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Functional Analysis of Spectinomycin Biosynthetic Genes from *Streptomyces spectabilis* ATCC 27741

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Abstract The function of genes related to spectinomycin biosynthesis (*spcD*, *speA*, *speB*, *spcS2*) from *Streptomyces spectabilis* ATCC 27741, a spectinomycin producer, was analyzed. Each gene was subcloned from a spectinomycin biosynthetic gene cluster and overexpressed in *E. coli* BL21 (DE3) using pET vector. After incubating each purified protein with its possible substrates, the final products were analyzed using high-performance liquid chromatography (HPLC). From these results, *spcD*, *speA*, and *speB* have been identified to be dTDP-glucose synthase, *myo*-inositol monophosphatase, and *myo*-inositol dehydrogenase, respectively. In addition, the results suggest that the *spcS2* gene product functions downstream of the *speB* gene product in the biosynthetic pathway of spectinomycin. Taken together, the present study elucidates the early steps of the biosynthetic pathway for 6-deoxyhexose (6-DOH) part (actinospectose) and aminocyclitol part (actinamine) of spectinomycin.

Key words: Spectinomycin biosynthetic gene cluster, dTDP-glucose synthase, *myo*-inositol monophosphatase, *myo*-inositol dehydrogenase

Many useful antibiotics from actinomycetes (macrolides, peptide antibiotics, β-lactams, etc.) have been studied by cloning their biosynthetic genes, analyzing the regulatory mechanisms, and creating recombinant antibiotics. However, due to the difficulties in purification and chemical synthesis, research on biosynthesis and genetic engineering of aminoglycoside antibiotics has been very slow, until recently when the biosynthetic gene clusters for streptomycin, spectinomycin, bluensomycin, kasugamycin, and butirosin were discovered [1, 7, 8, 9, 14, 18, 26].

Spectinomycin is an aminoglycoside antibiotic produced by several *Streptomyces* species such as *Streptomyces flavopersicus* [17], *Streptomyces hygroscopicus* [31], and *Streptomyces spectabilis* [15]. The antibiotic has a unique tricyclic structure in which a single sugar component, actinospectose, is linked to the diaminocyclitol moiety (actinamine) by β-glycosidic and hemiketal bonds [4, 30]. It blocks the translocation step of protein synthesis by inhibiting the binding of the elongation factor G to the ribosome [3]. It possesses a broad spectrum of activities against many Gram-positive as well as Gram-negative bacteria [29]. Specifically, it possesses potent activity against *Neisseria gonorrhoeae*, the gonorrhoea bacteria that have acquired resistance to penicillin and may be an alternate drug in such cases or for patients allergic to β-lactams [20]. However, the whole biosynthetic pathway for spectinomycin has not yet been elucidated, although there have been reports on the glycosylation process of the two subunits of spectinomycin [16] and the partial biochemical pathway using cell-free extract and labeling [5, 28].

Therefore, to study the spectinomycin biosynthetic pathways, a 45 kb DNA fragment containing the genes required for spectinomycin biosynthesis was isolated from *Streptomyces spectabilis* ATCC 27741, as previously described [7], and this study focuses on the functional analysis of the genes participating in the early steps of the biosynthetic pathway of each spectinomycin subunit using biochemical methods, since a stable transformation system is not available for *S. spectabilis*.

Objectives of this study were to investigate whether 1) *spcD* encodes dTDP-glucose synthase, 2) *speA* encodes *myo*-inositol monophosphatase, and 3) SpeB can convert *myo*-inositol to *scyllo*-inosose. Furthermore, it was found that *spcS2* plays a role following *speB* in the biosynthetic pathway of spectinomycin.

