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Molecules and Cells

Characterization of RbmD (Glycosyltransferase in Ribostamycin Gene Cluster) through Neomycin Production Reconstituted from the Engineered *Streptomyces fradiae* BS1

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Amino acid homology analysis predicted that *rbmD*, a putative glycosyltransferase from *Streptomyces ribosidificus* ATCC 21294, has the highest homology with *neoD* in neomycin biosynthesis. *S. fradiae* BS1, in which the production of neomycin was abolished, was generated by disruption of the *neoD* gene in the neomycin producer *S. fradiae*. The restoration of neomycin by self complementation suggested that there was no polar effect in the mutant. In addition, *S. fradiae* BS6 was created with complementation by *rbmD* in *S. fradiae* BS1, and secondary metabolite analysis by ESI/MS, LC/MS and MS/MS showed the restoration of neomycin production in *S. fradiae* BS6. These gene inactivation and complementation studies suggested that, like *neoD*, *rbmD* functions as a 2-*N*-acetlyglucosaminyltransferase and demonstrated the potential for the generation of novel aminoglycoside antibiotics using glycosyltransferases *in vivo*.

INTRODUCTION

The 2-Deoxystreptamine (DOS)-containing antibiotics constitute the largest subgroup of the aminoglycoside class of antimicrobial agents (Pearce and Rinehart, 1981). This class includes many clinically important antibiotics, among which neomycin (Nem) and gentamicin, are the best known. Nem and gentamicin are particularly useful for virulent Gram-negative organisms (Dax, 1997), for which few therapeutic options are available (Cho and Rando, 1999; Tok et al., 2001). However, the application of aminoglycosides is limited due to their intrinsic toxicities (nephrotoxicity and ototoxicity) and due to recent emergency of several aminoglycoside-resistant pathogens (Mingeot-Leclercq and Tulkins, 1999; Mingeot-Leclercq et al., 1999). Thus, there is great demand for the development of safer and more potent aminoglycoside-based antibiotics. A fundamental challenge in the field of aminoglycoside antibiotics is the identification of the exact functions of the glycosyltransferases involved in the biosynthesis of the glycan structures because the sugar components of aminoglycosides play key roles in determining the antibacterial activity and toxicity of the antibiotic (Rodriguez and McDaniel, 2001).

The biosynthetic gene clusters of many aminoglycoside antibiotics have been sequenced, and their biosynthetic pathways have been proposed (Huang et al., 2005; Kharel et al., 2003; 2004; Subba et al., 2005; Unwin et al., 2004). As shown in Fig. 1, Nem and ribostamycin (Rbm) are architecturally simple and share three common subunits containing DOS, neosamine and ribose sugar. Thus, the biosynthetic genes involved in the biosynthesis of these intermediates have been proposed and characterized (Huang et al., 2005; Kudo et al., 2005; Llewellyn and Spencer, 2006). Although many putative glycosyltransferase genes have been identified in biosynthetic gene clusters, only the function of the glycosyltransferase genes involved in Nem biosynthesis has been investigated in detail (*in vitro*), and it was shown that one glycosyltransfease is required for the attachment of each individual sugar (Yokoyama et al., 2008).

The Rbm gene cluster required for ribostamycin biosynthesis has been identified from the Rbm-producing strain, *Streptomyces ribosidificus* ATCC 21294; however, the glycosyltransferase (*rbmD*) required for ribostamycin biosynthesis has yet to be identified (Subba et al., 2005). Taking advantage of the structural similarity between Nem and Rbm, we report the reconstitution of Nem from the *neoD*-deleted mutant, *S. fradiae* BS6, by the successful expression of glycosyltransferase (*rbmD*) from the Rbm gene cluster. To the best of our knowledge, this is the first report to describe the functional characterization of a glycosyltransferase from aminoglycoside biosynthesis *in viv*o.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The Rbm-producing strain, *S. ribosidificus* ATCC 21294, and Nem-producing strain, *S. fradiae* ATCC 10745, were obtained from the Korean Culture Center of Microorganisms (KCCM).

