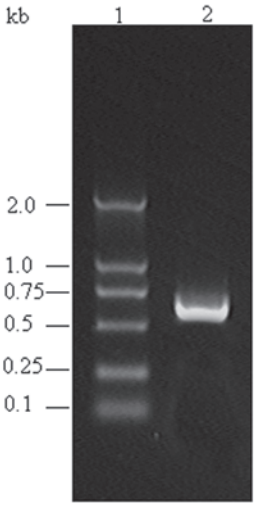


Fig. 1. Agarose gel analysis of PCR products. Lane 1, DNA marker DL2000; lane 2, PCR production.

site residue (S) (Fig. 2). This coincides with the invariant GXSXG and GXH, which is considered to be essential for TE activity *(21)*. The active-site Ser in motif GXSXG is coded by AGC (Fig. 3), which is the typical use for TE II (AGY, Y = C, T), while TCN (N = A, C, G, T) is used for coding Ser in TE I *(22)*. Thus, the cloned fragment was speculated to be part of TE II gene from *S. lydicus* AS 4.2501.

## *Construction of Mutant of Inactive TE*

The recombinant plasmid pKCTE was introduced into the protoplast of *S. lydicus* AS 4.2501. The system of transformation consisted of 100 mL of protoplast, 10 mL of plasmid, and 400 µL of T buffer containing 25% PEG1000 (w/v). One hundred microliters of mixed solution were spread on $R_2$YE plates. After incubating at 28°C for 30–35 h, 3 mL of apramycin (50 µg/mL) was added onto each plate, the cultivation continued for 4 to 5 d, and then the plates were incubated at 39°C for 7–10 d. The disruption mutants of TE II were selected by apramycin resistance and temperature sensitivity. Only the positive transformants survived under this condition *(19)*.

The primers were devised according to apramycin resistance gene (CAD97425) in synthetic construct for *S. coelicolor* transposon Tn5062 (AJ566337). The forward primer sequence is 5N-TCATCGGTCAGC TTCTCAAC-3N, and the reverse primer sequence is 5N-GGCATCGCA TTCTTCGCAT-3N. The PCR reaction system (25 µL) contained 2.5 µL of 10X buffer ($Mg^{2+}$ free), 2 µL of $MgCl_2$ (25 m*M*), 2 µL of dNTP (2.5 m*M* each), 1 mL of each primer (25 µ*M*), 1 µL of DNA (500 µg/mL), 0.25 µL of *Taq* (5 U/mL), and 15.75 µL of sterilized water. The reaction was conducted at