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MATERIALS AND METHODS

Bacterial Strains

Spectinomycin biosynthetic genes were isolated from *S. spectabilis* ATCC 27741, a spectinomycin producer. *E. coli* DH5αF' [F'/*endA1 hsdR17* ($r_K^-m_K^+$) *supE44thi-1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF) U169* (*φ80dlac Δ(lacZ)M15*)] was used as a host strain for transformation of plasmid harboring spectinomycin biosynthetic genes. *E. coli* BL21 (DE3) [F^-, *dcm*, *ompT*, *hsdS* ($r_B^-m_B^+$), *gal*] (ATCC 47092) was used to overexpress the transformants. The pET21a plasmid (Novagen, Germany) was used to express *spcD* and *spcS2* genes, and pET28a (Novagen, Germany) was used for *speA* and *speB*. All *E. coli* cells were grown on LB agar or broth media supplemented with 50 µg ml^{-1} ampicillin or kanamycin (Sigma-Aldrich corp., U.S.A.).

General Methods

General procedures for manipulating DNA were carried out according to Sambrook *et al*. [24]. DNA was isolated from agarose gels (BentechBio Co., Ltd. Korea) with the Qiagen kit (Chartworth, U.S.A.). Plasmid was purified using a Wizard plasmid kit (Promega, U.S.A.) following the manufacturer's instructions.

Cloning of *spcD* and *speA* Genes

PCR Amplification. For the functional analysis of *spcD*, *speA*, *speB*, and *spcS2* gene products, each gene was amplified using PCR from the cosmid template containing the spectinomycin biosynthetic cluster [7]. Oligoprimers included the restriction enzyme sites (underlined) to facilitate subcloning. The sequences of each primer used in the experiments were as follows; for *spcD* gene, forward primer was 5'-GCC<u>GAATTC</u>ATGCG CGGAATCATACTT-3'; and reverse primer was 5'-ATT<u>CTCGAG</u>CAACAGCGCCTC-CTCGTC-3', for *speA* gene, forward primer was 5'-GCC-<u>GAATTC</u>ATGTCTTTGGCACATGCC-3' and reverse primer was 5'-TT<u>CTCGAG</u>GGCATGGCCCACCAG-3'. For *speB* gene, forward primer was 5'-GCC<u>GAATTC</u>GTGCAGAA-AGAACGTGTC-3' and reverse primer was 5'-ATT<u>CTC-GAG</u>CATTCCTTTGAAGTT-3'. For *spcS2* gene, forward primer was 5'-ATT<u>GAATTC</u>ATGTCAGCGGTACGCAGT-3' and reverse primer was 5'-ATT<u>AAGCTT</u>TGCCCGG-CCCCTCTC-3'. PCR reactions were performed using the EX Taq DNA polymerase (Takara, Japan). The genes amplified by PCR were sequenced to confirm the absence of mutation during PCR reactions.

Construction, Overexpression, and Purification of Recombinant Proteins. Each PCR-amplified *spcD* and *spcS2* gene was cloned into the *E. coli* expression vector pET21a, and *speA* and *speB* genes were cloned into pET28a to give pETspcD, pETspcS2, pETspeA, and pETspeB, respectively. These plasmids were transformed into *E. coli* BL21 (DE3) to express his-tagged proteins. pETspcD and pETspcS2 were grown in LB medium supplemented with 50 µg ml^{-1} ampicillin at 37°C until OD_{600} of 0.6 was reached, then induced with 1 mM isopropyl β-D-thiogalactoside (IPTG), and allowed to grow at 28°C for 3 additional hours. pETspeA and pETspeB were grown in LB medium supplemented with 50 µg ml^{-1} kanamycin at 37°C until OD_{600} of 0.6 was reached, then induced with 1 mM IPTG and allowed to grow at 37°C for 3 additional hours [25]. The cells were harvested by centrifugation and resuspended in the binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9). Resuspended cells were lysed by sonication (Branson sonifer 450 sonicator), and cell debris was removed by centrifugation [10]. Each soluble protein was purified with a single nickel-affinity chromatography by using HisBind resin (Novagen, Germany). Protein concentration was determined by the Bradford [2] method, and the molecular weights were determined by SDS-PAGE [11].

