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Identification of 2-Deoxy-*scyllo*-inosose Synthase in Aminoglycoside Producer *Streptomyces*

KHAREL, MADAN KUMAR, BIMALA SUBBA, HEI CHAN LEE, KWANGKYOUNG LIOU, JIN SUK WOO[1], DONG HWAN KIM[2], YOUNG-HO MOON[2], AND JAE KYUNG SOHNG*

Institute of Biomolecule Reconstruction, Sun Moon University, 100 Kalsan-ri, Tangjeong-myun, Asan-si, Chungnam 336-708, Korea
[1]*GeneChem Inc., 461-6, Jeonmin-Dong, Yuseong-gu, Daejon 305-811, Korea*
[2]*GenoTech Corp., 461-6, Jeonmin-Dong, Yuseong-gu, Daejon 305-811, Korea*

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Abstract Although most of the DOS containing aminoglycosides are produced by *Streptomyces*, very little information is available about their biosynthesis. In the present paper, we report a method to isolate DOI synthase, a key enzyme in the biosynthesis of DOS, from aminoglycoside producer *Streptomyces*. PCR primers based on conserved region of DOI synthases were specific and reliable for the isolation of the biosynthetic genes of DOS containing aminoglycosides or the screening of the aminoglycoside producers. The use of DOI synthase as a probe could save both time and cost of cloning aminoglycoside biosynthetic genes.

Key words: Aminoglycoside biosynthetic genes, 2-deoxy-*scyllo*-inosose synthase, *Streptomyces*

Aminoglycosides antibiotics represent one of the most clinically important antimicrobial agents being used in practice today. They include gentamicin, kanamycin, streptomycin, spectinomycin, and tobramycin. The major structural feature of these antibiotics is the existence of characteristic aglycone [8-9]. Despite a large amount of research interest in the biosynthesis of aminoglycosides, little has been elucidated regarding the biosynthetic enzymes and genes for these compounds, especially for 2-deoxystreptamine (DOS) containing aminoglycosides. Given the fact that most of the clinically important antibiotics are produced by *Streptomyces*, their biosynthetic genes and enzymes are the subject of numerous current researches. Until now, some gene fragments of L-glutamine: 2-deoxy-*scyllo*-inosose aminotransferases have been reported [12], representing the first DOS biosynthetic enzyme from *Streptomyces*. Although various efforts have been made, not a single gene homologous to 2-deoxy-*scyllo*-inosose (DOI) synthase has been reported from *Streptomyces* species [12]. Recently, we have for the first time reported DOI synthase homologues from aminoglycoside producer *Streptomyces* strains and conserved regions that are valuable in the cloning of the biosynthetic genes of DOS. DOI synthase from the butirosin biosynthetic pathway (*btr*C) was isolated and its function was characterized by heterologous expression in *Escherichia coli* [6]. Similarly, DOI synthase was purified and characterized from the crude extract of neomycin producer *S. fradiae* [13]. It represents the first enzyme in the DOS biosynthetic pathway, which catalyzes the formation of carbocycle, using glucose-6-phosphate as a substrate and NAD^+ as a coenzyme [14]. The mechanism has been further verified by a labeling experiment [14]. Mechanistically, DOI synthase and dTDP-D-glucose 4,6-dehydratase are similar [11]. In this communication, we describe for the first time identification of a *btr*C homologous gene from several DOS containing aminoglycoside producer *Streptomyces* by PCR-based approach.

Genomic DNA of *Streptomyces ribosidificus* IFO13796 (ribostamycin producer), *S. tenebrarius* IFO 13396 (tobramycin producer), *S. kanamyceticus* IFO13414 (kanamycin producer), and *S. lividans* TK24 (aminoglycoside nonproducer) were prepared by following the standard protocol [3]. The bacteria were cultured in N-Z amine medium (in g/l): glucose, 10; soluble starch, 20; yeast extract, 5; N-Z-Amine A, 5; and calcium carbonate, 1; ISP medium 2 (in g/l): Bacto-yeast extract, 4; Malt extract, 5; glucose, 2; and GYM medium (in g/l): yeast extract, 4; malt extract, 10, and calcium carbonate, 2. *E. coli* XL1 Blue MRF (Stratagene) was cultured in LB medium or on LB-agar media containing 50 mg/ml of ampicillin whenever necessary.