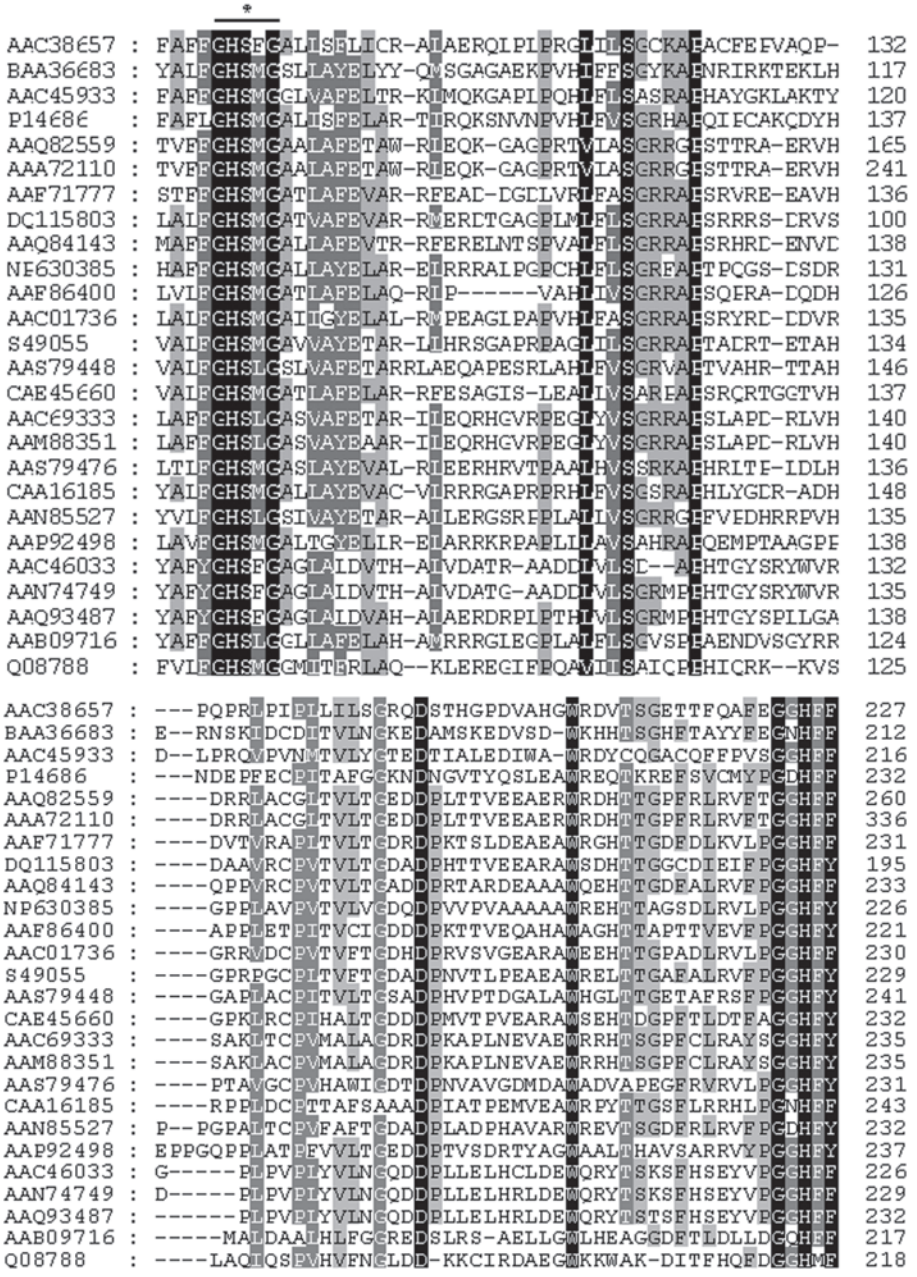

Fig. 2. Comparison of deduced TE II (DQ115803) from *S. lydicus* AS 4.2501 and TE IIs from other microorganisms producing natural products (*Pseudomonas syringae* pv. Glycinea [AAC38657], *Pseudomonas aeruginosa* [AAB09716], *S. coelicolor* [CAA16185], *S. venezuelae* [AAC69333], *S. fradiae* [S49055], *A. mediterranei* [CAD72413], *S. noursei* ATCC 11455 [AAF71777], *S. lydicus* AS 4.2501 [DQ115803], *Aneurinibacillus migulanus* [P14686], *Bacillus licheniformis* [BAA36683], *Brevibacillus brevis* [AAC45933], *B. subtilis* [Q08788], *P. syringae* pv. Glycinea [AAQ93487], *P. syringae* [AAC46033], *P. syringae pv. Actinidiae* [AAN74749], *S. coelicolor* A3[2] [NP630385], *S. parvulus* [CAE45660], *S. bikiniensis* [AAS79476], *S. bikiniensis* [AAS79448], *S. atroolivaceus* [AAN85527], *S.* sp. FR-008 [AAQ82559], *S.* sp. HK803 [AAQ84143], *S. vinaceus* [AAP92498], *S. narbonensis* [AAM88351], *S. hygroscopicus* var. *ascomyceticus* [AAF86400], *S. griseus* [AAA72110]). The asterisk indicates active-site residue, and the line below it indicates the motifs important to the function of TE.