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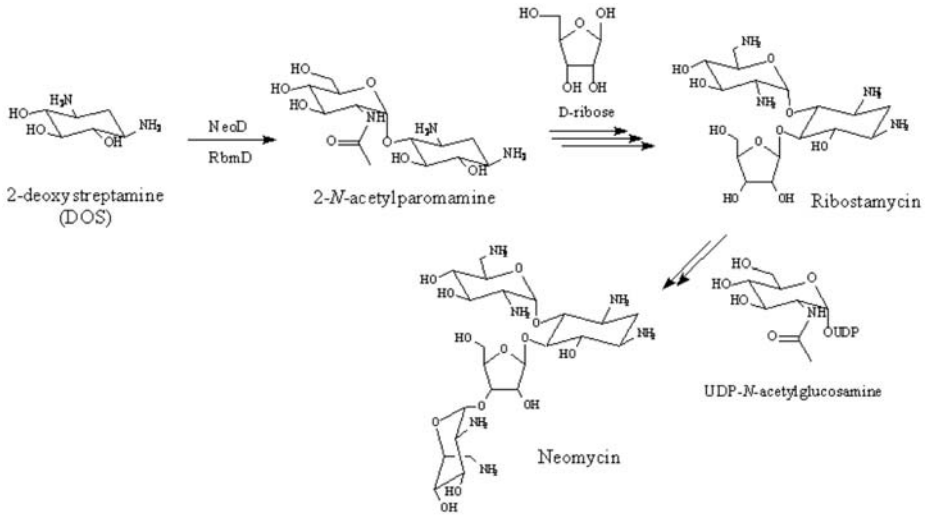

Fig. 1. Biosynthetic pathway of neomycin along with ribostamycin

Escherichia coli XL1 Blue MRF' (Stratagene, USA) was used to prepare recombinant plasmids. *E. coli* ET12567, *E. coli* ET12567 (pUZ8002), pKC1139, pOJ446 and pSET152 were all maintained in our laboratory (Basnet et al., 2006; Bierman et al., 1992).

S. ribosidificus and *S. fradiae* were grown in ISP2 (Difco, USA) media at 28°C. For protoplast preparation, *S. fradiae* was grown at 28°C in R2YE medium (Kieser et al., 2000) containing 0.4% glycine and 10.0% sucrose. For the production of secondary metabolites, all *S. fradiae* strains were grown in medium no. 167 (Bacto-yeast extract, 1 g; maltose, 10 g; N-Z amine, 2 g; beef extract, 1 g; and glucose, 1 g/L) for 5-8 d at 28°C. The required antibiotics including apramycin, thiostrepton and erythromycin were supplemented while inoculating during transformation and production.

General genetic manipulation

The routine manipulations of DNA were performed according to the standard procedures (Sambrook and Russell, 2001). Oligonucleotide primers were synthesized at Geno-Tech (Korea), and the enzymes were obtained from Takara (Japan). All chemicals were of molecular biology grade and commercially available. Southern hybridization was performed with Hybond N nylon membranes (Amersham). Probes were labeled with digoxigenin using a DIG labeling and detection kit (Boehringer Mannheim). Restriction mapping and other routine molecular biological methods were performed as described by Sambrook et al. (Sambrook et al., 1989). The conjugation from *E. coli* ET12567 (pUZ8002) into *S. fradiae* and its mutant was performed in a manner similar to the method of Kieser et al. with slight modification on MS agar medium (Kieser et al., 2000). Protoplast transformation and regeneration of protoplasts from *S. fradiae* BS1 were carried out in accordance with standard procedures (Hopwood et al., 1985).

Construction of *neoD*-deleted mutant in *S. fradiae*

For the disruption of *neoD*, an about 2.0 kb upstream region of *neoD* and 1.6 kb downstream region of *neoD* was amplified with two sets of primers (Table 1). These fragments were cloned into pKC1139 with the thiostrepton resistance gene (*tsr*), resulting in plasmid pGB1 (Fig. 2A). Plasmid pGB1 was then introduced into *S. fradiae* by conjugation, and gene replacement was confirmed by Southern blot analysis. Further confirmation was carried out by polymerase chain reaction (PCR) using *tsr* primer sets, Table 1. The 1.0-kb PCR product obtained from the disrupted mutants was cloned into the pGEM-T vector and sequenced from both ends, confirming that the mutant *neoD::tsr* was in fact obtained. The resulting *neoD* mutants were designated as *S. fradiae* BS1.

Construction of complementation plamids and transformation

To construct plasmids pGB5 and pGB6 to complement *S. fradiae* BS1 with *neoD* and *rbmD*, respectively, both genes were amplified from pNEM37 and pRBM4, respectively, using two sets of primers (Table 1). pGB5 was introduced into *S. fradiae* BS1 by protoplast method where as pGB6 was introduced by conjugation (Sambrook et al., 1989) resulted *S. fradiae* BS5 and *S. fradiae* BS6, respectively.

