J. Microbiol. Biotechnol. (2008), **18**(5), 866–873

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Heterologous Production and Detection of Recombinant Directing 2-Deoxystreptamine (DOS) in the Non-Aminoglycoside-Producing Host *Streptomyces venezuelae* YJ003

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Received: November 13, 2007 / Accepted: December 15, 2007

2-Deoxystreptamine is a core aglycon that is vital to backbone formation in various aminoglycosides. This core structure can be modified to develop hybrid types of aminoglycoside antibiotics. We obtained three genes responsible for 2-deoxystreptamine production, *neo7*, *neo6*, and *neo5*, which encode 2-deoxy-*scyllo*-inosose synthase, L-glutamine: 2-deoxy-*scyllo*-inosose aminotransferase, and dehydrogenase, respectively, from the neomycin gene cluster. These genes were cloned into pIBR25, a *Streptomyces* expression vector, resulting in pNDOS. The recombinant pNDOS was transformed into a non-aminoglycoside-producing host, *Streptomyces venezuelae* YJ003, for heterologous expression. Based on comparisons of the retention time on LC-ESI/MS and ESI-MS data with those of the 2-deoxystreptamine standard, a compound produced by *S. venezuelae* YJ003/pNDOS was found to be 2-deoxystreptamine.

Keywords: Aminoglycoside, 2-deoxystreptamine, neomycin, heterologous expression, *Streptomyces venezuelae*

Aminoglycoside-aminocyclitols (AmAcs) are an important class of antibiotics against Gram-positive and Gram-negative bacteria. They also show potentially useful activity against human immunodeficiency virus (HIV) and are mainly produced by *Streptomyces* and *Micromonospora* spp. [9, 11, 13, 24]. In general, AmAcs are a structurally diverse and relatively stable group that are comprised of two or more amino sugars joined to a hexose aglycon by glycosidic linkage. AmAcs are classified into two major groups, the streptamine-containing AmAcs and 2-deoxystreptamine (DOS)-containing AmAcs, depending upon their structural components. Substitutions of amino groups and deoxygenations of the usual sugar backbone have brought about structural diversities in AmAc antibiotics.

DOS is the central aglycon vital to backbone formation in many clinically important aminoglycosides and also shows low micromolar affinity to RNA hairpin loops for the biological activity [12]. Early studies on the incorporation of labeled substrates in neomycin showed that DOS is derived from D-glucose [17]. The biosynthetic studies of DOS-containing aminoglycosides such as neomycin, ribostamycin, butirosin, gentamicin, kanamycin, and tobramycin have been carried out, and the genes responsible for DOS biosynthesis have been studied and proposed [3, 4, 6–8, 13, 22, 27].

The *in vitro* characterization of genes responsible for the biosynthesis of DOS in the neomycin producer *Streptomyces fradiae* has been carried out by expressing each gene in *Escherichia coli* [8]. It has also been found that D-glucose-6-phosphate (G6P) is converted to DOS by *neo7* (430 aa), *neo6* (424 aa), and *neo5* (340 aa) in the neomycin biosynthetic pathway, which encode 2-deoxy-*scyllo*-inosose synthase (DOIS), L-glutamine: 2-deoxy-*scyllo*-inosose aminotransferase (GIA), and dehydrogenase, respectively [3, 14], as shown in the assembly of the DOS biosynthetic genes from the neomycin biosynthetic gene cluster (Fig. 1). However, none of the previously published reports have demonstrated the heterologous production of DOS *in vivo* using biosynthetic genes, and thus there are limits to such potential chemical modifications by attaching various substituents to the DOS ring [23].

We show here that heterologous expression of three genes (*neo7*, *neo6*, and *neo5*) from the neomycin biosynthetic gene cluster in a non-aminoglycoside-producing host, *S. venezuelae* YJ003 [1, 2], caused production of DOS.

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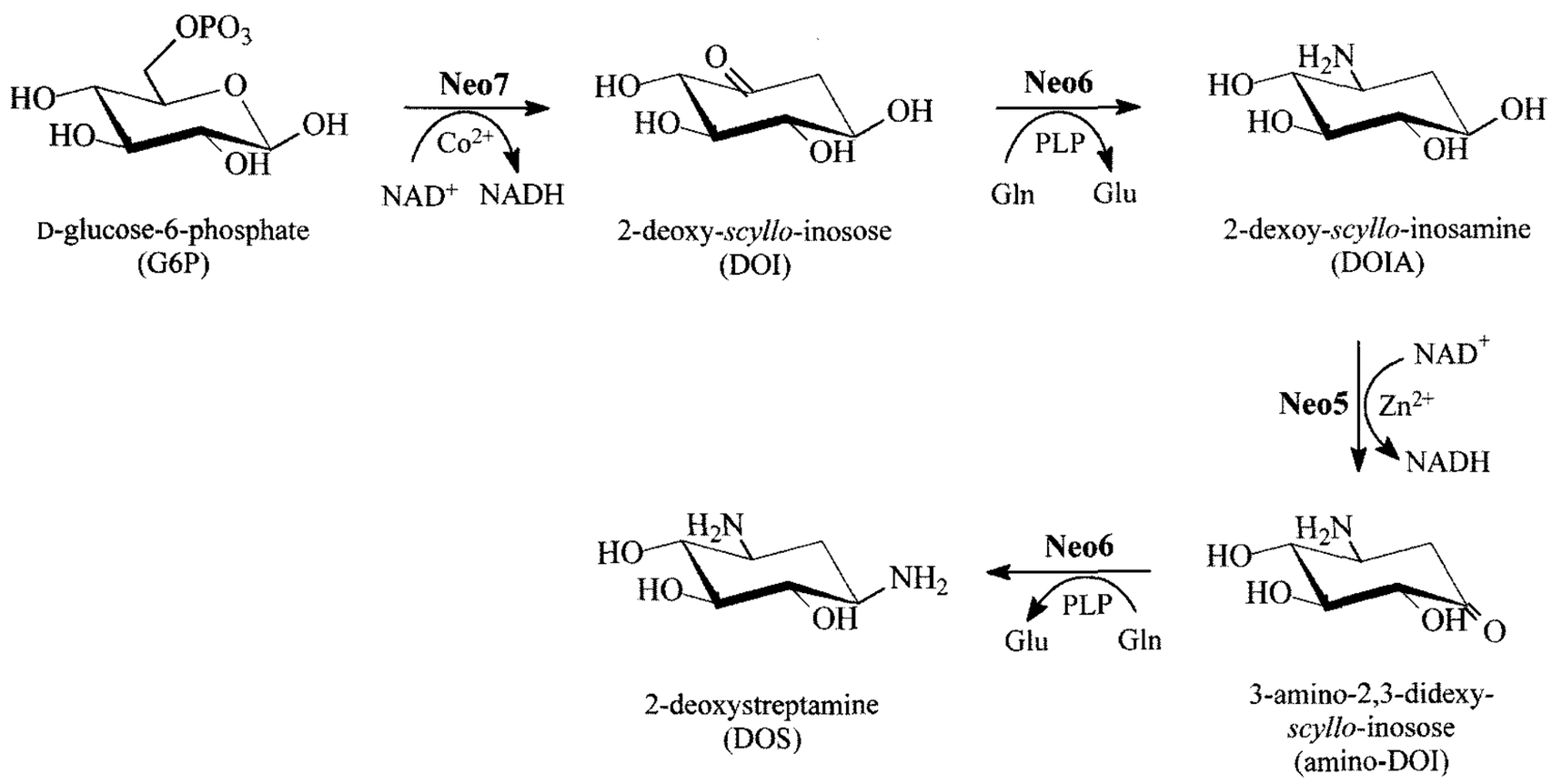

