A Self-defense Gene Homologous to Tetracycline Effluxing Gene Essential for Antibiotic Production in *Streptomyces aureofaciens*

Tohru DAIKI,
Kazuo AISAKA,
Ryoichi KATSUMATA,
and Mamoru HASEGAWA†

Tokyo Research Laboratories of Kyowa Hakko Kogyo Co., Ltd., 3–6–6, Asahimachi, Machida-shi, Tokyo 194, Japan

Received January 13, 1995

By Northern blot analyses with DNA probes carrying 6-demethylchlortetracycline (6-DCT) biosynthetic genes from *Streptomyces aureofaciens* NRRL3203, a highly expressed gene (terC) was detected in a high titer producing mutant derived from the parental strain NRRL3203 by NTG mutagenesis. The analysis of the nucleotide sequence of the 2.8-kb BamHI fragment containing terC gene showed that the predicted terC gene product is a protein consisting of 512 amino acids. The deduced amino acid sequence had a high level identity with that of the self-defense gene (ter347) of *Streptomyces rimosus*, known to mediate oxytetracycline efflux. The terC gene-inactivated strains generated from strain NRRL3203 by gene replacement had a 90% decrease in the level of resistance to tetracycline and the antibiotic productivity when compared with the parental strain.

A vast repertoire of naturally occurring antibiotics are produced by actinomycetes. Though these antibiotics are chemically well characterized, only limited information is available on the mechanism of regulation of their biosynthesis. To investigate this point, it is important to study the biochemical properties of enzymes participating in the biosynthesis. However, to date, only a few such studies have been done because of the lack of enzymatic assay systems and/or substrates. In contrast, recently developed genetic engineering techniques for actinomycetes enable us to clone the gene clusters for antibiotic biosynthesis, and to predict the primary structures of biosynthetic enzymes, and furthermore, to study the mechanism of regulation at least at the level of transcription.

In the case of chlortetracycline (including tetracycline and 6-DCT), which is mainly produced by *Streptomyces aureofaciens*, only the last three enzymes of at least 11 steps in the biosynthetic pathway have been studied. We had isolated a cosmids clone carrying a whole set of 6-DCT biosynthetic genes of *S. aureofaciens* NRRL3203. We therefore envisioned that this cosmids might be exploited for studying the regulation of chlortetracycline biosynthetic genes at the level of transcription.

In this study, we found by Northern blot analysis that mRNA of a self-defense gene that might mediate tetracycline efflux was highly expressed in the high producer of 6-DCT. It was also suggested that the self-defense gene is essential for tetracycline production.

**Materials and Methods**

*Bacterial strains and plasmids*. *Streptomyces aureofaciens* NRRL3203 was used as a 6-DCT-producing microorganism. A mutant designated strain HP351, which produces the antibiotic at a high level, was derived from strain NRRL3203 by N-methyl-N-nitro-N-nitrosoguanidine (NTG) mutagenesis (see below). *Streptomyces lividans* TK239 and a shuttle vector, pSE101, were used for the subcloning experiments. pSE101 was constructed as follows: pUC19 digested with *Aar*II was blunt-ended with T4 polymerase and followed by ligation to phosphorylated *Bgl*II linkers. The resulting plasmid pUC19-*Bgl* was digested with *Bgl*II and *Kpn*I, and the larger fragment was ligated to the *Bgl*II-*Kpn*I fragment prepared from pL7029 to generate pSE101. pL702 was also used as a vector for *S. aureofaciens*.

*Eschericia coli* XL1-Blue and plasmids, pUC118 and pUC119, were used for sequencing analysis. A cosmide clone, pGL2A, which carries a 6-DCT biosynthetic gene cluster of *S. aureofaciens* NRRL3203 was previously isolated.

*Media*. SK No. 2 and the soy-bean meal media were used for *S. aureofaciens* as the seed and production media, respectively. YEME was used for *S. lividans*. Growth conditions and manipulations of *E. coli* were as described by Maniatis et al. Transformants of *Streptomyces* strains and *E. coli* were cultivated in medium containing thiopropen (5 μg/ml), we used thiopropen instead of thioestroppen) and ampicillin (100 μg/ml), respectively.

*Isolation of high producer mutants*. A spore suspension (1 ml) of *S. aureofaciens* NRRL3203 (1× 10⁸ spores suspended in 0.85% NaCl) was treated with NTG (final concentration 1 mg/ml) at 30°C for 30 min. These spores were washed twice with 0.85% NaCl and spread on the Antibiotic Medium 4 (Difco). After incubation at 30°C for 7 days, the colonies were overlaid with nutrient agar broth (Difco) containing 10⁵ *E. coli* ATCC26 cells/ml. After incubation at 30°C for 20 h, colonies forming larger growth-inhibitory zones than that of strain NRRL3203 were selected and cultivated in the production medium to examine the productivity. One of the candidates, designated HP351, produced 6-DCT 1.4-fold higher than that of the parental strain.