Product isolation and analysis

All *S. fradiae* strains were grown in production medium as mentioned above. The pH of the whole culture broth was made acidic (pH 2-3), and mycelia were removed by centrifugation at 3,000 rpm for 20 min. The pH of the supernatant was adjusted to around 6.5, and it was passed through Amberlite IRC 50 ion-exchange resin (Arcos, USA). The resin was washed with 10 volumes of distilled water to remove most of the unbound impurities. Finally, the crude metabolites were eluted with 2 N ammonium hydroxide. The pH of the effluent was adjusted to 4.5 with 1 N sulfuric acid, and the crude metabolite was precipitated as sulfate salt with ice-cold methanol.

The electrospray ionization/mass spectrometry (ESI/MS), liquid chromatography/mass spectrometry (LC/MS) and electrospray ionization/mass-mass (ESI/MS-MS) spectra of these purified compounds were taken for the identification of molecular weight. The UV-visible derivatives of the isolated compounds were prepared in accordance with the previous report of Stead and Richards, with slight modifications (Stead and Richards, 1996). During derivatization, the compounds were mixed with 9-fluorenylmethyl chloroformate (FMO-Cl). The pH of the reaction sample was adjusted to around 9.5 using borate buffer (pH 10 and 0.37 mM), and the mixture was heated at 37°C for 1.5 h. The reaction was quenched by the addition of 0.1 M glycine. The sample was centrifuged for 20 min, and it was filtered with 0.2 μm nylon membrane filters (Whatman®, England). LC/MS analysis was carried out at 260 and 280 nm using a C18 column (Mightysil RP-18 Gp, Japan).

Table 1. List of the primers used in this study

Name	Sequence (5′ - 3′)	Restriction site	Purpose
NeoF1-F	CGGATCCAGCCGCCATCCTCA	*Bam*HI	Amplification of *neo*D
NeoF1-R	GGTCTAGAGGGGCGGGGCGGTTA	*Xba*I	Upstream region for disruption
NeoF2-F	GGTCTAGAGGGTGGGCGCCGTGACG	*Xba*I	Amplification of *neo*D
NeoF2-R	GGAAGCTTGGCCCGCGCCAGCGCCAG	*Hin*dIII	Downstream region for disruption
tsrM1-F	ACCATGACTGAGTTGGACACCATCGCA	-	To confirm replacement of
tsrM1-R	ACGGTTGGCCGCGAGATTCCTGTC	-	*neo*D::*tsr*
GTneo-F	CCGGATCCACGGCCCCGCGCC	*Bam*HI	Amplification of *neo*D
GTneo-R	CGGATCCACGTCACGGCGCCCAC	*Bam*HI	For self complementation
GTRB-F	TCGGATCCCGCCGTCCTCTTCCGT	*Bam*HI	Amplification of rbmD
GTRB-R	ACGCGGATCCGCAGGCGACGTCATG	*Bam*HI	For cross complementation

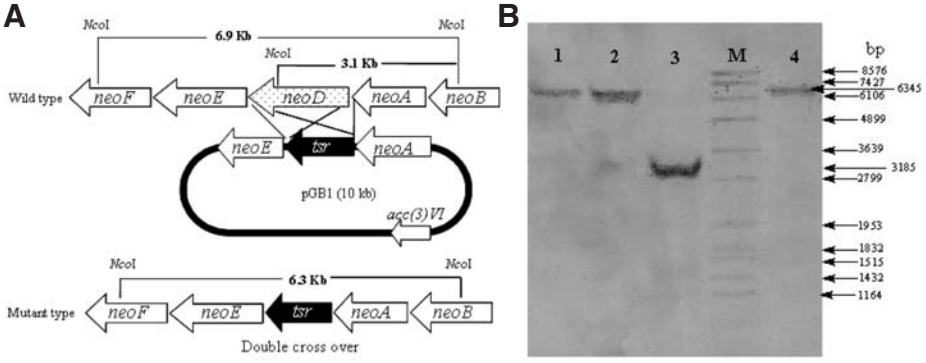

Fig. 2. Inactivation of *neoD* in *S. fradiae*. (A) Schematic representation of the *neoD* gene inactivation. (B) Southern blot analysis in the *neoD* gene inactivation experiment. Genomic DNA was restricted with *Nco*I from wild-type (lane 3) and double-crossover mutant (lanes 1, 2, and 4). Lane M, DNA dig labeling marker (Roche, Germay). The *neoA* (1.2 kb) was used as a probe.

