First Identification of *Streptomyces* Genes Involved in the Biosynthesis of 2-Deoxystreptamine-containing Aminoglycoside Antibiotics

—Genetic and Evolutionary Analysis of L-Glutamine:2-deoxy-*scyllo*-inosose Aminotransferase Genes—

Sir:

The biosynthesis of aminoglycoside antibiotics has long been attracting wide attention, however, little has been known for the biosynthetic enzymes and genes for these compounds, especially for 2-deoxystreptamine (DOS)-containing aminoglycosides. Particularly, clinically important aminoglycoside antibiotics are mostly produced by *Streptomycetes*, so that their biosynthetic enzymes and genes are the target of current studies.[1] Previously, we attempted to identify homologous genes of 2-deoxy-*scyllo*-inosose synthase[2] in *Streptomycetes* without success (unpublished results).

Quite recently, we have reported the identification of L-glutamine:2-deoxy-*scyllo*-inosose (DOI) aminotransferase gene (*btrS*) in the butirosin-biosynthetic gene cluster of *Bacillus circulans*, and confirmed its enzymatic activity with heterologously-expressed BtrS.[3] Because transamination of ketocyclitols is unique to the biosynthetic pathways of this class of aminoglycosides, *btrS*-homologous genes should obviously be included in other DOS-containing aminoglycoside-producers. In this communication, we describe the first identification of such *btrS*-homologous genes in several DOS-containing aminoglycoside producing *Streptomycetes* by PCR-based approach.

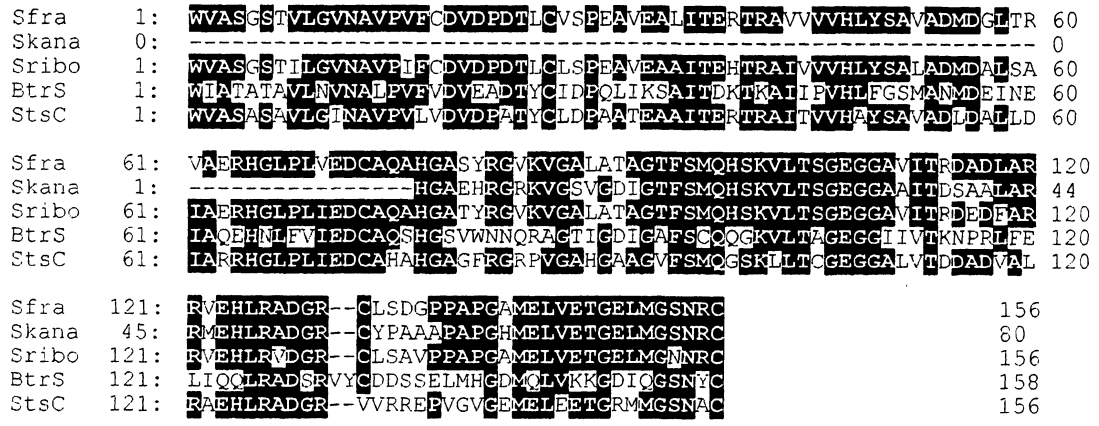
Streptomyces fradiae IFO12773 (neomycin-producer), *S. fradiae* IFO13147 (neomycin-producer), *S. kanamyceticus* JCM4433 (kanamycin-producer), *S. ribosidificus* JCM4923 (ribostamycin-producer) and *S. lividans* TK24 (non-producer) were separately cultured in Bennett's medium (1 g of yeast extract, 1 g of beef extract, 2 g of NZ amine, 10 g of glucose in 1 liter of water, pH 7.3). *Escherichia coli* JM109 was cultured in LB medium or on LB-agar containing 50 μg/ml of ampicillin, when necessary.

DNA manipulations were performed as described in the litrature.[4] DNA sequencing was carried out with LONG READIR 4200 (Li-Cor) according to the manufacturer's protocol. PCR was performed by GeneAmp PCR System 9700 (Amasham Pharmacia) using AmpliTaq Gold DNA polymerase (Applied Biosystems). Oligo DNAs for PCR primer were custom-synthesized by Amersham Pharmacia. Purification of plasmids was carried out with GFX Micro Plasmid Prep Kit (Amersham Pharmacia). Restriction enzymes and modification enzymes were purchased from TaKaRa (Japan). Genome preparations were carried out with Dr. GENTLE (TaKaRa). Genetic analysis was performed with GENETYX-WIN ver. 3 (Software Development, Japan), and database search was carried out by FASTA and BLAST2 on Internet. Evolutionary tree was constructed by the neighbor-joining method[5] using the CLUSTALW program on Internet. All other reagents were of the highest grade commercially available.

