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Isolation and Characterization of the Biosynthetic Gene Clusters for Aminoglycoside Antibiotics

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Abstract

The biosynthetic gene clusters for bluensomycin and spectinomycin were isolated and characterized from the bluensomycin producer, *Streptomyces bluensis* ATCC27420 and the spectinomycin producer, *Streptomyces spectabilis* ATCC27741, respectively. PCR primers were designed specifically to amplify a segment of dTDP-glucose synthase gene based on its conserved sequences of several actinomycete strains. By screening cosmid libraries using amplified PCR fragments, 30-kb and 45-kb DNA fragments were isolated from *Streptomyces bluensis* and *Streptomyces spectabilis,* respectively. Sequencing analysis of them revealed that each contains 15 open reading frames (ORFs). Some of these ORFs were turned out to be antibiotic resistance genes (*blmA* and *speN*), dTDP-glucose synthase genes (*blmD* and *spcD*), and dTDP-D-glucose 4,6-dehydratase genes (*blmE* and *spcE*), suggesting that the *blm* and *spec* gene clusters are likely involved in the biosynthesis of bluensomycin and spectinomycin, respectively.

Keywords: biosynthetic gene cluster; dTDP-glucose synthase; dTDP-D-glucose 4,6-dehydratase; spectinomycin; bluensomycin

1. Introduction

Most of aminoglycoside antibiotics containing sugar are the most commonly used and highly potent antibiotics for the treatment of life-threatening infections. More than 150 naturally occurring aminoglycoside antibiotics have been isolated from culture filtrates of *Streptomyces* species, other actinomycetes, and *Bacillus*. Aminoglycoside antibiotics are quite similar in their chemical characteristics, general biological properties, and mechanism of action. The aminocyclitol moiety of most aminoglycoside antibiotics is 2-deoxystreptamine, but those of bluensomycin and spectinomycin are bluensidine and actinamine, respectively (Hooper et

al., 1982). Aminoglycoside antibiotics bind directly to prokaryotic 16S rRNA at the decoding region A site inducing codon misreading and inhibiting translocation, resulting in interference with protein synthesis. Although the mechanism of antibiotic action by aminoglycosides is well understood, their biosynthetic pathways and genetic organizations remained to be elucidated (Moazed and Noller, 1987).

Bluensomycin produced by *Streptomyces bluensis* is a monoguanidinated analog of dihydrostreptomycin. In bluensomycin, the streptidine moiety of dihydrostreptomycin is replaced by bluensidine (Figure 1). Bluensidine and streptidine differ only in the substituent at C-1: bluensidine has a carbamoyl group while streptidine has a guanidine group. Bluensidine is synthesized from myo-inositol via scyllo-inosamine, which may undergo oxidation, transamination, phosphorylation, dephosphorylation, and transamidination by the enzyme system of streptomycin biosynthesis (Piepersberg, 1995). It has been suggested that the bluensomycin pathway is ancestral to that for streptomycin production.

Spectinomycin produced by *Streptomyces spectabilis* is an amiocyclitol antibiotic with the unique tricyclic structure (Figure 2). It has a broad spectrum of activity against Gram positive and Gram negative bacteria. In addition, it lacks the undesirable nephro- and ototoxic properties of the aminoglycosides (Wiley et al., 1963).