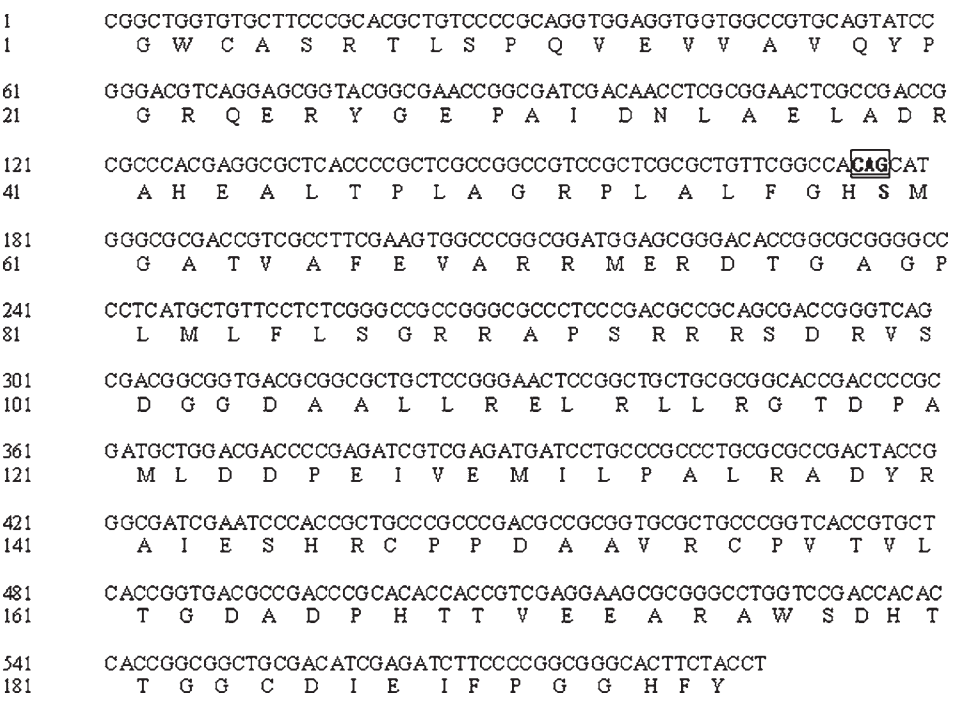


Fig. 3. Code of Ser in motif GXSXG of TE II in *S. lydicus* AS 4.2501.

95°C for 5 min followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and finally at 72°C for 10 min.

Positive results were observed when plasmid pKC1139 and genomic DNA of the mutant were used as template, respectively. However, no product was amplified when genomic DNA of *S. lydicus* AS 4.2501 was used (Fig. 4). The results showed that plasmid pKCTE was inserted into the genomic DNA, instead of just existing as plasmid.

## *Functional Analysis of TE II in Streptolydigin Biosynthesis*

At present, most of the information about the function of TE II in polyketide biosynthesis comes from gene disruption and complementation experiments. When TE II gene of *Amycolatopsis mediterranei* was deleted, the yield of polyketide rifamycin B dropped to 30–60% *(23)*. In addition, disruption of TE II gene of *S. fradiae* reduced antibiotic accumulation by at least 85% *(13)*.

*S. lydicus* AS 4.2501 and the disruption mutant were inoculated into seed medium. After incubating for 48 h, 5 mL of seed culture was transferred into 45 mL of production medium and cultivated for 96 h. The results of LC-MS analysis showed that the metabolites produced by the mutant were different with *S. lydicus* AS 4.2501. The original strain produced streptolydigin, which had a peak of m/z 599 in mass spectrum (Fig. 5A,B). The mutant mainly accumulated some other compounds (–c ESI Full Ms

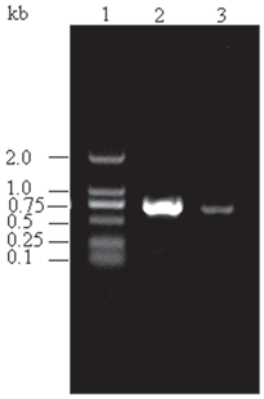


Fig. 4. Amplification of apramycin resistance gene by PCR. Lane 1, DNA marker DL2000; lane 2, plasmid pKC1139 was used as template; lane 3, genomic DNA of the mutant strain was used as template; lane 4, genomic DNA of *S. lydicus* AS 4.2501 was used as template.