Fig. 1. Biosynthetic pathway of 2-deoxystreptamine.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Culture Conditions

S. fradiae was used to amplify the three genes responsible for DOS. *S. venezuelae* YJ003, which is deficient in biosynthesis of the endogenous desosamine, was used as a heterologous host for the expression of DOS biosynthetic genes [1, 2] and was grown on R2YE liquid medium (5% sucrose, 0.02% potassium sulfate, 1% magnesium chloride, 1% glucose, 0.5% yeast extract, and 0.01% Difco casamino acid) and an R2YE agar supplemented with thiostrepton (500 μg/ml) and kanamycin (1 mg/ml). *E. coli* XL1 blue (Stratagene, U.S.A.) and pGEM-T Easy (Promega, U.S.A.) were used for subcloning and DNA manipulation. *E. coli* ET12567 was used as a host to obtain demethylated DNA before transformation into a heterologous host. All *E. coli* strains were grown at 37°C for 6 h to overnight in Luria Bertani (LB) supplemented with ampicillin (100 mg/ml), tetracycline (25 μg/ml), chloramphenical (100 μg/ml) and kanamycin (100 μg/ml) when required. *Streptomyces* strains were cultivated at 28°C for 4 days in four different kinds of media. R2YE, TSB (3% tryptic soy broth, 10.3% sucrose, 20.33% magnesium chloride hexahydrate, and 20% glycine) [15, 19], and N-Z amine (1% glucose, 2% soluble starch, 0.5% yeast extract, 0.5% N-Z amine, and 0.5% calcium carbonate) [5] were used for DOS production analysis, and SPA (0.1% yeast extract, 0.1% beef extract, 0.2% tryptose, 1.0% glucose, and 0.01% ferrous sulfate) was used for seed culture. The authentic standard DOS used in this study was provided by the GenChem Co. (Daejeon, Korea).

PCR Amplification of DOS Biosynthetic Genes

Two pairs of deoxyoligonucleotide primers were designed from three genes responsible for DOS. Two genes, *neo5* (dehydrogenase) and *neo6* (GIA), with a total size of 2.4 kb, were obtained using the forward primer Neo1 (5'-CGG ATC CCG GAG GAT TCG GCA CGA TGA AG-3') and reverse primer Neo3 (5'-GTT CTA GAG GGC GGT CAA GTG GCC AGG-3') (Geno-Tech, Korea). Similarly, the third gene, *neo7* (DOIS), having a size of 1.3 kb, was obtained with the forward primer Neo4 (5'-ATC TAG AGA GGA CCG GGA AGC ATC ATG C-3') and reverse primer Neo2 (5'-CGA AGC TTG GCG GTT ACG GCA CGG GTC C-3'). The restriction sites in the primers are underlined. Polymerase chain reaction (PCR) was performed as follows: 1 cycle at 94°C for 1 min and 30 cycles at 94°C for 1 min, 60 to 70°C for 1 min, and 72°C for 1 min. The PCR products were purified and cloned in the pGEM-T Easy vector for DNA sequencing.

Construction of Recombinant Plasmids

General procedures, such as DNA isolation, restriction endonuclease digestion, DNA ligation, and other DNA manipulations in *E. coli*, were performed according to the standard protocols [18]. Plasmid DNA was isolated from *Streptomyces* as described by Kieser *et al.* [5]. The *Streptomyces* expression vector pIBR25, which has a strong *ermE** promoter system, was used for the cloning and expression of three genes [21]. As shown in Fig. 2, pIBR25 was digested with the restriction sites BamHI/XbaI corresponding to the sites engineered in the primers designed to amplify *neo5* and *neo6*. The BamHI/XbaI fragment containing *neo5* and *neo6* was then obtained and cloned into pIBR25, resulting in pNK101. The recombinant pNK101 was used to clone the third gene by digestion with restriction enzymes XbaI/HindIII. The XbaI/HindIII fragment containing *neo7* was then obtained and cloned into pNK101, resulting in pNDOS.