*Protoplast transformation of *S. aureofaciens*. A recently developed protocol was used to prepare and to transform the protoplasts of *S. aureofaciens* NRRL3203.

*Measurement of antibiotic resistance*. About 10⁹ spores of strain NRRL3203 were spread on ATCC5 plates containing 0, 25, 50, 125, 250, 500, or 1000 μg/ml each of chlorotetracycline. Antibiotic resistance levels were observed after incubation at 30°C for 3 days.

*DNA manipulation*. Plasmids from *Streptomyces* strains were isolated by the method of Hopwood et al. Plasmids from *E. coli* were prepared by using Qiagen Plasmid Kit (Qiagen). All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Takara (Kyoto, Japan). Isolation of RNA and Northern blot analysis were done as described previously.

*Sequence analysis*. The 2.8-kb *Bam*HI fragment carrying terC were prepared from pGL2A, and subcloned into the *Bam*HI site of pUC119 to generate the plasmid pTCRB3. After we constructed a series of plasmids subcloned from pTCRB3, these single-stranded DNAs were prepared with helper phage M13 KO. Sequence analyses were done by the dideoxy chain termination method with Sequenase version 2 (U.S. Biochemical

*Present address*: Toyama Prefectural University, 5180 Kurokawa, Kurogi, Toyama 939-03, Japan.

† To whom correspondence should be addressed.
Co.). The nucleotide sequence of the 2.8-kb *BamHI* fragment will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D38215.

**Results**

**Temporal expression of the 6-DCT biosynthetic genes**

As one approach to study the regulation of expression of 6-DCT biosynthetic genes, temporal expression of mRNA of each biosynthetic gene was examined by Northern blot hybridization. Previously, we had isolated a cosmid clone designated pGLA2 (Fig. 1) carrying a gene cluster of 6-DCT biosynthesis of *S. aureofaciens* NRRL3203.³⁹ In this study, we used the DNA fragments carried on pGLA2 as probes for Northern blot analyses. Total RNA was isolated from the strain NRRL3203 cultivated in the production medium for 1, 4, and 7 days, and used for Northern blot analyses using ³²P-labeled probes of seven kinds of fragments in pGLA2 (Fig. 1, fragments a–g). Although a few signals could be detected in each blot analysis with the respective probes, there were no significant differences as to the intensity and the initiation of the transcription among each of the detectable messages (Fig. 2A, lanes 1, 3, and 5).

**A highly expressed gene (terC) in the high-titer producer**

In the production of actinorhodin in *Streptomyces coelicolor*, it is known that an increasing concentration of a putative transcriptional activator (actII ORF4) caused overproduction of the actinorhodin.¹³ As concerns chlorotetracycline biosynthesis, there are no reports about the gene(s) participating in antibiotic overproduction. To search for such genes in the biosynthetic gene cluster, a mutant strain, HP351, which produces 6-DCT at a high level was isolated by NTG mutagenesis. The expression profile of genes in the cluster was compared with that of the parental strain NRRL3203 by the method described above. There were no differences between strains NRRL3203 and HP351 as to the numbers and the sizes of detected messages. However, in HP351, every detectable message was expressed at a high level from days 1 to 7 (Fig. 2A, lanes 2, 4, 6) when compared with that of the parental strain. Furthermore, surprisingly, a transcript of about 1.5 kb that hybridized to the fragment-c probe (Fig. 1) was produced to a high level in the high-titer producer HP351 throughout the cultivation (Fig. 2A, c, lanes 2, 4, 6).

To discover the function of this gene product, the nucleotide sequence of the 2.8-kb *BamHI* fragment used as a probe was analyzed (Fig. 3). Two ORFs (ORF1 and ORF2), which could encode proteins consisting of 512 and 191 amino acids, respectively, were predicted by FRAME ANALYSIS.¹⁴ These two ORFs have high G+C contents (71% and 75%, respectively) and a marked bias in codon usage. Codons with G or C in the third positions in ORF1 and ORF2 are 95% and 96%, respectively. The stop codon of ORF2 is followed by a GC-rich region containing a long palindromic sequence (underlines in Fig. 3). Although it had been suggested to be a transcriptional terminator of the 7-chlorinating gene neighboring ORF2 (Figs. 1 and 3),¹⁶ this sequence might act as a transcriptional terminator of both the 7-chlorinating and ORF2-specifying genes. Searches in the database found that ORF1 had significant homology with a self-defense gene mediating tetracycline efflux of oxytetracycline-producing *S. rimosus*¹⁵ (ter347), (Fig. 4, 43% amino acid identity), tetracycline resistance gene of a transposon, Tn10¹⁶ (22% identity), actII ORF2