Antibacterial assay

The antibacterial activities of authentic Nem (Sigma) and the isolated compounds against *Bacillus subtilis* ATCC 14893 were determined using a paper disc-diffusion assay, as described elsewhere. *B. subtilis* was initially grown on Luria-Bertani (LB) medium, and aliquots of the grown culture were dispensed on LB agar-based medium. Each compound from *S. fradiae* BS1, *S. fradiae* BS5 and *S. fradiae* BS6 and standard Nem) were dispensed into paper disks. The dried disks were placed on agar plates and incubated at 37°C for about 8-10 h.

RESULTS AND DISCUSSION

Sequence analysis

Having cloned and sequenced the Rbm biosynthetic gene cluster (Subba et al., 2005), our work was aimed at further characterizing the genes involved in Rbm biosynthesis. Database comparison with the deduced product of *rbmD* using the BlastX program revealed similarities between RbmD and several glycosyltransferases from aminoglycoside producers. The most similar protein found was NeoD from *S. fradiae* (91% amino acid identity, accession no. AB211959.1) (data not shown). The *N*-acetylglucosaminyltransferase activity of NeoD has been demonstrated (Yokoyama et al., 2008). Due to the close structural similarity between Rbm and Nem, *rbmD* can be expected to function similarly in Rbm biosynthesis (Baud et al., 1977).

Generation of a *S. fradiae* BS1 mutant with the disrupted *neoD*

For the generation of a stable Nem non-producing mutant, two DNA fragments located up and downstream of *neoD* were amplified by PCR and cloned into the temperature-sensitive vector pKC1139 (Fig. 2A). This plasmid, named pGB1, was introduced into *S. fradiae*. Apramycin/thiostrepton-sensitive and Nem non-producing colonies were obtained. Strains ΔGT8, ΔGT253 and ΔGT258 were obtained after screening for apramycin-sensitive colonies. Genomic DNA was extracted from wild *S. fradiae* and mutants ΔGT8, ΔGT253 and ΔGT258 digested with *Nco*I and probed by Southern hybridization with a Dig-labeled 1.2 kb *neoA* (*Bam*HI/*Hin*dIII). A strong single signal was detected at 3.1 kb when probing wild-type DNA, whereas a 6.3 kb signal was obtained from the mutants (Fig. 2B). This 6.3 kb signal corresponds to an integrated 1.0 kb *tsr* cassette within the *Nco*I fragment of the Nem cluster. PCR analysis also confirmed the disruption of the *neoD* by replacement with *neoD::tsr* (data not shown). The productions of Nem from *S. fradiae* BS1 and the wild-type strain were compared using an antibacterial assay. As expected, wild *S. fradiae* produced Nem, but it was not detected in *S. fradiae* BS1 (Fig. 3).

Complementation of mutant *S. fradiae* BS1

To clearly determine whether the mutation event caused a polar effect or not, plasmid pGB5, having *neoD* in pSET152, was introduced in *S. fradiae* BS1 by protoplast transformation. Analysis of the extracts of complemented strain by ESI/MS and high performance liquid chromatography (HPLC)-UV clearly showed the restoration of Nem production (data not shown). Thus, we could rule out any upstream or downstream effects.

Expression of *rbmD* glycosyltransferase in *S. fradiae* BS1

The high sequence similarity between *rbmD* from *S. ribosidificus* and *neoD* from *S. fradiae* suggested that these two enzymes might play an identical role in the paromamine biosynthetic pathway. In order to test this hypothesis, a 1.2-kb *Bam*HI

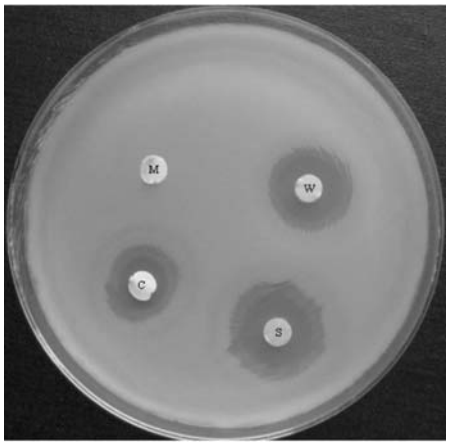

Fig. 3. Antibacterial assay of isolated compounds and authentic neomycin against *B. subtilis*. M, mutant *S. fradiae* BS1 metabolites (20 μl); W, wild *S. fradiae* metabolites (20 μl); C, complement *S. fradiae* BS6 metabolites (20 μl); and S, authentic neomycin (Sigma) (50 μg). Samples were isolated under identical condition and amount.