Protoplast Transformation

Protoplast preparation, transformation, and selection of thiostrepton-resistant transformants were performed using previously described methods [5]. The host strain *S. venezuelae* YJ003 was cultured in 50 ml of R2YE liquid medium at 28°C for 36 h with vigorous shaking and selecting in 1 mg/ml of kanamycin. Proper mycelial growth was observed under a light microscope. The culture broth was transferred to a tube and washed with 10.3% sucrose. The protoplasts were generated by incubating the mycelia at 37°C for 45 min with the addition of 3 ml of lysozyme (5 mg/ml). The demethylated recombinant DNA (pNDOS) using *E. coli* ET12567 was then transformed into the protoplast of *S. venezuelae* YJ003 using polyethylene glycol (PEG, 40%). The condition was optimized

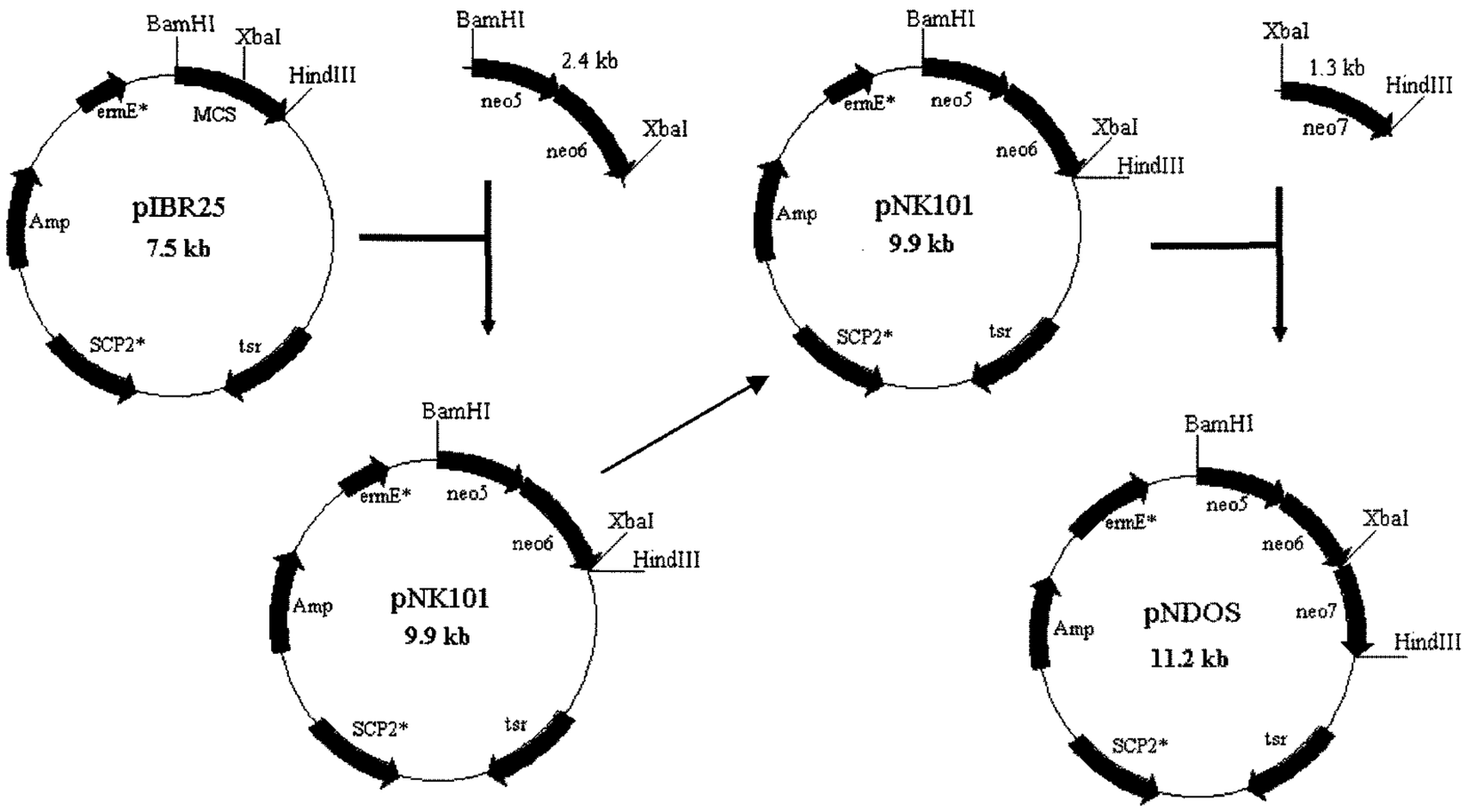

Fig. 2. Construction of recombinant pNDOS.
Transformation was confirmed by digestion with BamHI/HindIII to separate the inserted genes (3.7 kb) and pIBR25 vector (7.5 kb).

for protoplast transformation. R2YE plates were used for plating in different conditions. Overlay was done after 12 h with 500 μg/ml of thiostrepton in 0.3% agar and then incubated again at 28°C for 4 to 5 days. Colonies were selected in an R2YE plate supplemented with different concentrations of kanamycin/thiostrepton and several transformants obtained. Some of the transformants were selected in an R2YE plate with the required antibiotics and cultured in liquid media in order to isolate the plasmid DNA. The corrected colonies that were referred to as *S. venezuelae* YJ003/pNDOS were then confirmed by restriction enzyme digestion and by performing PCR with the plasmid DNA from *S. venezuelae* YJ003/pNDOS as template and primers for the genes investigated in this study. As a control, pIBR25 was transformed, yielding *S. venezuelae* YJ003/pIBR25.

Production, Isolation, and Analysis of DOS

Transformants *S. venezuelae* YJ003/pNDOS were cultured in 50 ml of seed media containing 1 mg/ml of kanamycin and 500 μg/ml of thiostrepton. *S. venezuelae* YJ003/pIBR25 was also cultured in 50 ml of seed media containing 1 mg/ml of kanamycin. After 36 h incubation at 28°C, these seed cultures were inoculated into 400 ml of main media in Erlenmeyer flasks. The culture flasks were incubated for 5 days at 28°C. The culture broth was transferred to a beaker and a few drops of antifoam reagent were added. The pH of the broth was adjusted to 2.5 with 2 N H_2SO_4, and the mycelial cake was removed by centrifugation. The pH of the supernatant was adjusted to approximately 6.5–7.0 with 2 N NaOH and passed through Amberlite IRC 50 ion-exchange column (Arcos, U.S.A.). The column was washed with several volumes of distilled water to remove unbound impurities. Finally, our target compound was eluted with 1 N, 2 N, and 3 N ammonium hydroxide solutions, respectively. The effluent was concentrated under reduced pressure using a SpeedVac centrifuge, and the dried compound was dissolved in distilled water. The isolated compound was purified by the methanol precipitation method using ice-cold methanol. For this, the compound was acidified to pH 4.5 with dilute sulfuric acid and treated with activated charcoal. The mixture was slowly poured into ice-cold methanol, drop by drop, to precipitate the desired compound. Then, the methanol was filtrated out and the remaining solute was dissolved again in distilled water.