---

**Fig. 1.** Restriction Map of the Gene Cluster for 6-DCT Production in *S. aureofaciens* NRRL3203.

Solid bars under the upper physical map show the DNA fragments carried on cosmids pGLA2 and pGLA11 and fragments carrying self-defense genes inserted into pTCRB1 and pTCRB11 as reported previously.⁹⁰ The 2.8-kb *BamHI* fragment (fragment c) used for nucleotide sequence analysis in this study and the 4.4-kb *SacI-BamHI* fragment, the nucleotide sequence of which was analyzed,⁹⁰ are enlarged. Hatched boxes indicate the fragments used for Northern blot analysis. The restriction enzyme sites used for frame-shift mutations are marked with asterisks. 6-OH, CH₂, and 7-Cl represent the genes encoding hydroxylating enzyme at C-6 position, a putative methylation enzyme, and a chlorination enzyme at the C7-position, respectively.
A Self-defense Gene Essential for Tetracycline Biosynthesis

(a putative transmembrane export protein) from *S. coelicolor*\(^{17}\) (28% identity), and the *tecM* gene, mediating the metabolite export pump of tetracyclomycin C resistance\(^{18}\) (27% identity). On the other hand, the deduced amino acid sequence of ORF2 had no significant similarity to any proteins.

**Construction of highly expressed gene-inactivated strain**

To find whether the highly expressed transcript was derived from either ORF1 or ORF2, and detailed Northern blot analysis was done. The *EcoRI–SacI* and *SphI–BamHI* fragments containing ORF1 and ORF2 sequences, respectively (Fig. 1, fragments i and h, respectively), were prepared from the *BamHI* fragment c (2.8-kb), and used as probes. The highly expressed transcript (1.5 kb) could be specifically detected with the 1.4-kb *EcoRI–SacI* fragment (Fig. 2B, lane 1). On the other hand, a 0.8-kb transcript, which was also detected using fragment c as a probe (Fig. 2A, c), could be detected with the 0.4-kb *SphI–BamHI* fragment (Fig. 2B, lane 2), showing that the highly expressed transcript was derived from ORF1 but not ORF2.

On the basis of the sequence homology, the highly expressed gene (*terC*) was suggested with a high probability to encode a self-defense enzyme that mediates tetracycline
Fig. 3. Nucleotide Sequence of the 2.8-kb BamHI Fragment Carrying tcrC. The deduced amino acid sequences of ORF1 and ORF2 are also shown. Putative Shine-Dalgalno sequences and transcriptional terminator are indicated by double underlines and underlines, respectively. The nucleotide sequence from 2806 to 3000 has been reported previously.32 Nucleotides are numbered from the BamHI site upstream from ORF1.
Fig. 4. Alignment of the ORF1 Product with Homologous Proteins.

Numbers indicate positions in the predicted protein sequence of ORF1, with 1 corresponding to the putative N-terminus. Dashes within the sequences indicate gaps giving optimal alignment. Deduced amino acid sequences of ORF1 (A), the self-defense enzyme of S. rimous (B), and ORF2 (C) from Streptomyces coelicolor (C), icos gene mediating tetramycolicin C efflux (D) and tetracycline resistance gene from transposon 10 (E) are aligned using the DNASIS program (Hitachi, Japan) and indicate identical and similar residues, respectively.