Enzymatic Assay for SpcD and SpeA

For the functional analysis of SpcD, a reaction mixture (300 µl) containing 50 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 6 mM dTTP (deoxythymidyltriphosphate), 24 mM D-glucose-1-phosphate, 1.8 U of inorganic pyrophosphatase, and 1 µg of purified SpcD was incubated at 37°C for 0, 30, or 60 min, then the reaction was terminated by adding 50 mM potassium phosphate (pH 3.0) [12]. The reaction product was analyzed by HPLC with a $N(CH_3)_2$-1101-N column (0.46×10 cm) using a stepwise gradient of 50 to 600 mM potassium phosphate buffer (pH 4.0) at 30°C. The flow-rate was 1.5 ml min^{-1}, and the products peaks were detected by a UV detector at 254 nm. Standard reagents, dTTP, and dTDP-glucose were purchased from Sigma-Aldrich Corp (U.S.A.). To determine the substrate specificity of SpcD, dCTP, dATP, and dGTP were tested as substrates and the products from the reaction were analyzed by the method described above.

For the functional analysis of SpeA, a reaction mixture (300 µl) containing 20 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 0.7 mM *myo*-inositol-1-phosphate, and 10 mg SpeA cell extract was incubated at 37°C for 0, 30, or 60 min, and the reaction was then terminated by freezing at –20°C [13]. As a control experiment to eliminate the effect of the *E. coli*'s primary metabolism of *myo*-inositol monophosphatase, the cell extract from *E. coli* cells harboring only the vector plasmid was reacted instead of the SpeA cell extract. The reaction product was analyzed by HPLC with a mBondapak™C_{18} column (0.46×10 cm) using 20% acetonitrile. The flow-rate was 1.0 ml min^{-1}, and the product peaks were detected by a UV detector at 254 nm. The standard reagent used was the commercial *myo*-inositol (Sigma-Aldrich Corp., U.S.A.).

Enzymatic Assay for SpeB and SpcS2

For the functional analysis of SpeB, a reaction mixture (300 µl) containing 50 mM Tris-HCl (pH 9.5), 4 mM NAD^+,

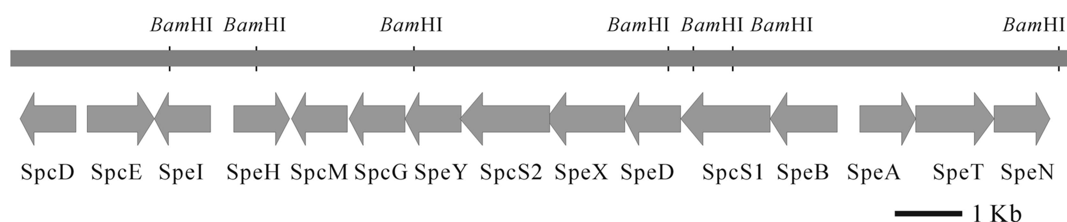

Fig. 1. Organization of gene cluster for spectinomycin biosynthesis, and speculated gene products.
The gene cluster consists of the genes for SpcD (dTDP-glucose synthase), SpcE (dTDP-glucose 4,6-dehydratase), SpeI (putative keto-isomerase), SpeH (putative dehydrogenase), SpcM (putative methyltransferase), SpcG (putative glycosyltransferase), SpcS2 (putative L-glutamin:*scyllo*-inosose aminotransferase), SpcS1 (putative PLP-dependant dehydrogenase or aminotransferase), SpeB (putative *myo*-inositol dehydrogenase), SpeA (*myo*-inositol-monophosphatase), SpeN (putative resistance protein), SpeY, SpeX, SpeD, and SpeT (unknown) proteins, involved in spectinomycin biosynthesis.

200 mM *myo*-inositol, and 10 μg *E. coli* cell extract containing SpeB was incubated at 30°C for 0, 30, or 60 min, then the reaction was terminated by freezing at -20°C [23]. Since the predicted reaction product by SpeB was not commercially available, *myo*-inositol dehydrogenase considered to react the same way as SpeB was purchased and used in the control experiment. The reaction product was analyzed by HPLC with a $N(CH_3)_2$-1101-N column (0.46×10 cm) using a linear gradient formed with 100% of 50 mM potassium phosphate buffer (pH 4.0) and 50% of 600 mM potassium phosphate buffer (pH 4.0) at 30°C. The flow-rate was 1.0 ml min^{-1}, and the product peaks were detected by a UV detector at 254 nm.