The molecular weight of the compound was evaluated by electrospray ionization-mass spectrometry (ESI-MS). To observe the UV absorbance spectra, the isolated compound was derivatized with the UV-active reagent 9-fluorenylmethyl chloroformate (FMOC-Cl) according to the process reported by Stead and Richards [20], with slight modifications. The derivatized compound was analyzed by liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI/MS). Analyses of the products detected at 260 and 280 nm were carried out using a reversed-phase column (Mightysil RP-18 Gp, Japan) with a linear gradient of acetonitrile (0–100%) in acidified water (0.01% trifluoroacetic acid).

Results and Discussion

Heterologous Expression of pNDOS in *S. venezuelae* YJ003

Amino acid sequence analysis of the DOS-producing genes revealed that Neo7 of neomycin, which converts G6P to 2-deoxy-*scyllo*-inosose (DOI) in the presence of NAD^+ and Co^{2+} as a cofactor, has high homology with the well-characterized BtrC (DOIS) of butirosin [7]. Furthermore, Neo6 and Neo5, which act as a PLP-dependant aminotransferase and alcohol dehydrogenase, respectively, show high homology with BtrR (also called BtrS) and BtrE, the respective functional genes of butirosin. Multiple sequence alignment of the amino acids of the DOS-forming enzymes of neomycin and butirosin showed that some regions were highly conserved between the two gene clusters (data not shown).

We chose *S. venezuelae* YJ003 as a heterologous expression host for the production of DOS. It is an engineered strain constructed with the kanamycin resistance gene by deleting the entire D-desosamine gene cluster (*desI* to *desVIII* and *desR*) [1, 2]. This strain has some important characteristics, such as rapid growth, high transformation efficiency, and complete dispersal of mycelia, which make it suitable as a heterologous host [10] and allow for large quantities of cell mass and metabolites to be harvested relatively quickly in comparison with other user-friendly hosts, such as *S. lividans* and *S. coelicolor*. Three genes, *neo7*, *neo6*, and *neo5*, which encode DOIS, GIA, and dehydrogenase, respectively, are located in the neomycin gene cluster of *S. fradiae*. For the heterologous expression of these genes, we used the *Streptomyces* expression vector pIBR25, which contains a strong *ermE** promoter, a very compatible and reliable SCP2* origin of replication [26], and thiostrepton/ampicillin-resistant markers (Fig. 2).

Heterologous expression was also confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). The standard protocol described in the RNeasy Mini Kit was followed (QIAGEN). The transcription level of *neo7* was found only from *S. venezuelae* YJ003/pNDOS but not from *S. venezuelae* YJ003/pIBR25, which was used as the control (data not shown).

Production, Isolation, and Analysis of DOS from *S. venezuelae* YJ003/pNDOS

S. venezuelae YJ003/pNDOS and *S. venezuelae* YJ003/pIBR25 transformants were cultured in the seed culture. These seed cultures were inoculated into 400 ml of defined media R2YE, and incubated at 28°C for 5 days, under the same condition. The compound was isolated by ion-exchange column chromatography as described in the Materials and Methods section. The isolated product was first analyzed by ESI-MS. The molecular weight of DOS, which corresponds exactly to the authentic standard (*i.e.*, 163 in the positive mode), was detected from the extract of *S. venezuelae* YJ003/pNDOS (Fig. 3). The compound was also analyzed by LC-ESI/MS. The extracts and authentic standard DOS were derivatized with FMOC-Cl for UV visualization, and thus the molecular weight of the compound increases by 222 if derivatized with one free NH_2 group of DOS. Furthermore, the total molecular weight of derivatized DOS with FMOC-Cl is 385 in the positive mode. We were able to detect the peak corresponding to the UV-visible derivative of DOS isolated from *S. venezuelae* YJ003/pNDOS as well as the authentic standard DOS (Fig. 4), but we were not able to detect this peak in the control strain *S. venezuelae* YJ003/pIBR25. These results confirm that *S. venezuelae* YJ003/pNDOS can produce DOS derivatives and that the heterologous production of DOS is possible in the aminoglycoside nonproducing host *S. venezuelae* YJ003.

In order to increase the production yield of DOS, we observed the production of DOS in different media (R2YE, N-Z amine, and TSB), which varied in their composition. The analysis of liquid chromatography-mass spectrometry (LC-MS) peaks with reference to the concentration of standard DOS showed that the amount of DOS production in TSB media was about 0.2 mg/l. It was also found that among the three media used, the best production was obtained in