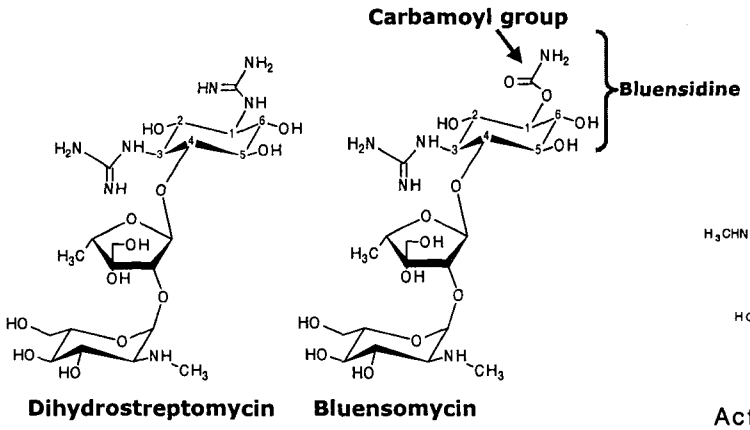

Figure 1. Structures of dihydrostreptomycin and bluensomycin

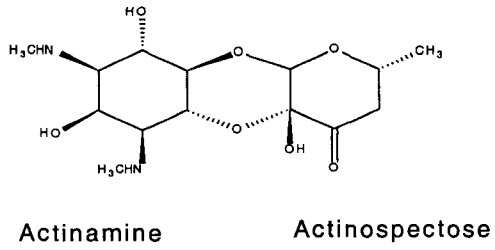

Figure 2. Structures of spectinomycin

The aim of this study is to isolate the biosynthetic gene clusters and then elucidate the spectinomycin and bluensomycin biosynthetic pathways. Oligonucleotide primers were designed based on the dTDP-glucose synthase gene as bluensomycin and streptomycin contain a deoxysugar moiety. Using amplified DNA fragments of the dTDP-glucose synthase gene from spectinomycin or bluensomycin producers, we have isolated 30-kb and 45-kb DNA fragments from *Streptomyces bluensis* and *Streptomyces spectabilis,* respectively. Fifteen open reading frames (ORFs) were found in 30kb and 45kb DNA fragments (Figure 3,4): *blmA* and *speN* are the antibiotic resistant genes; *blmD* and *spcD* encode dTDP-glucose synthase; *blmE*

and *spcE* encode dTDP-D-glucose 4,6-dehydratase.

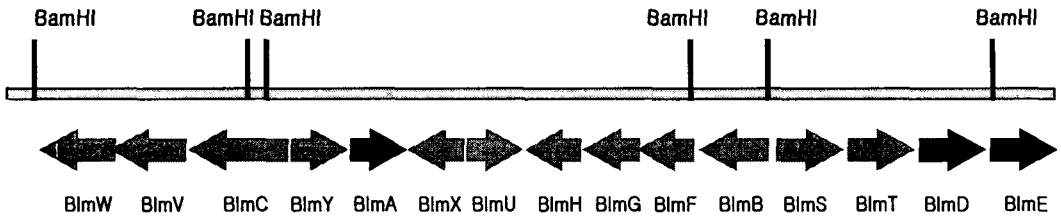

Figure 3. Organization of the gene cluster for bluensomycin biosynthesis. The 15 ORFs was sequenced. The transcription is indicated as arrows and the characterized genes are indicated as black arrows.

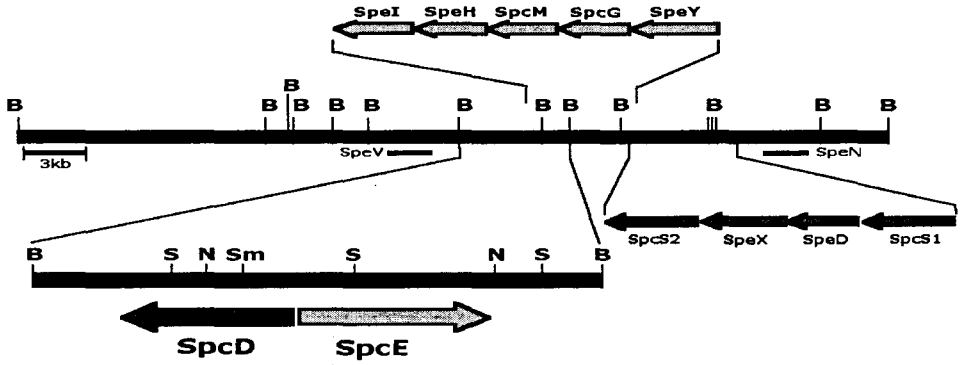

Fig. 4. Restriction map of the 45kb insert region contained the *spcD* and *spcE* genes.