fragment containing the *rbmD* gene was cloned into the integrative vector pSET152 (pGB6) and introduced into *S. fradiae* BS1, and the result was designated as *S. fradiae* BS6. ESI/MS was used to analyze extracts of culture supernatants of the wild-type, *S. fradiae* BS6 and authentic Nem (Sigma). Extracts of the wild-type and complement showed peaks at 615 $[M + H]^+$ (Fig. 4). One peak was detected in the HPLC chromatogram for the derivatives of standard Nem. An identical peak was also observed with the products of wild *S. fradiae* and *S. fradiae* BS6 (data not shown). This compound may be the result of an incomplete derivatization of the amino groups of Nem. In order to verify this, both derivatized samples were subjected to LC/MS analysis, which showed an identical peak (Fig. 5). This compound corresponded to the $[M + H]^+$ for one amide derivative (products of amino group reactions with FMO-Cl) of Nem. The production of Nem by *S. fradiae* BS6 was further confirmed by MS/MS (Fig. 6). The control experiment (the pSET152 in *S. fradiae* BS1) did not show any antibiotic activity (data not shown).

CONCLUSION

The successful generation of Nem from a *neoD*-deleted mutant after complementation by *rbmD* suggested that *rbmD* seems to be responsible for the transfer of UDP-*N*-acetylglucosamine, the abundant primary metabolite in paromamine biosynthesis. Finally, the mutant *S. fradiae* BS1 can be complemented with glycosyltransferase genes from other strains to generate novel types of antibiotics in the aminoglycoside family.

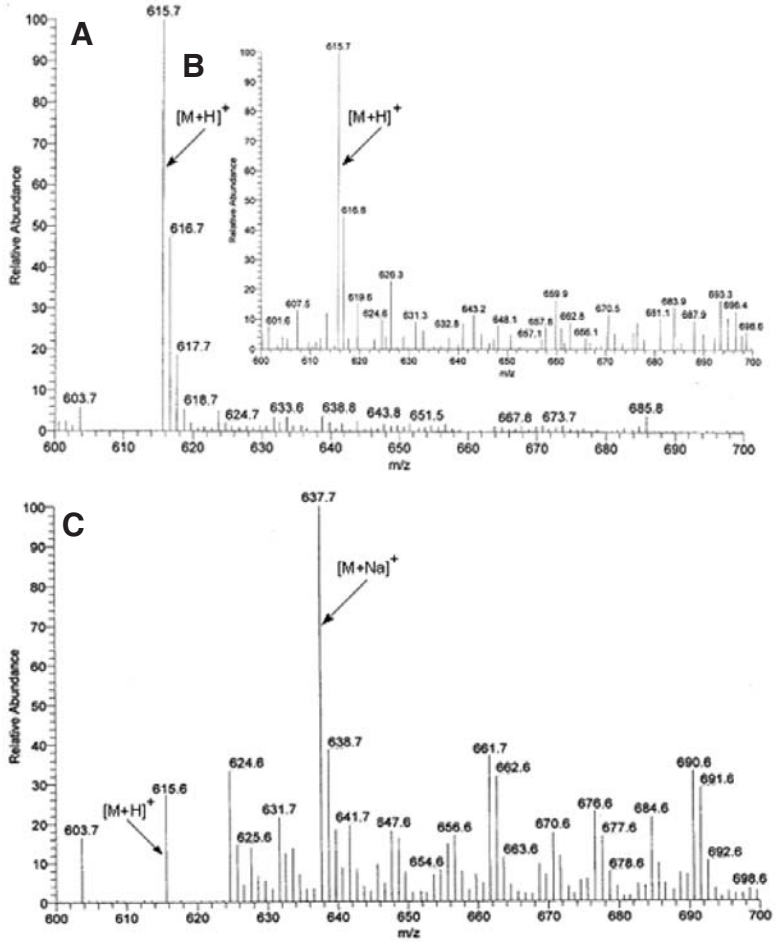

Fig. 4. ESI/MS spectra of authentic neomycin (A), extracts of wild *S. fradiae* (B) and complement strain *S. fradiae* BS6 (C), showing signals characteristic for neomycin (*m/z* = 615)