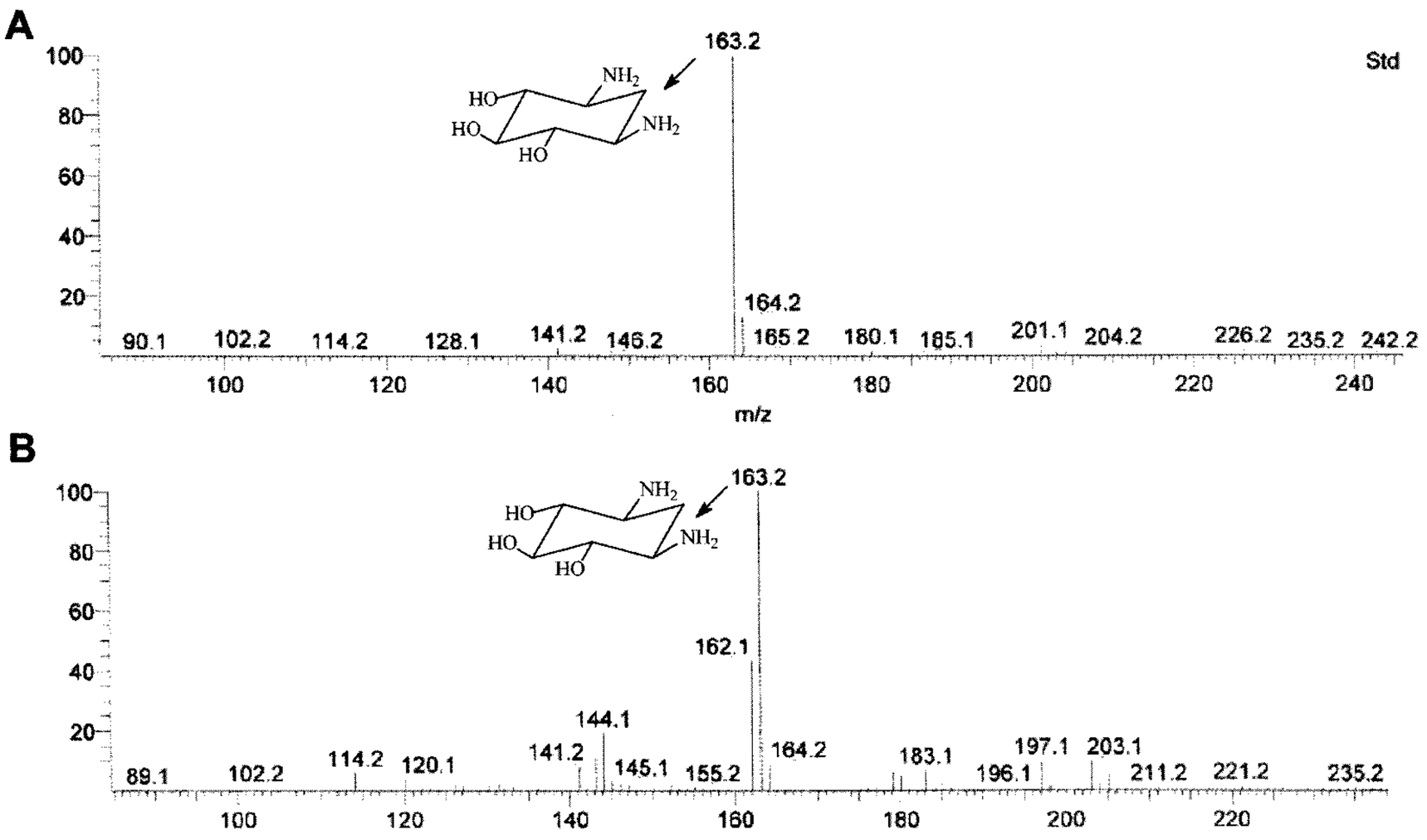

Fig. 3. ESI-MS of authentic 2-deoxystreptamine (**A**) and the isolated compound from the extract of *S. venezuelae* YJ003/pNDOS (**B**) in the positive mode.

the N-Z amine media, which was calculated to be 5 times greater than the production in TSB media and nearly 2 times greater than the production in R2YE media (data not shown). We also observed the dry cell weight of *S. venezuelae* YJ003/pNDOS in liquid media only and with soluble starch feeding. The dry cell weight at an interval of 24 h was measured for 9 days, and data showed that cell growth remained nearly consistent from 4 to 6 days when growth was at a maximum, which indicated that the appropriate time for harvesting secondary metabolites was between 4 to 6 days. It was also found that growth was enhanced by feeding with 1% soluble starch after 24 h in the culture media. The dry cell weight of the 1% starch-fed sample was found to be higher than that of the unfed sample cultured under identical conditions (data not shown).

DOS is an important moiety present in many clinically important aminoglycosides. This core moiety plays a critical role in the biological function of this class of antibiotics and has been shown to interact directly with the 16S-rRNA subunit [12]. The positively charged nature of this group of antibiotics combined with their high degree of flexibility facilitates their interaction with the negatively charged RNA backbone. Because of this effect, the majority of antibiotics containing this moiety mediate their action by disrupting mRNA processing and causing the misreading