*Corresponding author
Phone: 82-41-530-2246; Fax: 82-41-544-2919;
E-mail: sohng@email.sunmoon.ac.kr

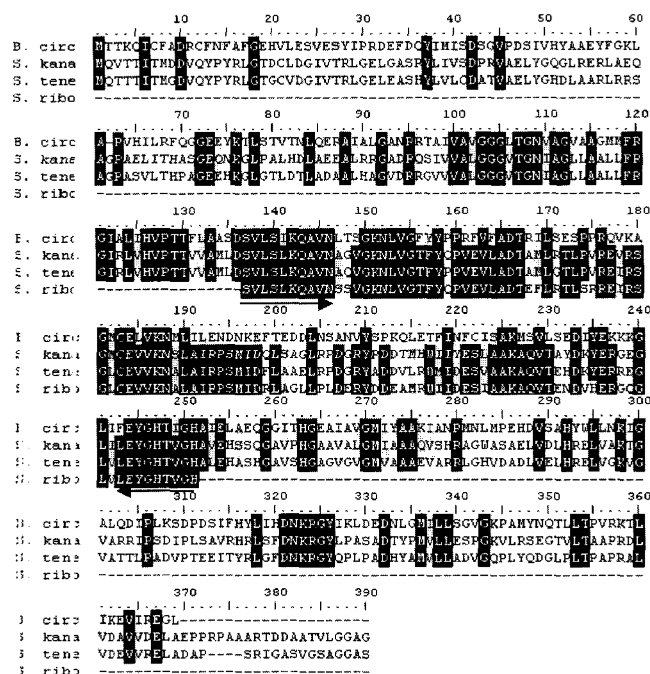

Fig. 1. Multiple sequence alignment of DOI synthases from *Streptomyces* and *Bacillus* species.

S. tene, S. ribo, S. kana, and *B. circ* stand for the *Streptomyces tenebrarius, Streptomyces ribosidificus, Streptomyces kanamyceticus*, and *Bacillus circulans*, respectively. The oppositely oriented arrows indicate the conserved region taken to design the primers.

DNA manipulation was carried out by following the standard protocol [10], the DNA sequencing by the dideoxy chain termination method using automatic sequencer, and polymerase chain reaction (PCR) by Eppendrof Mastercycle gradient. Oligonucleotide primers were synthesized at GenoTech (Korea), PCR premix used in the experiment was obtained from Bioneer (Korea), and pEZ-T vector (RNA Co. Korea) was used to clone the PCR amplified product. All the restriction enzymes used in this experiment were obtained form TAKARA (Japan). A database search was performed using BLAST and FASTA. Multiple alignment of sequence and construction of phylogenetic tree were carried out using ClustalW software. All the chemicals used in this study were of molecular biology grade and commercially available.

In order to identify *btr*C homologues from *Streptomyces*, two primers were designed by selecting the highly conserved region (SVLSLKQAV for the forward primer 5'-TCGGT-GCTGTCCCTGAAGCA-3' and EYGHTVGH for the reverse primer 5'-CCCGACGGTGTGGCCGTACT-3') of the DOI synthases from the butirosin biosynthetic pathway [4] and the gentamicin biosynthetic pathway (Fig. 1). Considering the high G/C codon preference in *Streptomyces* genes, G or C was used for the third nucleotide base, wherever possible. PCR experiment was carried out using the diluted genomic DNA template and DMSO (5% final concentration). Melting, annealing, and polymerization conditions were 95°C for 7 min, 54°C for 1 min, and 74°C for 1 min, respectively, for the first cycle, followed by 40 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min, and then 72°C for 9 min for the last cycle. Genomic DNA of aminoglycoside nonproducer *S. lividans* TK24 was used as a template for the reference. In all cases except *S. lividans*, we found the PCR product (345 bp) with expected size. The PCR product was subcloned into pEZ-T vector and sequenced. To avoid the false PCR product, three replicates of each were sequenced and analyzed. *E. coli* XL1-Blue MRF (Strategene) and *E. coli* BL21(DE3) (Invitrogen) were used as cloning and expression host, respectively.

The sequence result of PCR products revealed the highest similarities with BtrC from the butirosin biosynthetic pathway, which is the sole DOI synthase available in the databases. Identities of 41-45% with BtrC were found for all PCR-amplified fragments (Table 1). Other closer homologues are the 3-dehydroquinate synthases, a key enzyme in the shikimate acid pathway from various organisms: e.g., *Yersinia pestis, Campylobacter jejuni, Bacillus subtilis*, and *Escherichia coli*. This is probably due to the similar reaction mechanisms conferred by those enzymes [1, 2, 4, 13]. Multiple alignments of amino acids showed that DOI synthases of *Streptomyces* species have higher homologies with each other than with that of *Bacillus circulans* (Fig. 1). This indicates that DOI synthases in those *Streptomyces* have a common origin and similar diversification, as verified in the phylogenetic tree (Fig. 2). It is not well known whether DOI synthases and AroB have convergent origins or the reverse. The PCR-amplified DOI synthase (345 bp) was used to screen the biosynthetic genes of 2-deoxystreptamine from the genomic library of *S. tenebrarius* and *S. kanamyceticus*. Out of several positive cosmids, pST51 and pSKC2 were isolated from the genomic library of *S. tenebrarius* and *S. kanamyceticus*, respectively, and sequenced. The sequence revealed

Table 1. Summary of *Streptomyces* DOI synthases and their closest homologues in the databases.