2. Materials and methods

2. 1. Bacterial strains and plasmids

Streptomyces spectabilis ATCC27741 and *Strptomyces bluensis* ATCC27420 strains were used for isolation of spectinomycin and bluensomycin biosynthetic gene clusters, respectively. *Streptomyces lividans* TK23 (Hopwood et al., 1985) and *Streptomyces steffisbergensis* IMSNU 21902 (ATCC 27466) were used for heterologous expression of *Streptomyces* plasmids.

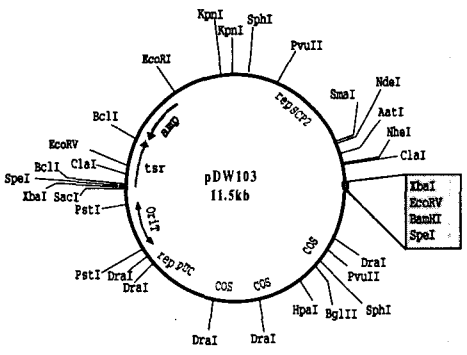

Figure 5. Map of pDW103 cosmid vector

Escherichia coli XL1-Blue MRF' was used as a host strain for the recombinant plasmids and gene library. *E. coli* BL21 (DE3) was used to overexpress the *spcE, speN, spcD, blmE, blmA, and blmD* genes. Plasmids pGEM-T easy (Promega Biotech, Madison, WI) or pT7Blue (Novagen, Madison, WI) were used as vectors for subcloning of the PCR products. Cosmid libraries of *S. spectabilis* ATCC27741 and *S. bluensis* ATC27420 were prepared using the pDW103 vector (Figure 5).

2. 2. Polymerase chain reaction

The PCR reaction was performed in a 50$\mu\ell$ reaction mixture containing 100 pmol of each primer, 300ng of template DNA, 5$\mu\ell$ of 10X PCR buffer, 0.2 mmol of dNTPs, and 2.5 units of Taq polymerase (Takara, Shiga, Japan). After overlaying with mineral oil, the reaction mixtures were preheated at 94℃ for 3 min, thereafter, 30 amplification cycles were carried out. Each cycle consisted of 20 sec at 98℃ and 1 min at 67℃. Finally, an additional 10 min of extension reaction was performed at 72℃ for complete extension. Amplification was performed in a Perkin-Elmer thermal cycler model 480 (Perkin-Elmer Cetus, CT).

2. 3. Construction of the *S. spectabilis* and *S. bluensis* genomic libraries

A cosmid library of *S. spectabilis* genomic DNA was constructed in pDW103, an *E. coli-Streptomyces* shuttle cosmid. Most cosmid vectors for *Streptomyces* contained the apramycin resistance gene to select the transformants in both *Streptomyces* and *E. coli*. For the library construction, the pDW103 DNAs were digested with *Bam*HI/*Hpa*I. Chromosomal DNAs isolated from *S. spectabilis* and *S. bluensis* were partially digested with *Sau*3AI to produce 30-40 kb fragments which were ligated into the dephosphorylated vectors. The ligated DNAs were packed in vitro using the Gigapack II-XL lambda extracts from Stratagene (La Jolla, CA) as recommended by the manufacturer. The packaged phages were transfected into *E. coli* XL1-Blue MRF'.

2. 4. DNA sequencing and analysis

The nucleotide sequences of both strands were determined using an ABI model 373 DNA Sequencer (Applied Biosystems, Inc., Foster City, CA). The sequences were analyzed by codon preference (Bibb et al., 1984). Several subclones were constructed using convenient restriction sites to facilitate sequencing of both strands. The criteria for revealing ORFs were a typical codon usage as detected by a CODONPREFERENCE program, and identification of a possible ribosom-binding site (RBS) that is complementary to the 3 end of *S. lividans* 16S rRNA at about 10 bp upstream of each potential translational start codon (Bibb and Cohen, 1982). Possible functions of the genes were deduced from results of a computer-aided homology search using the FASTA and TFASTA programs. Comparisons of the nucleotide and amino acid sequences with databases were performed by the BLAST network service (Altschul et al., 1990). Protein sequences were aligned with a Genetic Computer Group software package or a CLUSTAL program (Higgins and Sharp, 1988).