To study the biochemical activity of SpcS2, a reaction mixture (400 μl) containing 50 mM potassium phosphate buffer (containing 6.5 mM EDTA, 4 mM pyridoxal phosphate, pH 7.4,), 2.5 mM NAD^+, 20 mM glutamine, 125 mM *myo*-inositol, and of 10 μg each of *E. coli* cell extracts containing SpeB and SpcS2 were incubated at 37°C for 0, 30, or 60 min, and the reaction was then terminated by freezing at -20°C [1]. Also, commercial *myo*-inositol dehydrogenase instead of SpeB was incubated under the same conditions. The enzyme reaction products were analyzed by the same method used for SpeB analysis. In addition, as controls, cell extracts from *E. coli* cells containing only the pET28a plasmid were also reacted in the same conditions, and the retention times for the reaction products were compared in an HPLC analysis.

Results and Discussion

SpcD has dTDP-Glucose Synthase Activity

The reaction in which glucose-1-phosphate is converted into dTDP-D-glucose can be found in the biosynthesis of many aminoglycoside antibiotics containing 6-DOH (deoxyhexose), and it is the first step for 6-DOH biosynthesis [21]. Yuichi *et al*. [32] genetically elucidated the biosynthetic pathway of dTDP-rhamnose, including the 6-DOH moiety in *Streptococcus mutants*. But the biosynthetic pathway of actinospectose, the 6-DOH moiety of spectinomycin, has not yet been elucidated, even though the spectinomycin gene cluster (Fig. 1) had already been isolated by Lyutzkanova *et al*. [7, 14].

Among the genes isolated from the putative spectinomycin biosynthetic cluster, the *spcD* gene product showed more than 70% identity to the gene encoding dTDP-glucose synthase involved in the biosynthesis of a variety of antibiotics according to the GenBank. To verify the function of *spcD* gene product, SpcD was purified in soluble form from *E. coli*, and the molecular mass of the purified protein was estimated at 32 kDa on SDS-PAGE (data not shown). α-D-glucose-1-phosphate and dTTP (deoxythymidyltriphosphate) as substrates were incubated with purified SpcD, and the reaction products were analyzed by HPLC. Only the peak of dTTP used as a substrate was detected at 0 min reaction time. However, the peak of dTTP decreased as the reaction proceeded, and a new peak was detected at the same retention time (5.3 min) as the standard dTDP-glucose (Fig. 2). From these results, it was concluded that *spcD* encodes the enzyme dTDP-glucose synthase. When SpcD was incubated with dATP, dCTP, and dGTP instead of dTTP, the concentrations of these nucleotides did not change as the reaction progressed, which indicates that dTTP is a specific substrate for SpcD (data not shown). In addition, from the kinetic studies of SpcD reactions, it was found that the K_m value was 33.95 μM and the V_{max} was 188.64 μM min^{-1} (mg $protein)^{-1}$ for dTTP (data not shown).

It was previously reported that SpcE forms dTDP-4-keto-6-deoxy-D-glucose only from dTDP-glucose [7]. Therefore, together with the current results, it is increasingly obvious that SpcD and SpcE function continuously in the initial biosynthesis of the actinospectose, 6-DOH portion of spectinomycin.

speA, *speB*, *spcS2* are Utilized in Actinamine Biosynthesis

Spectinomycin has actinamine as part of aminocyclitol, which is common for the structure of aminoglycoside antibiotics. From aminocyclitol biosynthetic studies, it has been confirmed that *myo*-inositol is a precursor of the streptidin moiety, through a feeding test from a streptomycin producer [6]. In addition, the streptidin biosynthetic pathway from glucose-1-phosphate to streptidine-6-phosphate has been proposed by Walker [27] using a cell extract in *S. griseus*,