efflux as described previously.\(^{15}\) To explore the possibility that terC codes for a self-defense enzyme, pTCRA2 was constructed in E. coli by inserting fragment c into a shuttle vector pSE101 replicable in both E. coli and S. lividans, and introduced into S. lividans TK23 to examine whether pTCRA2 could confer chlorotetacycline resistance on S. lividans. However, no transformants were obtained in spite of the fact that pSE101 itself transformed S. lividans TK23.
Thus, to assess the problem, we construct a mutant, in which ORF1 was inactivated by the gene replacement technique originally developed for Streptomyces by Anzai et al.\textsuperscript{19)} Plasmid pTCSRBA3, which had been constructed by inserting fragment c (2.8-kb BamHI fragment) into the BamHI site of pUC119, was digested with Stul and ligated to a phosphorylated BglII linker to construct pTCSRFSI. ORF1 in which was inactivated by a frame-shift mutation. The BamHI fragment of pTCSRFSI as then inserted into the BglII site of pIJ702 (thiopetpin\textsuperscript{®}), and the resultant plasmid pTCSRFS2 was introduced into Streptomyces RRL3203. Protoplasts from thiopetpin-resistant colonies were prepared and regeneratated on RA medium. Genomic DNAs of the regeneratated thiopetpin-sensitive colonies (plasmid free) were prepared, digested with BglII, and hybridized by Southern blotting using fragment c as a probe (Fig. 5). In our experiment, about one tenth of the regeneratated colonies lost the plasmids, and the ORF1-inacivated strains emerged from plasmid-cured colonies at the frequency of about 5%. Three strains was isolated at random and used to examine sensitivity to chlortetracycline. The minimum growth-inhibitory concentrations of chlortetracycline of the parental strain RRL3203 and of all of the ORF1-inactivated strains were 200 and 25 (μg/ml), respectively, indicating that the ORF1-specifying protein might function in vivo as a self-defense enzyme. Furthermore, it was found that all of these mutants had a 90% decrease in the productivity of the antibiotic relative to the parental strain, suggesting that the ORF1-specified product is essential for tetracycline production.

**Discussion**

In this work, we used Northern blot hybridization to study the regulation of gene expression for 6-DCT biosynthesis. By using DNA fragments containing the cloned gene cluster as probes, the following results were obtained: (i) a highly expressed gene (terC) was detected in the mutant of high-titer (HP351) derived from the parental strain RRL3203 by NTG mutagenesis. terC seemed to encode a self-defense gene mediating tetracycline efflux based on the significant sequence similarity with proteins that were known to confer resistance by export of various agents in other microorganisms (Fig. 4). To explore whether terC encoded a self-defense enzyme, the recombinant plasmid (pTCSRBA2) carrying terC was constructed and introduced into S. lividans. However, no transformants were obtained. This observation was consistent with the result of our previous shotgun cloning experiment, that is, although isolated at a high frequency two recombinant plasmids designated pTCSRBA1 and pTCSRBC1 (Fig. 1) carry tetracycline-resistance determinants different from terC, recombinant plasmids harboring terC were obtained. Considering these results, the presence of either terC or the ORF2-specifying gene, or of both genes on the high copy plasmids such as pIJ702 might be lethal to S. lividans. The following observation support the idea that terC itself might be lethal to S. lividans. Sloan et al.\textsuperscript{20) c} have cloned from Clostridium perfringens a tetracycline resistance determinant tetA(P), mediating tetracycline efflux. They found that when tetA(P) gene on a high copy plasmid, pKK223-3, was introduced into E. coli, the growth of transformants was significantly inhibited.\textsuperscript{20) c} Only low level expression of the tetA(P)- and terC-specifying proteins might be required to confer tetracycline resistance in E. coli and S. lividans respectively. In this study, however, terC was confirmed to be a self-defense gene since all of the three terC-inactivated strains had a 90% decrease in the level of resistance relative to the parental strain. (ii) every detectable message was expressed at high levels from day 1 to 7 (Fig. 2A, lane 2, 4, 6) in mutant HP351 when compared with that in the parental strain. Thus we do not know the meaning of this difference at this stage, it is possible that the highly expressed terC-specifying protein reduced the intracellular concentration of tetracycline in the high-titer strain.
Besides tcrC, we had isolated two distinct self-defense genes cloned into pTCRBA1 and pTCRBC1,\(^\text{60}\) revealing that S. aureofaciens NRRL3203 has at least three distinct self-defense genes in contrast to the reports on S. rimosus, from which two self-defense genes have been isolated.\(^\text{15,21–24}\) Until now, three mechanisms of tetracycline resistance have been known.\(^\text{25}\) The first is tetracycline efflux, which is the dominant mechanism of tetracycline resistance among Enterobacteriaceae. The second is ribosomal protection, which is found in mycoplasmas, and Gram-positive and Gram-negative bacteria. The third is tetracycline modification, which has been found only in the genus Bacteroides. However, the question of where the tetracycline resistance gene came from is not fully understood. Based on the fact that many tetracycline resistance genes have been found on plasmids,\(^\text{25}\) often as a transposable element, an idea that tetracycline resistance genes have been evolved from the self-defense genes of tetracycline producers has been proposed.\(^\text{25}\) The analysis and characterization of the self-defense genes on pTCRBA1 and pTCRBC1 might shed light on the origin of tetracycline resistance.

Acknowledgments. We thank Mrs Michiko Nakagawa (Kyowa Hakko Kogyo Co., Ltd.) for her excellent technical assistance.

References