2. 5. Overexpression of the *spcE* and *blmE* genes in *E. coli* and an enzyme assay for dTDP-glucose 4,6-dehydratase

To confirm that the putative *spcE* and *blmE* gene products actually utilize TDP as a

substrate, we constructed overexpresson plasmids by inserting the PCR products of the *spcE* and *blmE* coding region under the control of the strong T7 promoter of expression vector pET22b and pET28a yielding plasmid pHCG122 and pBLME, respectively. *E. coli* BL21 (DE3) was transformed with these plasmids or with pET22b and pET28a alone as a control by a $CaCl_2$ method (Sambrook et al., 1989). One of transformant colonies selected from a LB medium containing 50 ㎍/㎖ of kanamycin (for pET28a derivatives) or ampicillin 50 ㎍/㎖ (for others) was inoculated into 3 ㎖ of LB medium containing proper antibiotics and grown overnight at 37℃. For induction of the genes encoding SpcE and BlmE, fresh 200 ml of LB was inoculated with 1/100 volume of overnight cultures and shaked vigorously at 37℃ until OD_{600} reached 0.6 (for pET22b and pET28a derivatives) or OD_{660} reached 1.2 (for pET32a). IPTG was added up to a final concentration of 1 mM and shaked further for 3 hours. Cells were lysed by sonication.

The dTDP-glucose 4,6-dehydratase activity of the expressed protein was examined using ADP-glucose, CDP-glucose, GDP-glucose, and TDP-glucose. The standard assay system of dTDP-glucose dehydratase contained 150 mM Tris-HCl, pH7.6, 1 mM NAD+, 0.5 mM TDP-glucose, and enzyme in a final volume of 160㎕. After incubation at 37℃ for 30 min, the reaction was terminated by addition of 0.75㎖ of 0.1 M NaOH. The amount of product formed was determined by measuring absorbance at 340 nm. The control reaction was set without TDP-glucose (Vara & Hutchinson, 1988).

2. 6. Overexpression of the *blmD* and *spcD* genes in *E. coli* and an enzyme assay for dTDP-glucose synthase

The PCR products of *spcD* and *blmD* were cloned into pET24a and then introduced into *E. coli* BL21 (DE3). One of transformant colonies selected from LB medium cotaining 50 ㎍/㎖ of ampicillin for pET21a was inoculated into 3 ㎖ of LBA medium and grown overnight at 37℃. For induction of the genes encoding *spcD* and *blmD*, fresh 10 ml of LB were inoculated with 1/100 volume of overnight cultures and shaked vigorously at 37℃ until OD_{600} reached 0.5. IPTG was added up to a final concentration of 1 mM and shaked further for 3 hours. Cells were lysed by sonication. BlmD and SpcD in cell extract were purified using His-resin columns (Novagen, Madison, WI).

The 300㎕ reaction mixture for dTDP-glucose synthase cotained 50mM Tris-HCl (pH 8.0), 12mM $MgCl_2$, 24mM α-D-glucose-1-phosphate, 6mM dTTP ,1.8U of inorgranic pyrophosphatase and 150㎕ cell extract. After incubation at 37℃ for 0, 1, 2, 5, 16 hours, the reaction was terminated by addition of 1ml of 50mM potassium phosphate (pH3.0). The reaction mixture was stored at -20℃ until used. The amount of dTDP-glucose was determined by HPLC with a $N(CH_3)_2$-110-N column at 254nm.

2. 7. Resistance determination of the *blmA* and *speN* genes to aminoglycoside

antibiotics.