Source	Gene fragment/full gene sequence (bp)	Typical homology (%)	Accession numbers of selected homologous proteins
S. ribosidificus	345/-	41/-	ABO3391 (BtrC)
S. tenebrarius	342/1,161	43/32	ABO3391 (BtrC)
S. kanamyceticus	345/1,173	45/35	ABO3391 (BtrC)

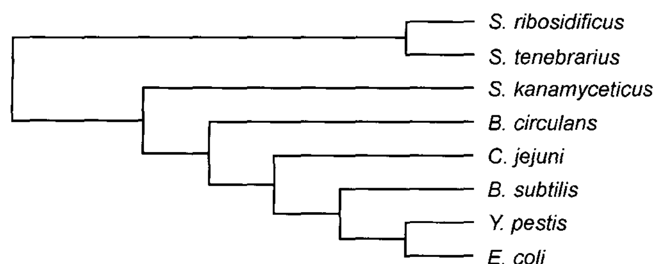

Fig. 2. Phylogenetic tree of BtrC homologues.
DOI synthases from *S. ribosidificus*, *S. tenebrarius*, *S. kanamyceticus*, and *B. circulans*, and 3-dehydroquinate synthase from *C. jejunii*, *B. subtilis*, *Y. pestis*, and *E. coli* were taken for the construction of the phylogenetic tree.

several DOS biosynthetic genes including DOI synthase, L-glutamine: *scyllo*-inosose aminotransferase, dehydrogenase, glycosyltransferase, and aminoglycoside resistance ribosomal methylases. A comparison of the full sequences of DOI synthases from *S. kanamyceticus* and *S. tenebrarius* revealed 32% and 35% identities with BtrC from *B. circulans*. Unlike 3-dehydroquinate synthases, S-137, V-138, L-139, S-140, and E-244, Y-245 residues are conserved in DOI synthases. Thus, the primers based on these conserved regions yield the DOI synthase-specific PCR products, which can be used to screen the DOS biosynthetic genes or DOS containing aminoglycoside producers. DOI synthases from *S. tenebrarius* (TbrA, 40 kDa) were overexpressed in *E. coli* BL21(DE3), the soluble protein was assayed for the conversion of glucose-6-phosphate to DOI, and the coversion by TbrA was detected in TLC, HPLC, and ESI-mass spectra (unpublished result), thus proving its active role in the biosynthesis of DOS.

Cloning of biosynthetic genes is the bottleneck for the study of biosynthetic pathways or their modulations. Application of core sequences of the keto-acyl synthase (KS) domain for screening polyketide synthase (PKS), and dTDP-D-glucose synthase or dTDP-D-glucose 4,6-dehydratase for screening deoxy sugar genes can be found elsewhere. Suh and coworkers have demonstrated the isolation of nonribosomal peptide synthase (NRPS) genes by the screening probe generated from conserved sequences of peptide synthases [5]. In contrast, no systematic approach has so far been found or proposed regarding the cloning of DOS-containing aminocyclitol antibiotics biosynthetic genes. In this context, we were successful to find that the DOI synthases from various *Streptomyces* showed their specificities in DOS containing aminoglycoside producers. To the best of our knowledge, this is the first report on DOI synthase sequences from *Streptomyces*. Furthermore, it is shown to be a good probe to screen the biosynthetic genes of DOS containing aminoglycosides, and can be used similarly to L-glutamine: *scyllo*-inosoe aminotransferase proposed by Piperbergs and Distler [7]. Thus, the use of DOI synthase or both DOI synthase and L-glutamine: DOI aminotransferase reduces formation of falsely hybridized products, and it is more beneficial for sorting out aminoglycosides biosynthetic gene clusters or screening aminoglycoside producers.

The sequences determined in the present study have been deposited in EMBL nucleotide sequence databases with the accession numbers AJ544586 for *S. kanamyceticus*, AJ544584 for *S. tenebrarius*, and AJ544585 for *S. ribosidificus*.

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