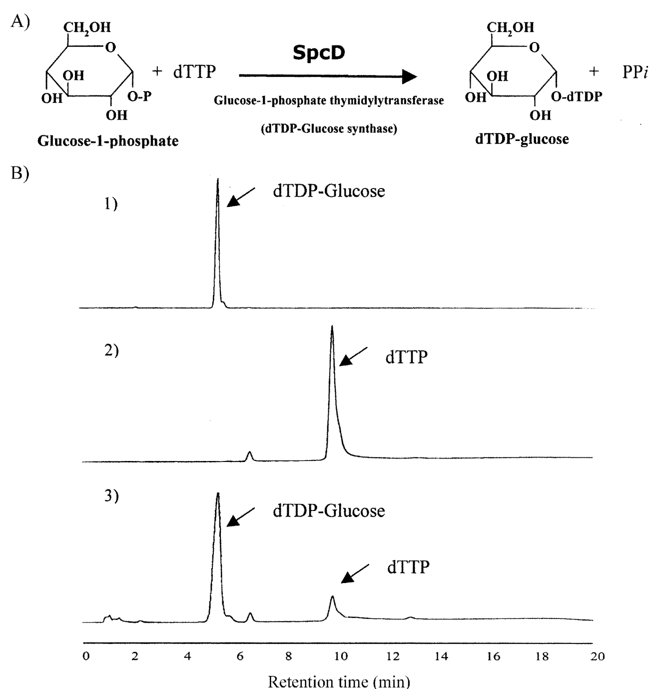

Fig. 2. SpcD protein has dTDP-glucose synthase activity.
(A) The purified SpcD protein produced dTDP-glucose when incubated with glucose-1-P and dTTP. (B) HPLC analysis of the reaction products. The dTDP-D-glucose, product of SpcD action was detected by HPLC. 1) dTDP-glucose standard. 2) Reaction with cell extract harboring only the pET21a plasmid. 3) Reaction with purified SpcD. All reactions were stopped after 60 min of incubation.

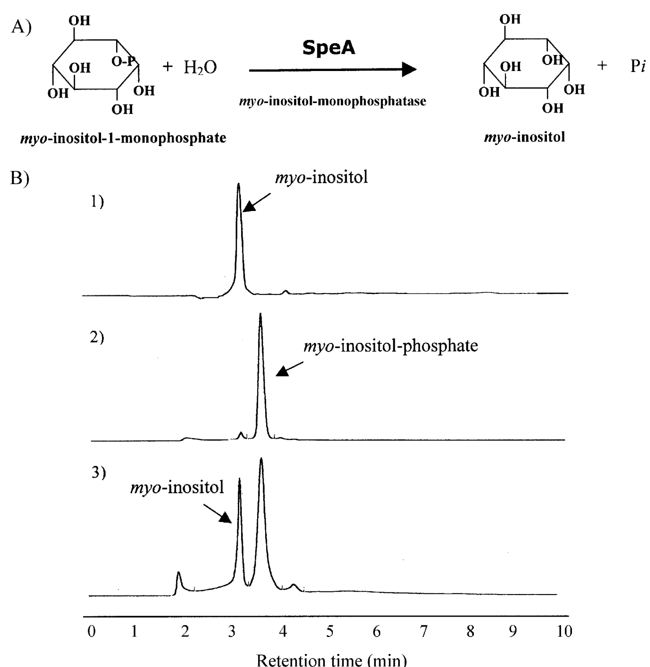

Fig. 3. SpeA catalyzes the formation of *myo*-inositol from *myo*-inositol-1-phosphate.
(A) Incubation of *myo*-inositol-1-P with cell extract of *speA* expressed in *E. coli* produced *myo*-inositol. (B) HPLC analysis of the reaction products, The *myo*-inositol, a product of SpeA activity, was detected by HPLC. 1) *myo*-inositol standard. 2) Reaction with cell extract harboring only pET28a plasmid. 3) Reaction with cell extract harboring *speA* gene. All reactions were stopped after 30 min of incubation.

S. bikiniensis, and *S. glebosus*. However, genetic studies on the aminocyclitol biosynthetic pathway have not yet been performed, even though the biosynthetic gene cluster of several aminoglycoside antibiotics has already been isolated [1, 7, 8, 14, 18, 19, 22, 26].