The coding regions of the *speN* and *blmA* were amplified by PCR from pHCG1242 and pHCG5844, respectively. The PCR product was ligated into the *Bam*HI and *Sac*I sites of the expression vector, pET32a, and the recombinant plasmid was transformed into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) strains containing pSPEN(speN) and pBLMA(blmA) were grown at 37℃ in the presence of spectinomycin and other aminoglycoside antibiotics such as streptomycin, gentamycin, sisomicin, apramycin, and tobramycin. Growth was scored after overnight incubation. In addition, the *Bam*HI 5.6-kb DNA fragment containing the *speN* gene was subcloned into the *E. coli-Streptomyces* shuttle vector pWHM3 to yield pHCG705.

3. Results

3. 1. Organization of the bluensomycin and spectinomycin biosynthetic gene clusters

The bluensomycin biosynthetic gene cluster was composed of 15 ORFs (Figure 3). The derived amino acid sequences of proteins encoded by *blm* genes showed the sequence identity to streptomycin biosynthetic enzymes. However, the sequence identity varid ranging from 88% (e.g., BlmB) to 55% (e.g., BlmA) (Table 1), indicating that the development of bluensomycin was subject to very different evolution from that of streptomycin despite of their similarity in components in a conserved gene cluster.

Table 1. Comparison of enzymes involved in streptomycin- related antibiotic production.

Enzyme name			(Postulated) Function	Identity	Positive
Gene product	Strains	*S.bluensis*			
AphE (272aa)	*S.griseus*	BlmA (269aa)	Resistance	55%	64%
StrB1 (347aa)	*S.griseus*	BlmB (348aa)	Amidonotransferase	88%	84%
NovN (677aa)	*S.spheroides*	BlmC (666aa)	O-Carbamoyltransferase	55%	64%
StrD (356aa)	*S.glaucescens*	BlmD (356aa)	dTDP-Glucose Synthase	78%	88%
StrE (328aa)	*S.glaucescens*	BlmE (327aa)	dTDP-Glucose 4,6-Dehydratase and Epimerase	75%	83%
StrF (281aa)	*S.glaucescens*	BlmF (280aa)	NDP-Hexose Epimerase	78%	84%
strG (201aa)	*S.glaucescens*	BlmG (198aa)	NDP-Hexose Epimerase	76%	87%
StrH (385aa)	*S.glaucescens*	BlmH (384aa)	Glycosyltransferase	75%	83%
StrS (378aa)	*S.glaucescens*	BlmS (379aa)	Aminotransferase	76%	82%
StrT (318aa)	*S.glaucescens*	BlmT (319aa)	Putitive aldo/keto redutase	74%	82%
StrU (428aa)	*S.glaucescens*	BlmU (partially sequenced)	NDP-Hexose Oxidoreductase	83%	82%
StrV (587aa)	*S.glaucescens*	BlmV (587aa)	ABC Transporter	65%	70%
StrW (591aa)	*S.glaucescens*	BlmW (606aa)	ABC Transporter	74%	80%
StsG (253aa)	*S.griseus*	BlmX (258aa)	Methyltransferase	76%	88%
		BlmY (291aa)	Unknown		

Sequencing analysis of the spectinomycin gene cluster of the 16.2-kb DNA revealed fifteen complete ORFs (Figure 4). The deduced function of each ORF is summarized in Table 2.

Table 2. The deduced functions of *spec* gene cluster.

Gene	Enzyme	Gene	Enzyme
spcD	dTDP-glucose synthase	*speX*	Unknown
spcE	dTDP-glucose-4,6-dehydratase	*speD*	Unknown
spcI	Keto-isomerase	*spcS1*	PLP-dependant dehydrogenase
spcH	Dehydrogenase	*speB*	*myo*-inositol dehydrogenase
spcM	Methyltransferase	*speA*	*myo*-inositol monophosphatase
spcG	Glycosyltransferase	*speT*	ABC transporter
speY	Unknown	*speN*	Resistance
spcS2	L-glutamin : *syllo*-inosose aminotransferase		

3. 2. Gene Characterization

3. 2. 1. Characterization of the *blmA* and *speN*, bluensomycin and spectinomycin resistance genes.