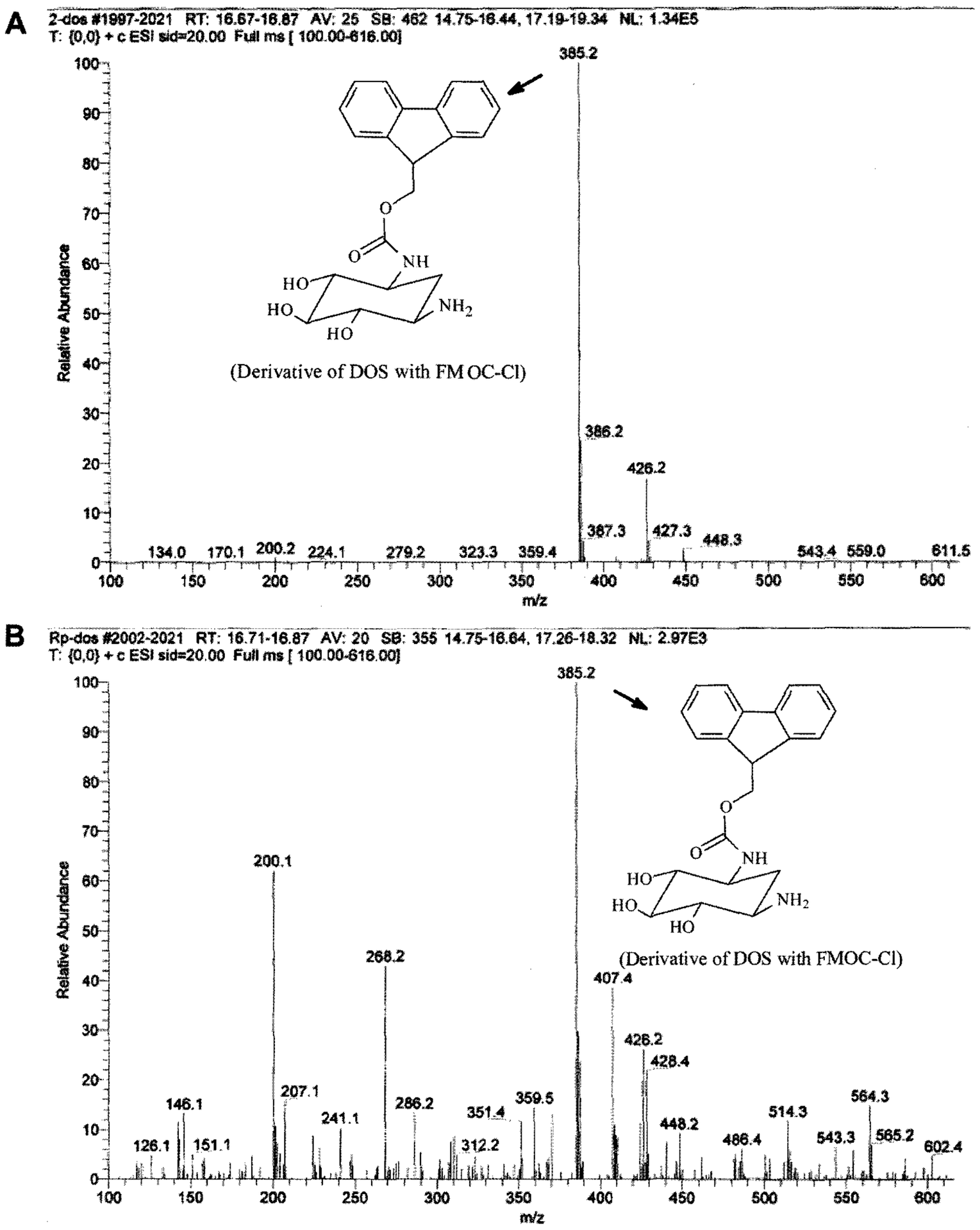

Fig. 4. LC-ESI/MS of authentic 2-deoxystreptamine (**A**) and the isolated compound from the extract of *S. venezuelae* YJ003/pNDOS (**B**) after derivatization with FMOC-Cl.

of the genetic code, thus resulting in a bactericidal effect [16]. Therefore, DOS itself is an important moiety, and the reason behind its production is that, by having the core structure in hand, it should be possible to develop a new and hybrid type of aminoglycoside antibiotics in the future.

We initially investigated the heterologous production of DOS by using different production media. We found that the production level was highest in N-Z amine, because this medium is rich not only in the carbon source (like in other media) required as the precursor, but also in the amine source required for the formation of DOS. The precursor of the desired product is often a limiting factor, because multiple enzymes compete for the same substrate. D-Glucose, which is the precursor in DOS biosynthesis, is also a major substrate in the primary metabolic pathway and is thus acted upon by multiple enzymes, which causes it to be relatively scarce in the substrate pool. Considering this factor, we fed 1% soluble starch to the culture broth after 24 h. As expected, the growth of starch-fed *S. venezuelae* YJ003/pNDOS was enhanced, resulting in a higher dry cell biomass production than in the unfed culture sample of the same strain in liquid media. Enhanced growth and higher dry cell biomass production direct our expectation towards the higher production of any secondary metabolite, and thus a higher level of DOS production can be expected after adding starch as the carbon source.

Butirosin is a DOS-containing aminoglycoside in which the enzymes responsible for DOS formation are well characterized [8], and it is the first aminoglycoside found to be produced by a non-actinomycete bacterium, *i.e.*,

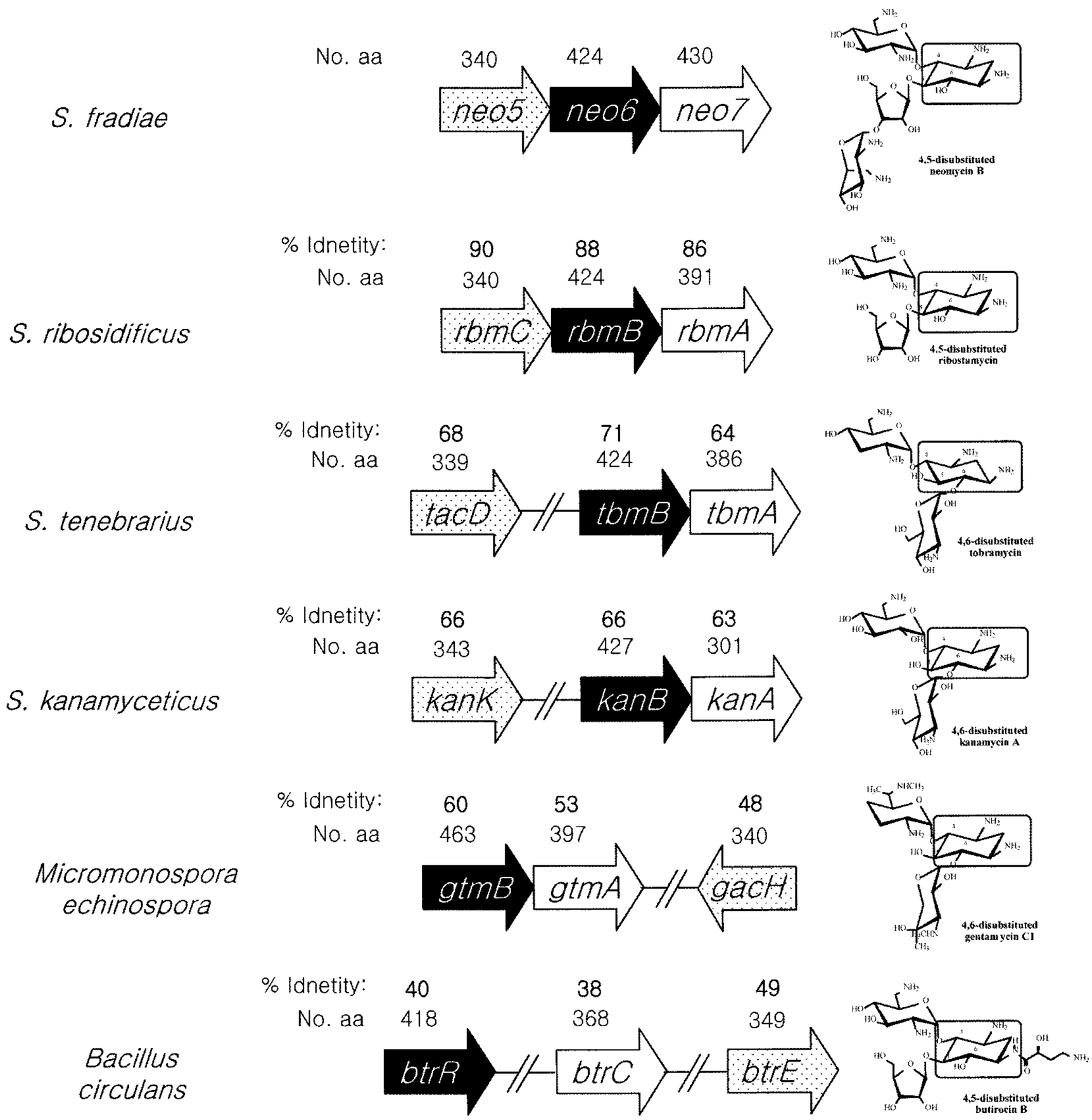

Fig. 5. Organization of the 2-deoxystreptamine biosynthetic gene set in *S. fradiae* and other species.
The percentages of amino acid identity of *S. fradiae* ORFs with other respective ORFs are indicated in bold. The sizes in amino acids are given in regular numerals above the respective ORF.