It seems that the biosynthetic pathway of actinamine in spectinomycin resembles that of streptidin, the aminocyclitol of streptomycin. It was speculated that *speA* in the spectinomycin biosynthetic cluster catalyzes the formation of *myo*-inositol from *myo*-inositol-1-phosphate, based on the fact that the *speA* gene product has conserved domains of the inositol-monophosphatase family which forms *myo*-inositol, the precursor of the aminocyclitol moiety. To verify whether the cloned *speA* gene encodes *myo*-inositol monophosphatase, SpeA was also overexpressed in *E. coli*. Expressed SpeA had a molecular mass of 28 kDa on SDS-PAGE (data not shown). SpeA or *E. coli* cell extract harboring only the plasmid pET28a (control) was incubated with *myo*-inositol-1-monophosphate, and the products were analyzed by HPLC (Fig. 3). The reaction product was dramatically increased in the reaction with SpeA compared to the one with the control cell extract, and the product was detected at the same retention time (3.1 min) as the standard *myo*-inositol. Therefore, through its *myo*-inositol monophosphatase activity, it was concluded that SpeA forms *myo*-inositol from *myo*-inositol-1-phosphate by removing the phosphate group, which is consistent with the results of Horner [6] and Walker [27] who had reported that *myo*-inositol was the precursor of the streptidin moiety.

It was also hypothesized that SpeB had *myo*-inositol dehydrogenase activity, which converts *myo*-inositol to *scyllo*-inosose. To test this possibility, SpeB was incubated with *myo*-inositol, and the reaction products were analyzed by HPLC [23]. Since *scyllo*-inosose was not commercially available, *myo*-inositol dehydrogenase as a control was used instead, which was expected to catalyze the same reaction as SpeB. As shown in Fig. 4, SpeB showed *myo*-inositol dehydrogenase activity, producing a reaction product which had a retention time (10.5 min) identical to the one from the *myo*-inositol dehydrogenase reaction.

In addition, the SpcS2 protein was similar to the putative L-glutamine:*scyllo*-inosose aminotransferase (StsC) of *S. griseus*, according to the database from GenBank [1]. Thus, it follows that the SpcS2 transfers an amino group from glutamine to *scyllo*-inosose. Since it was thought that SpcS2 uses *scyllo*-inosose as a substrate, a coupled assay was performed simultaneously with SpeB, so that SpcS2 can react with the *scyllo*-inosose produced *in situ* by SpeB. The new peak corresponding to the products was detected at 12.7 min by HPLC (Fig. 5). Although the structure of

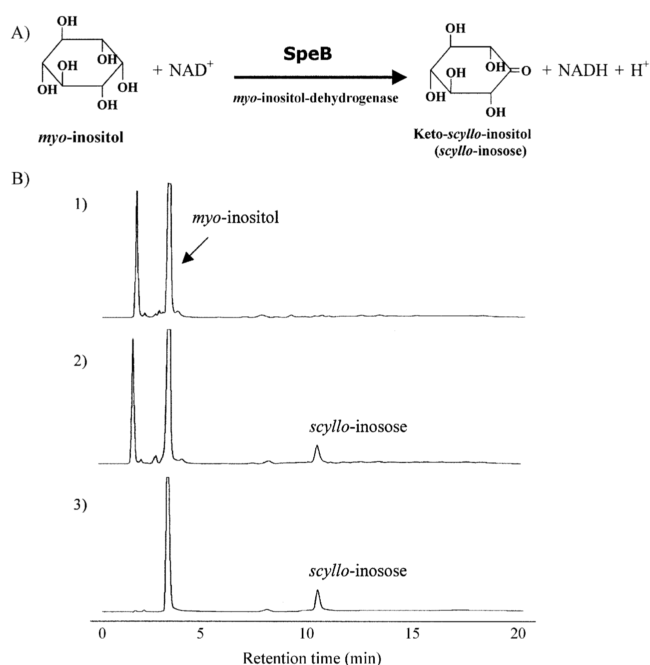

Fig. 4. Isolated SpeB protein produced the same reaction product as the commercial *myo*-inositol dehydrogenase.
(A) SpeB, *speB* product is proposed to be *myo*-inositol-dehydrogenase. (B) HPLC analysis of the reaction products. The *myo*-inositol dehydrogenase, a product of SpeB activity, was detected by HPLC. 1) Reaction product with cell extract containing only the plasmid pET28a. 2) Reaction product with cell extract containing SpeB. 3) Reaction product with commercial *myo*-inositol dehydrogenase. All reactions were stopped after 60 min of incubation.