E. coli transformants with pBLMA which is the derivative of pET28a and contains the *blmA* gene showed resistance to streptomycin to a substantial degree of 300 ug/ml. However, introduction of the *blmA* gene into *S. lividans* TK23 and *S. steffisbergensis* did not confer the streptomycin resistance. Since many proteins are known to be reglulated by the pathway specific regulator, StrR, in the streptomycin biosynthesis, the expression the *blmA* gene might be not enough for conferring resistance.

Introduction of pHCG705 containing the *speN* gene into *S. lividans* TK23 and *S. steffisbergensis* conferred the spectinomycin resistance (100 ug/ml). An *E. coli* strain transformed with pSPEN (the *speN* gene in the *E. coli* expression vector pET32a) grew on LB broth containing 300ug/ml of spectinomycin. However, no transformant exhibited enhanced resistance to other aminoglycoside antibiotics such as streptomycin, gentamicin, sisomicin, apramycin, and tobramycin (data not shown).

3. 2. 2. Characterization of the *blmE* and *spcE* genes

Biosynthesis of deoxyhexoses involves the conversion of dTDP-glucose to TDP-4-keto-6-deoxy-glucose by dTDP-glucose 4,6-dehydratase. After induction of the *blmE* and *spcE* genes in *E. coli*, an approximately 2.5 to 3 times increase in dTDP-glucose 4,6-dehydratase activity was observed over the controls (Table 3, 4). In addition, the expressed proteins showed the dTDP-glucose 4,6-dehydratase activity when only TDP-glucose was used as a substrate, demonstrating that these enzymes are highly specific for TDP-glucose.

Table 3. The dehydratase activity of BlmE.

After enzyme reaction using *E. coli* cell extract, the amount of dTDP-4-keto-6-deoxy-D-glucose was determined by measuring absorbance at 340 nm.

Gene construction	dTDP-D-glucose 4,6-dehydratase (nmol/min/mg of protein)	
	-IPTG	+IPTG
pET28a(vector)	10.06±1.95	10.21±0.64
pBLME(plus *blmE*)	10.65±1.31	28.3±1.92

Table 4. The dehydratase activity of SpcE.

After enzyme reaction using *E. coli* cell extract, the amount of dTDP-4-keto-6-deoxy-D-glucose was determined by measuring absorbance at 340 nm.

Gene construction	dTDP-D-glucose 4,6-dehydratase (nmol/min/mg of protein)	
	-IPTG	+IPTG
pET22b(vector)	9.63±1.92	10.25±0.642
pHCG122(plus *spcE*)	11.54±1.25	32.1±1.283

3. 2. 3. Characterization of the *blmD* and *spcD* genes.

To verify that the cloned genes encode the putatively assigned enzymes, dTDP-glucose synthase, the *blmD* and *spcD* genes were expressed in *E. coli*. When the purified proteins of transformants including *blmD* and *spcD* genes were included in the enzyme reaction, the amount of dTDP-glucose that is a product of blmD and spcD reaction increased according to reaction time (0, 1, 2, 5, 16 hours)(Figure 6, 7). This result indicates that both the *blmD* and *spcD* genes encode dTDP-glucose synthase.

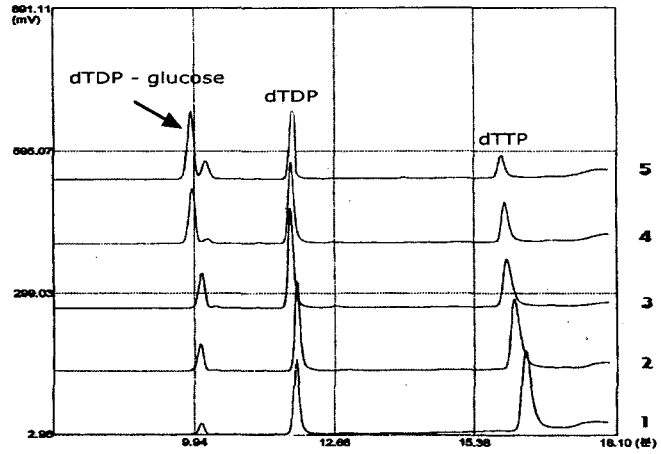

Figure 6. HPLC Analysis of the dTDP-glucose.