[m/z] = 321, 483, 525, 275, 511, and 553 corresponding to peak 1, 2, 3, 4, 5, and 6, respectively, in Fig. 6A) and produced only a small amount of streptolydigin (peak 7), which was judged by retention time and mass spectrum (Fig. 6A,B). MS-MS analysis also demonstrated that the compound of m/z 599 produced by the mutant is streptolydigin (Figs. 5C and 6C).

## Discussion

Streptolydigin, discovered in 1955, is an acyl tetramic acid antibiotic. There is substantial evidence that the acyl groups were derived from a polyketide, and the structure of streptolydigin has been determined *(7,9)*. Although the biosynthesis pathway of streptolydigin is unclear, its precursors have been confirmed using isotope labeling and nuclear magnetic resonance. Previous work indicated that PKS (type I) took part in the biosynthesis of streptolydigin, but there was no evidence from molecular biological study and no report about streptolydigin biosynthesis gene cluster up to now. To control streptolydigin biosynthesis, PKS and other tailored enzymes must be mastered.

When working out a metabolic or biosynthetic pathway for the first time, scientists usually rely on biochemical analysis to predict the steps in the pathway. However, to determine whether a pathway actually works as predicted, it is necessary to disrupt genes encoding enzymes or other proteins in the pathway to demonstrate their functions in the intact organism *(24)*. Driven by the needs of functional genomics, DNA engineering by homologous recombination in *E. coli* has emerged as a major addition to existing technologies *(25)* and has been applied in other organisms. For an organism whose genomic sequence is known, the information of genomic sequence facilitates the metabolic reconstruction to predict the catabolic