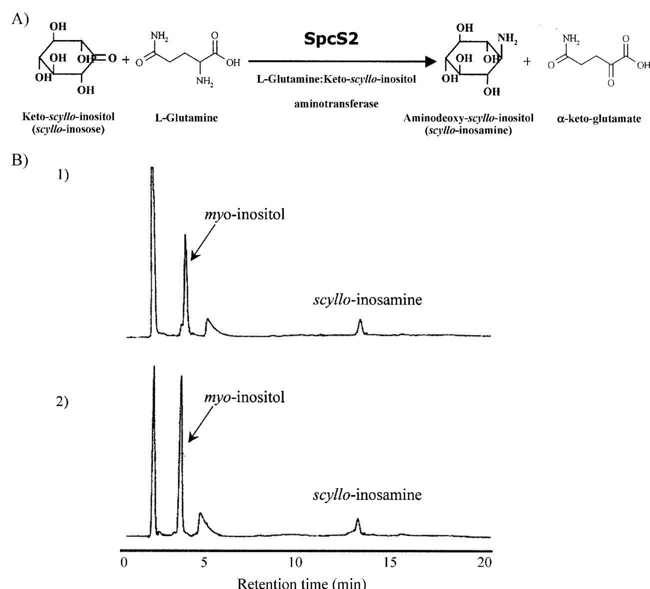

Fig. 5. SpcS2 functions downstream of SpeB in the biosynthetic pathway of spectinomycin.
(A) SpeS2, *spes2* product is proposed to be L-glutamin:scyllo-inosose aminotransferase. (B) HPLC analysis of the reaction products. The *scyllo*-inosamine, the reaction product of SpcS2, was detected by HPLC. 1) Reaction product with cell extracts containing SpeB and SpcS2. 2) Reaction product with commercial *myo*-inositol-dehydrogenase and cell extract containing SpcS2. All reactions were stopped after 60 min of incubation.

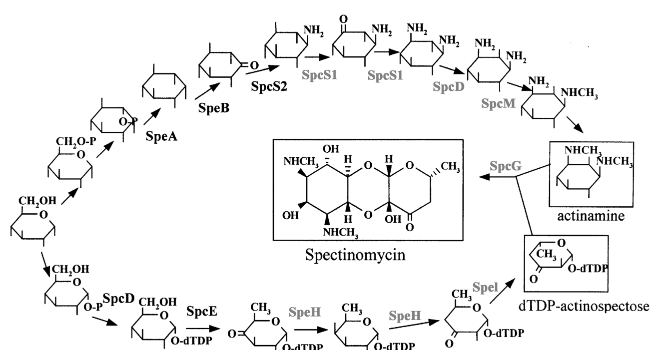

Fig. 6. The proposed biosynthetic pathway of spectinomycin based on the deduced functions of the isolated gene products.

the product at 12.7 min retention time was not definitively elucidated, it was possible to confirm that SpcS2 produced a new reaction product using the *scyllo*-inosose, an enzyme reaction product of SpeB.

Therefore, these results verified the hypothesis that SpeA, SpeB, and SpcS2 are involved in this order during the initial biosynthesis of actinamine.

Based on this study, a biosynthetic pathway of actinamine and actinospectose (Fig. 6) is suggested, as well as the putative function of the genes in the spectinomycin biosynthetic gene cluster (Fig. 1) [7]. The results clearly show that gene products from this study are involved in the early steps of the biosynthesis of aminoglycoside antibiotics containing 6-DOH and aminocyclitol moieties.

To further understand the system, it is planned to investigate whether other gene products from this cluster are also used in the downstream processing of spectinomycin biosynthesis, through gene disruption studies by homologus recombination in *S. spectabilis* when a stable transformation system is established for *S. spectabilis*, and to carry out subsequent biochemical analysis.

Acknowledgments

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