In order to identify *btrS* homologues in DOS-containing aminoglycoside-producing *Streptomycete*, five different PCR primers (btrS1f, btrS2f, btrS3f, btrS3r and btrS4r) were designed from the well-conserved regions between *btrS* and *stsC* (from *S. griseus*, involved in the streptomycin biosynthesis). Degenerate PCR (1 cycle at 95°C for 10 minutes, followed by 40 cycles of 95°C for 1 minute, 35°C or 30°C for 1 minute, and 72°C for 1 minute, and then 72°C for 7 minutes) was carried out by using two of these primers, with the chromosomes of aminoglycoside-producers (*S. fradiae* IFO12773, *S. fradiae* IFO13147, *S. kanamyceticus* JCM4433 or *S. rirosidificus* JCM4923) or a non-producer (*S. lividans* TK24) as template. PCR amplification was observed by using btrS1f and btrS4r as primers with the chromosome of *S. fradiae* IFO12773, *S. fradiae* IFO13147, or *S. ribosidificus* JCM4923 as template, and the same was true by using btrS3f and btrS4r as primers in all cases of aminoglycoside-producers. In contrast, similar experiments using the chromosome of aminoglycoside non-producing *S. lividans* TK24 as template gave no amplification at all in any case. Each PCR product was subcloned into pT7-blue T vector (Novagen), and the plasmid thus obtained was sequenced. To avoid incorporation of mismatch in PCR, at least 3 individual clones were analyzed.

By sequencing these products, *btrS* homologues of these *Streptomycetes* were clearly identified (Fig. 1). PCR fragments from the genome of *S. fradiae* IFO12773 and *S. fradiae* IFO13147 gave the same sequence. Based on the results, phylogenetic tree was construcrted, which showed that BtrS homologues of DOS-containing aminoglycoside-producing organisms were closely related. In addition, other related genetic information including streptomycin- and spectinomycin-biosynthetic genes and hypothetical ORF

Fig. 1. Alignment of btrS homologues.



Sfra; *S. fradiae*, Skana; *S. kanamyceticus*, Sribo; *S. ribosidificus*, BtrS; *B. circulans*, StsC (Y08763); from *S. griseus* (streptomycin-producer).

from *Streptomyces coelicolor* were also compared (Fig. 2). As a results, closer relationships of these genes were observed to streptomycin- and spectinomycin-biosynthetic enzymes than to BtrS of *B. circulans*. These secondary metabolism-specific genes seem to be derived from a rather old gene, and may be evolved in complex ways, vertically and horizontally.

In the present study, we found successfully the secondary metabolism-specific L-glutamine: DOI aminotransferase genes from various organisms and also showed that these genes are specific in DOS-containing aminoglycoside-producers. It should be emphasized here that this is the first confirmation of a biosynthetic gene of 2-deoxystreptamine-containing aminoglycosides in *Streptomycetes*. It appears further that this gene can be a useful marker for screening of aminoglycoside-producers or exploration of its biosynthetic genes, as PIPERSBERG and DISTLER suggested previously.[1] Our results should provide useful resources for engineered biosynthesis of aminoglycosides.

The sequences determined in the present study have been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers of AB066368 for *S. fradiae* IFO12773, AB066369 for *S. fradiae* IFO13147, AB066370 for *S. kanamyceticus* JCM4433, and AB066371 for *S. ribosidificus* JCM4923.

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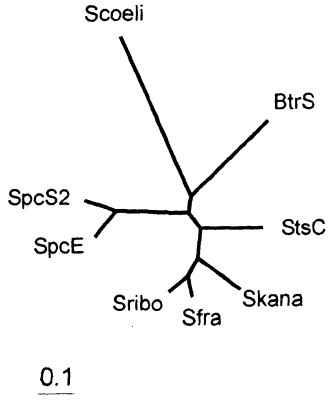
Fig. 2. Phylogenetic tree of BtrS homologues.



BtrS; *B. circulans*, Sfra; *S. fradiae*, Skana; *S. kanamyceticus*, Sribo; *S. ribosidificus*, StsC (Y08763); from *S. griseus* (streptomycin-producer), SpcE (U70376); from *S. flavopersicus* (spectinomycin-producer), SpcS2 (AF145039); from *S. spectabilis* (spectinomycin-producer), Scoeli; SC7C7.01 from *S. coelicolor* (http://www.sanger.ac.uk/Projects/S_coelicolor/).

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