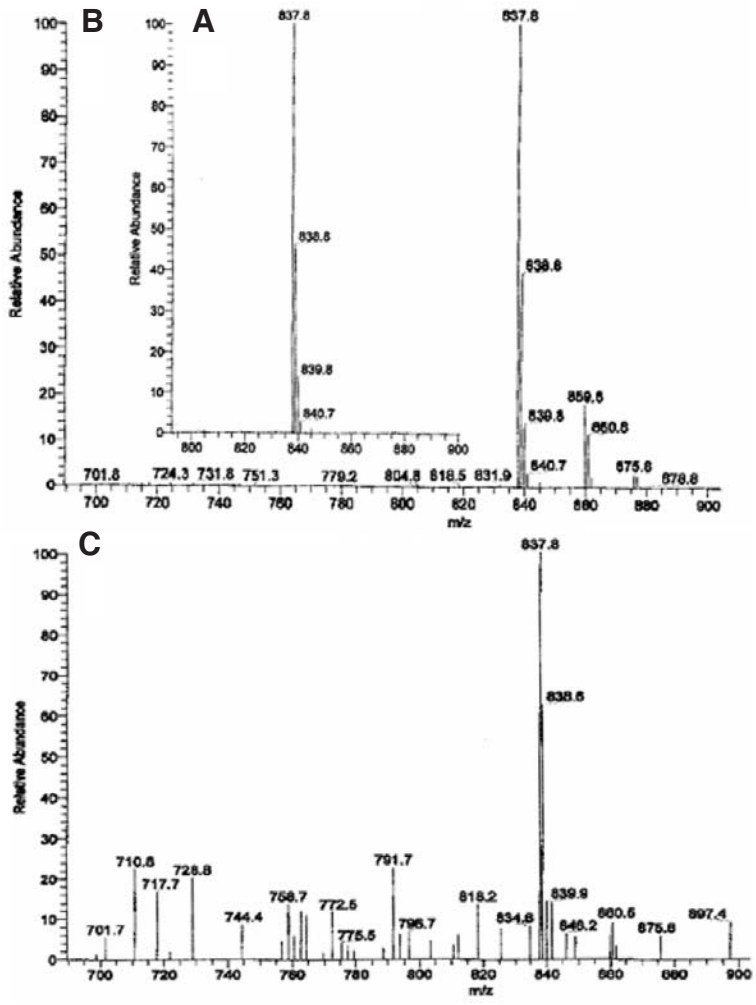

Fig. 5. LC/MS spectra of authentic neomycin (A), compounds isolated from wild *S. fradiae* (B) and compounds isolated from complement strain *S. fradiae* BS6 (C)

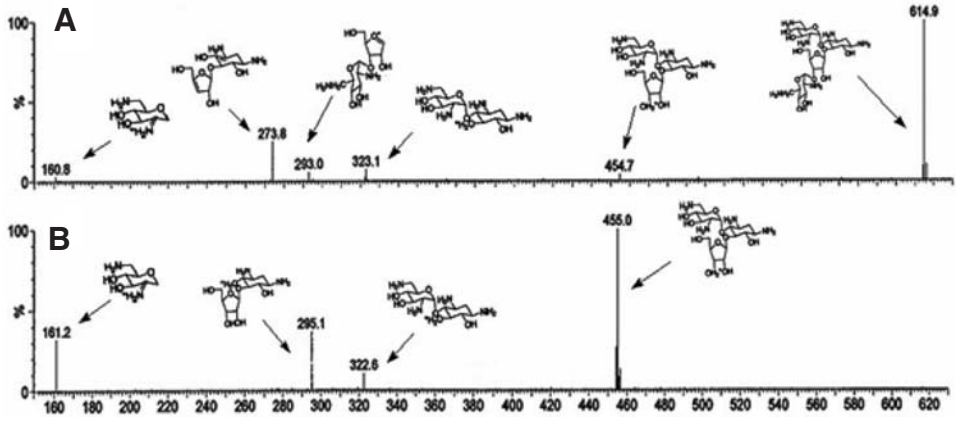

Fig. 6. ESI/MS-MS spectra of isolated compounds from *S. fradiae* BS6. (A) Ion products spectra formed from neomycin (*m/z* = 615). (B) Ion products spectra formed from ribostamycin (*m/z* = 455).

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