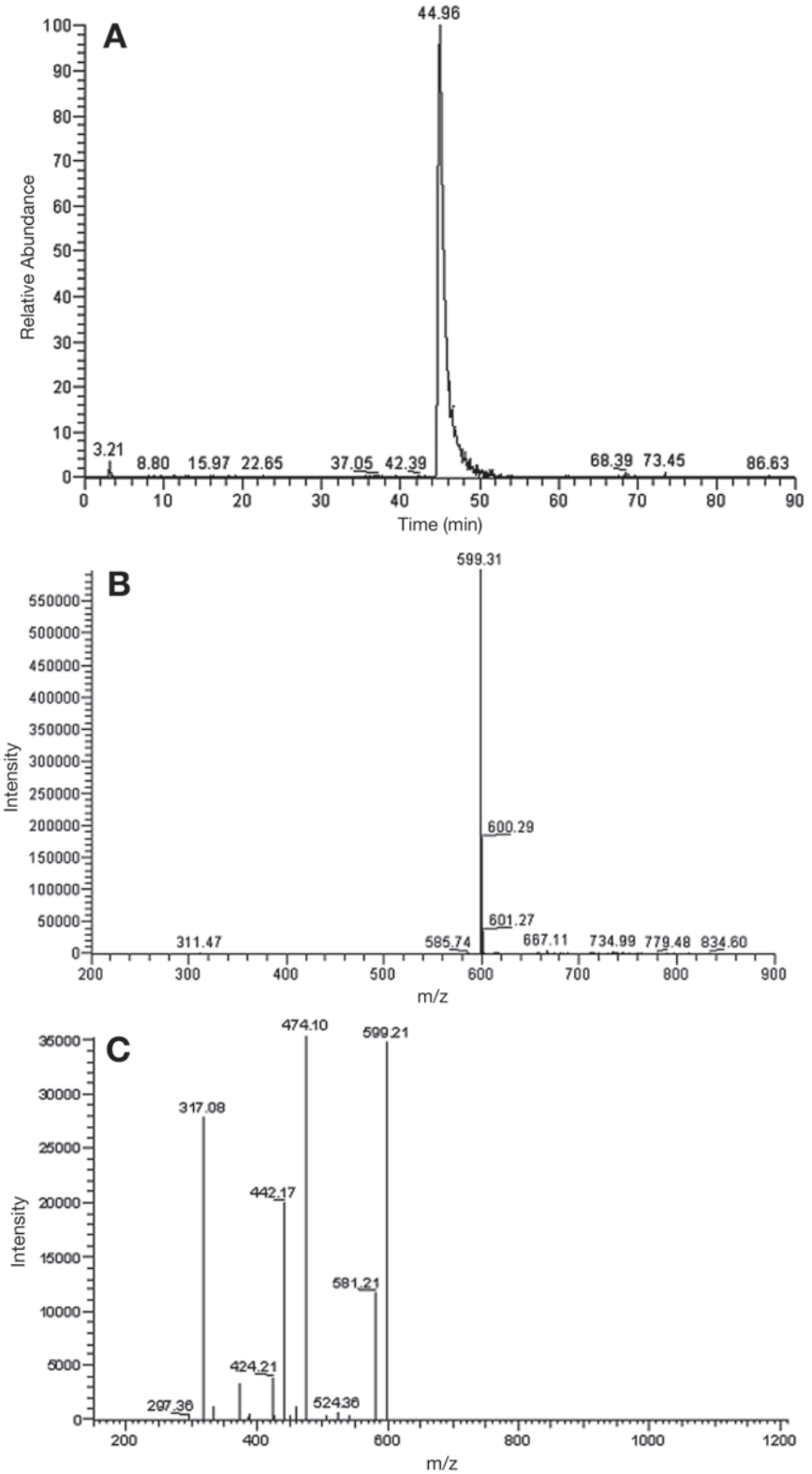


Fig. 5. LC-MS/MS analysis of fermentation product of *S. lydicus* AS 4.2501: **(A)** TIC spectrum; **(B)** negative ESI-MS spectrum of streptolydigin; **(C)** MS-MS product ion spectrum obtained from $[M-H]^-$ (m/z 599.31) ions of streptolydigin.