Bacillus circulans [25]. Therefore, we compared the amino acid sequence of the DOS-producing ORFs of neomycin with that of butirosin and found 38% identity between Neo7 and BtrC, 40% identity between Neo6 and BtrR (BtrS), and 49% identity between Neo5 and BtrE. Despite the well-characterized aminoglycosides, the DOS-producing ORFs of the neomycin gene cluster also share very high homology with the putative DOS-producing ORFs of other DOS-containing aminoglycoside gene clusters (Fig. 5). For example, Neo7, which catalyzes G6P to DOI in the presence of NAD^+ and Co^{2+} as cofactors, has 86%, 64%, 63%, and 53% identities with RbmA of ribostamycin, TbmA of tobramycin, KanA of kanamycin, and GtmA of the gentamicin gene cluster, respectively. The homology study was carried out using the BLAST program (NCBI). Similarly, Neo6, which catalyzes the two steps of DOS biosynthesis (the conversion of DOI to 2-deoxy-*scyllo*-inosamine [DOIA] and the conversion of 3-amino-2,3 dideoxy-*scyllo*-inosose [amino DOI] to DOS), has 88%, 71%, 66%, and 60% identities with RbmB of ribostamycin, TbmB of tobramycin, KanB of kanamycin, and GtmB of the gentamicin gene cluster, respectively. Moreover, Neo5, which acts as an alcohol dehydrogenase, shares 90%, 68%, 66%, and 48% identity with RbmC of ribostamycin, TacD of tobramycin, KanK of kanamycin, and GacH of the gentamicin gene cluster, respectively.

From these data, it is clear that the highest homology is between neomycin and ribostamycin, which can be expected because of their structural similarity and similar ORF arrangement. The structure varies by only one additional aminosugar of neomycin in the parent ribostamycin. They also belong to the same class of DOS-containing 4,5-disubstituted aminoglycosides, and they are both produced by *Streptomyces*. The ORFs of DOS-producing tobramycin and kanamycin are in a similar pattern and share high homology, as both of them belong to the DOS-containing 4,6-disubtituted aminoglycoside produced by *Streptomyces*. However, gentamicin, which also belongs to the same DOS-containing 4,6-disubstituted aminoglycoside subclass of antibiotics, varies in the arrangement of the DOS-producing ORFs because this antibiotic is produced by another class of microorganism, *i.e.*, *Micromonospora*. On the other hand, butirosin is structurally similar to ribostamycin and varies by only a single side chain, but shows less amino acid sequence homology because the biosynthetic gene cluster of butirosin belongs to the non-actinomycete bacterium, *i.e.*, *Bacillus*. As shown in Fig. 5, the organization of the ORFs for DOIS, GIA, and alcohol dehydrogenase is nearly identical in all these organisms. In addition, the high sequence homology data indicate the possibility for the replication of this technique of heterologous expression in other homologous aminoglycoside antibiotics, which can sometimes yield a better result, and further study of its role may lead to a deeper understanding of DOS.

In summary, three genes, *neo7*, *neo6*, and *neo5*, which encode DOIS, GIA, and alcohol dehydrogenase, respectively, are necessary and sufficient for DOS formation. They were heterologously expressed in the non-aminoglycoside-producing host *S. venezuelae* YJ003, and the metabolites produced from the *S. venezuelae* YJ003/pNDOS transformant contained DOS as the desired product. Three media, R2YE, TSB, and N-Z amine, were used for the production of DOS and the best DOS production was observed in the N-Z amine medium. Feeding of 1% soluble starch in the culture broth after the lag phase of cell growth (24 h) enhanced the biomass production, which may correlate to secondary metabolite production. Cell growth was nearly constant at the maximum level from 4 to 6 days at 28°C. Our result suggests the possibility of the heterologous production of DOS from an engineered host, which often produces a lucrative amount of target secondary metabolites. Therefore, the recombination of various DOS-related genes from structurally different aminoglycosides is expected to lead to the production of novel aminoglycosides by combinatorial biosynthesis.

Acknowledgments

This work was supported by the Basic Research Program of the Korea Science and Engineering Foundation, Grant No. R01-2006-000-10234-0 and a grant (#20050401034682) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea. The authors thank to Korea Research Foundation (KRF) for providing Ph D (graduate) research scholarship to Nagendra Prasad Kurumbang.

References

1. Hong, J. S., S. H. Park, C. Y. Choi, J. K. Sohng, and Y. J. Yoon. 2004. New olyvosyl derivatives of methymycin/pikromycin from an engineered strain of *Streptomyces venezuelae*. *FEMS Microbiol. Lett.* **238:** 392–399.
2. Hong, J. S., W. S. Jung, S. K. Lee, W. S. Koh, H. S. Park, S. J. Park, Y. S. Kim, and Y. J. Yoon. 2005. The role of a second protein (DesVIII) in glycosylation for the biosynthesis of hybrid macrolide antibiotics in *Streptomyces venezuelae*. *J. Microbiol. Biotechnol.* **15:** 640–645.
3. Huang, F., S. F. Haydock, T. Mironenko, D. Spiteller, Y. Li, and J. B. Spencer. 2005. The neomycin biosynthetic gene cluster of *Streptomyces fradiae* NCIMB 8233: Characterization of an aminotransferase involved in the formation of 2-deoxystreptamine. *Org. Biomol. Chem.* **3:** 1410–1419.
4. Kharel, M. K., B. Subba, D. B. Basnet, J. S. Woo, H. C. Lee, K. Liou, and J. K. Sohng. 2004. A gene cluster for biosynthesis of kanamycin from *Streptomyces kanamyceticus*: Comparison with gentamicin biosynthetic gene cluster. *Arch. Biochem. Biophys.* **429:** 204–214.

5. Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. *Practical Streptomyces Genetics*. John Innes Foundation, Norwich, U.K.
6. Kudo, F., Y. Hosomi, H. Tamegai, and K. Kakinuma. 1999. Purification and characterization of 2-deoxy-*scyllo*-inosose synthase derived from *Bacillus circulans*: A crucial carbocyclization enzyme in the biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics. *J. Antibiot.* **52:** 81–88.
7. Kudo, F., M. Numakura, H. Tamegai, H. Yamamoto, T. Eguchi, and K. Kakinuma. 2005. Extended sequence and functional analysis of the butirosin biosynthetic gene cluster in *Bacillus circulans* SANK 72073. *J. Antibiot.* **58:** 373–379.
8. Kudo, F., Y. Yamamoto, K. Yokoyama, T. Eguchi, and K. Kakinuma. 2005. Biosynthesis of 2-deoxystreptamine by three crucial enzymes in *Streptomyces fradiae* NBRC 12773. *J. Antibiot.* **58:** 766–774.
9. Kwon, Y. J. 2006. Studies on the binding affinity of aminoglycoside antibiotics to the HIV-1 Rev responsive element for designing potential antiviral agents. *J. Microbiol. Biotechnol.* **16:** 109–117.
10. Lee, S.-K., J.-W. Park, S.-R. Park, J.-S. Ahn, C.-Y. Choi, and Y. J. Yoon. 2006. Hydroxylation of indole by PikC cytochrome P450 from *Streptomyces venezuelae* and engineering its catalytic activity by site-directed mutagenesis. *J. Microbiol. Biotechnol.* **16:** 974–978.
11. Litovchick, A., A. Lapidot, M. Eisenstein, A. Kalinkovich, and G. Borkow. 2001. Neomycin B-arginine conjugate, a novel HIV-1 Tat antagonist: Synthesis and anti-HIV activities. *Biochemistry* **40:** 15612–15623.
12. Liu, X., J. R. Thomas, and P. J. Hergenrother. 2004. Deoxystreptamine dimers bind to RNA hairpin loops. *J. Am. Chem. Soc.* **126:** 9196–9197.
13. Li, Y., N. M. Llewellyn, R. Giri, F. Huang, and J. B. Spencer. 2005. Biosynthesis of the unique amino acid side chain of butirosin: Possible protective-group chemistry in an acyl carrier protein-mediated pathway. *Chem. Biol.* **12:** 665–675.
14. Llewellyn, N. M. and J. B. Spencer. 2006. Biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics. *Nat. Prod. Rep.* **23:** 864–874.
15. Park, N.-S., H.-J. Park, K. Han, and E.-S. Kim. 2006. Heterologous expression of novel cytochrome P450 hydroxylase genes from *Sebekia benihana*. *J. Microbiol. Biotechnol.* **16:** 295–298.
16. Patricia, M. F. and T. Mahmud. 2006. Biosynthesis of aminocyclitol-aminoglycoside antibiotics and related compounds. *Nat. Prod. Rep.* **24:** 358–392.
17. Rinehart, K. L., J. M. Malik, R. S. Nystrom, R. M. Stroshane, S. T. Truitt, M. Taniguchi, J. P. Rolls, W. J. Haak, and B. A. Ruff. 1974. Carbon-13 as a biosynthetic tool. IV. Biosynthetic incorporation of glucosamine-1-^{13}C and glucose-6-^{13}C into neomycin. *J. Am. Chem. Soc.* **96:** 2263–2265.
18. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. Seo, M.-J., E.-M. Im, D. Singh, A. Rajkarnikar, H.-J. Kwon, C.-G. Hyun, J.-W. Suh, Y.-R. Pyun, and S.-O. Kim. 2006. Characterization of D-glucose α-1-phosphate uridylyltransferase (VldB) and glucokinase (VldC) involved in validamycin biosynthesis of *Streptomyces hygroscopicus* var. *limoneus* KCCM 11405. *J. Microbiol. Biotechnol.* **16:** 1311–1315.
20. Stead, D. A. and R. M. E. Richards. 1997. Sensitive high-performance liquid chromatography assay for aminoglycosides in biological matrices enables the direct estimation of bacterial drug uptake. *J. Chromatogr. B* **693:** 415–421.
21. Sthapit, B., T.-J. Oh, R. Lamichhane, K. Liou, H. C. Lee, C. G. Kim, and J. K. Sohng. 2004. Neocarzinostatin naphthoate synthase: An unique iterative type I PKS from neocarzinostatin producer *Streptomyces carzinostaticus*. *FEBS Lett.* **556:** 201–206.
22. Subba, B., M. K. Kharel, H. C. Lee, K. Liou, B. G. Kim, and J. K. Sohng. 2005. The ribostamycin biosynthetic gene cluster in *Streptomyces ribosidificus*: Comparison with butirosin biosynthesis. *Mol. Cells* **20:** 90–96.
23. Sztaricskai, F. 2001. The fight against bacteria: The antibiotics policy. *Acta Pharm. Hung.* **71:** 89–98.
24. Tok, J. B., L. J. Dunn, and R. C. Des Jean. 2001. Binding of dimeric aminoglycosides to the HIV-1 rev responsive element (RRE) RNA construct. *Bioorg. Med. Chem. Lett.* **11:** 1127–1131.
25. Woo, P. W. K., H. W. Dion, and Q. R. Bartz. 1971. Butirosins A and B, aminoglycoside antibiotics. III. Structures. *Tetrahedron Lett.* **12:** 2625–2628.
26. Xue, Y., L. Zhao, H. W. Liu, and D. H. Sherman. 1998. A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity. *Proc. Natl. Acad. Sci. USA* **95:** 12111–12116.
27. Zhao, X. Q., K. R. Kim, L. W. Sang, S. H. Kang, Y. Y. Yang, and J. W. Suh. 2005. Genetic organization of a 50-kb gene cluster isolated from *Streptomyces kanamyceticus* for kanamycin biosynthesis and characterization of kanamycin acetyltransferase. *J. Microbiol. Biotechnol.* **15:** 346–353.