The dTDP-glucose was detected by HPLC with N(CH3)2-110-N column at 25 nm after 0,1,2,5,16 hours of enzyme reaction - 1,2,3,4,5.

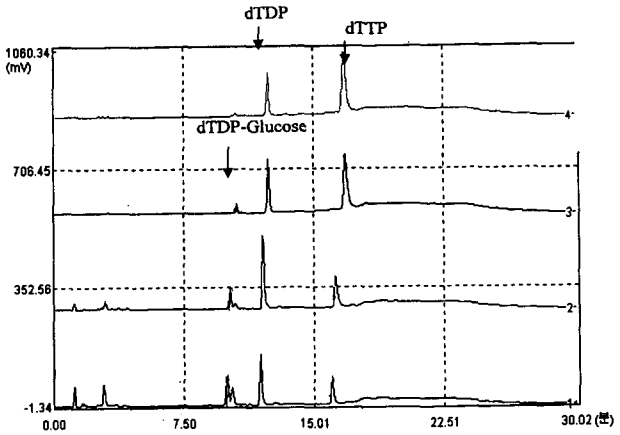

Fig. 7. Characterization of SpcD (dTDP-Glucose synthase)
A. Reaction time 0 hr B. Reaction time 1 hr
C. Reaction time 2 hr D. Reaction time 5 hr

4. Discussion

Bluensomycin and spectinomycin, which are aminoglycoside antibiotics, have a great advantage and commercial potential. For example, bluensomycin has a lower antimicrobial activity but also lower toxicity than dihydrostreptomycin (Munro et al, 1975). Spectinomycin has a broad spectrum of activity against Gram positive and Gram negative bacteria but lacks the undesirable nephro- and ototoxic properties of the aminoglycosides (Wiley et al., 1963). In addition, it possesses a potent activity against *Neisseria gonorrhoeae*, the gonorrhoea bacteria. However, bluensomycin and spectinomycin biosynthetic pathways are still unknown. Therefore, we ioslated their biosynthetic gene clusters from *Streptomyces bluensis* and *Streptomyces spectabilis,* respectively, to study the biosynthesis of bluensomycin and spectinomycin.

The bluensomycin and spectinomycin gene clusters were composed of 15 ORFs. It has been suggested that the bluensomycin pathway is ancestral to the streptomycin pathway and its biosynthetic enzymes are similar to those of streptomycin. However, a comparison of the *blm* genes with the known *str* genes from *S. griseus* showed many differences in organization and sequence identity, suggesting that the development of bluensomycin is subject to very different evolution from that of streptomycin. The functions of some genes could be deduced based on sequence homology. So far, three ORFs of each gene cluster have been characterized biochemically. The *blmA* and *speN* were antibiotic resistance genes. The *blmD* and *spcD* genes coded for dTDP-glucose synthase, and the *blmE* and *spcE* genes for dTDP-D-glucose 4,6-dehydratase.

Once charcterized, biosynthetic genes of bluensomycin and spectinimycin might be used for creating new hybrid antibiotics. A new hybrid antibiotic can be formed by cloning heterologous antibiotic biosynthesis genes from a producing strain into another strain producing a similar compound. There are two basic approaches in the application of this principle: (1)

cloning of single or multiple genes encoding structural modification reactions (2) cloning or mixing and matching of core biosynthetic enzymes, yielding completely new core molecules (Strohl, 1997). Since it has been reported that the biosynthetic pathway of aminoglycoside antibiotics are similar to each other, some genes encoding key enzymes such as BlmD (carbamoyltransferase), BlmH (glycosyltransferase), SpcM (methyltransferase) and SpcG (glycosytranferase) might be useful.

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