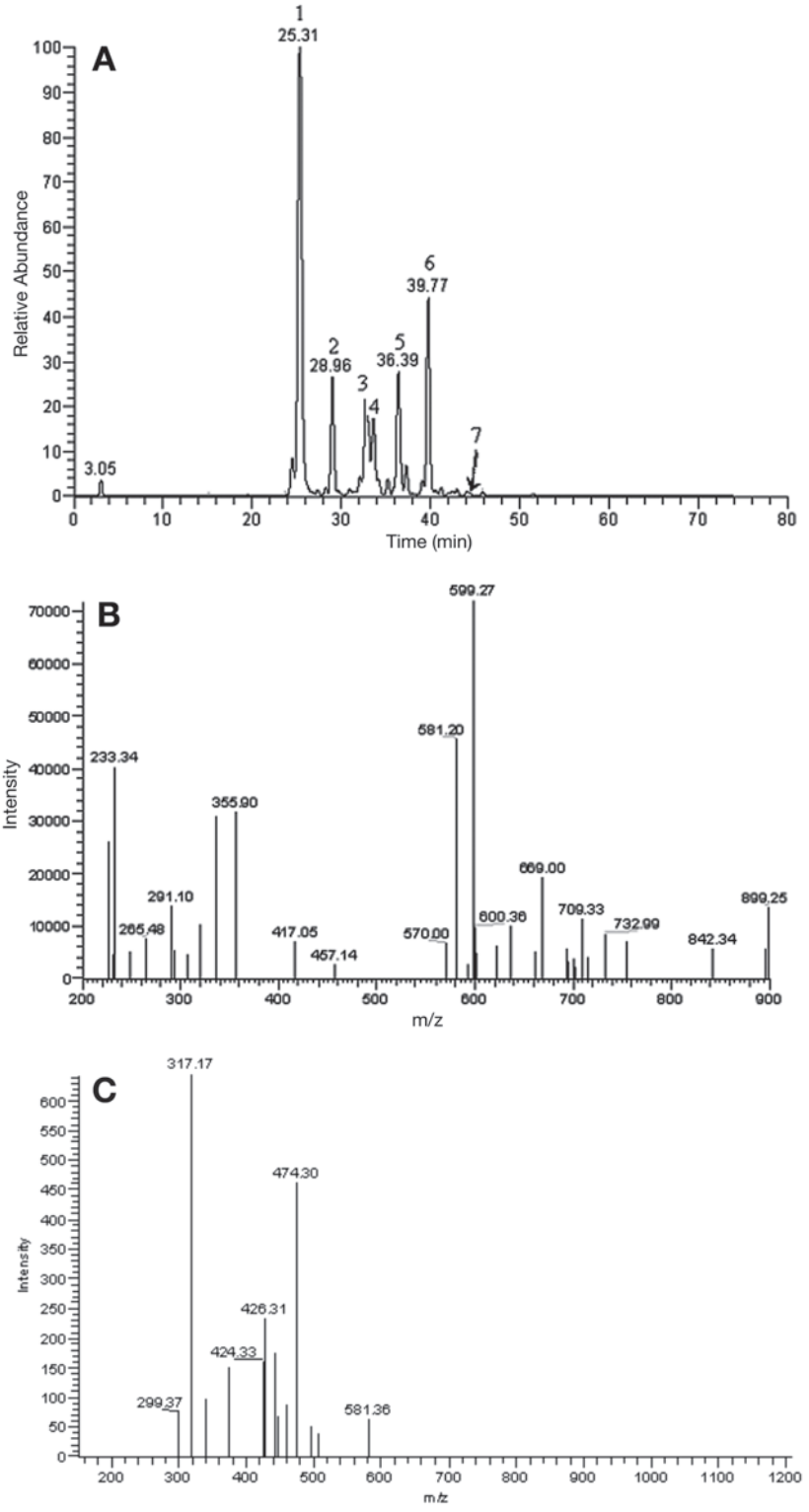


Fig. 6. LC-MS/MS analysis of fermentation products of inactive TE II mutant: **(A)** TIC spectrum; **(B)** Negative ESI-MS spectrum of peak 7 (probable streptolydigin); **(C)** MS-MS product ion spectrum obtained from $[M\text{-}H]^-$ (m/z 599.27) ions of probable streptolydigin.

and biosynthetic pathway associated with secondary metabolite biosynthesis *(26)*. If a gene is important for secondary metabolite production, gene disruption would cause a reduction in yield. If a gene is a negative regulatory element, gene disruption might increase productivity. Thus, there is a potential for improvement of the target secondary metabolite yield by genetic manipulation *(27,28)*. Regarding *S. lydicus* AS 4.2501, only 16S rRNA, cytochrome monooxygenase P450, transglutaminase, RNA polymerase gene, and some fragments of the findings are published in databases besides our reports. Under this condition, genes of *Streptomyces* are usually obtained by shotgun method, which is tedious and time-consuming. According to the homology among genes, fragments could be obtained by PCR *(29)*. Furthermore, PCR has been widely used in gene cloning and analysis *(30,31)*. In the present study, a 588-bp fragment was cloned and sequenced. It was deduced to be part of TE II gene of *S. lydicus* AS 4.2501 according to the conservative motif and active site of TE II amino acid composition. The sharp reduction in streptolydigin yield by inactivating TE II gene further confirmed that this fragment coded TE II, which was involved in PKS and played a role in streptolydigin biosynthesis.

Fungal PKSs differ from the very large bacterial modular PKSs in many aspects *(32)*. Acyl tetramic acid antibiotics such as fusarin C and equisetin were also discovered in fungi. It has been found that there was no type I TE in fusarin C and equisetin biosynthesis gene clusters *(32,33)*. The fully elaborated polyketide of fusarin C was proposed to be held by the ACP domain as a thiol ester and cleaved from PKS by reduction *(32)*. Thus, it is especially interesting to see what the PKS for an acyl tetramic acid from a *Streptomyces* will look like. Our group is working on cloning the full gene cluster of streptolydigin biosynthesis, and this fragment could be used as a specific probe.

So far, the total genomic sequences of *S. coelicolor* and *S. averimitilis* as well as the partial gene sequences of some important strains producing antibiotics have been reported. Furthermore, molecular biology has been central in the production of novel products, as well as in the improvement of existing processes *(34)*. Because of the existing homology among the same functional genes of different strains, PCR-based gene clone and targeted gene manipulation is a powerful tool for studying gene function and secondary metabolite biosynthesis pathway. In addition, genetic manipulation of polyketide biosynthesis genes could become a promising tool for constituting superstrains to produce novel active metabolites or synthesize novel antibiotics *(35–39)*, even for metabolomics study *(40)*.

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