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Cloning and Sequence Analysis of the Aminoglycoside Resistance Gene from a Nebramycin Complex Producer, *Streptoalloteichus hindustanus*

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Abstract The aminoglycoside multiple-resistance determinant from *Streptoalloteichus hindustanus* was cloned into *Streptomyces lividans* and named *nbrB*. The 1.2-kb *ApaI-BclI* fragment encompassing *nbrB* was located within a 2.6-kb *ApaI* fragment by successive subcloning experiments. The complete DNA nucleotide sequence of 1.2-kb containing *nbrB* was determined. The sequence contains an open reading frame that putatively encodes a polypeptide of 281 amino acids with a predicted molecular weight of 30,992. The deduced amino acid sequence of *nbrB* shows identities of 85.1% to *kgmB* of *S. tenebrarius*, 59.6% to *sgm* of *Micromonospora zionensis*, and 57.7% to *grm* of *M. rosea*. The similarity of *nbrB* to *kgmB* suggests that *nbrB* encodes a 16S rRNA methylase similar to that encoded by *kgmB* and that both genes might be derived from a common ancestral gene.

Key words: Nebramycin complex, aminoglycoside antibiotic, multiple-resistance gene, 16S rRNA methylase

Antibiotic-producing organisms must be able to protect themselves against their own toxic products [4, 5]. The most common protective mechanisms include modification of the antibiotic, alteration of the target sites to which the drugs normally bind, export of the antibiotic, and induction of an alternative cellular component which is insensitive to the antibiotic [10, 11, 16, 17, 19, 21, 23, 26]. Antibiotic resistance (mainly self-resistance) of antibiotic producers has been studied with regards to antibiotic biosynthesis [26], possible origin of clinically-occuring antibiotic resistance [24], resistance mechanism [7, 33], and gene evolution [28].

A number of experimental observations indicate that the 3' terminal region of 16S rRNA is involved in the binding of aminoacyl tRNAs, and that aminoglycoside antibiotics interfere with this binding [7, 33]. There are multiple binding sites for these antibiotics. In this respect, it is noteworthy that the microorganisms producing aminoglycoside antibiotics protect themselves from the action of the antibiotic through methylation of a specific base of the 16S rRNA [10, 17, 19, 26, 27].

Nebramycin is an aminoglycoside antibiotic complex produced by *Streptoalloteichus hindustanus* and *Streptomyces tenebrarius* [18, 35]. The morphological and cultural characteristics of *Stall. hindustanus* are similar to those of *S. tenebrarius* except for the production of sporangium in the substrate mycelium [18]. However, the chemical composition of the cell-wall peptidoglycan of *Stall. hindustanus* is entirely different from that of *Streptomyces* and different from any of the known cell-wall types [18]. All components in the nebramycin complex are basic, water-soluble compounds that fall in the general class of aminoglycoside antibiotics. The anti-pseudomonas portion of the broad antimicrobial spectrum is of particular interest [34].

The genes for antibiotic biosynthesis are invariably found clustered and tightly linked to the genes encoding resistance to the antibiotic and genes involved in the regulation of biosynthesis of that antibiotic [6, 14, 26]. Therefore, as a first step in studying the genetic organization of nebramycin biosynthetic genes in *Stall. hindustanus*, the resistance determinant from this strain was cloned and the DNA nucleotide sequence was determined for the region.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Stall. hindustanus ATCC31219 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

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Escherichia coli DH5αF' was used as a host for generating the double-stranded plasmid and single-stranded M13 DNA (for sequencing) was isolated after the strain was grown at 37°C on Luria-Bertani (LB) media [31]. *Streptomyces lividans* TK24 and *Stall. hindustanus* were grown on R2YE plates at 30°C and 37°C, respectively [12, 18]. *S. lividans* transformation was carried out by standard procedures [12] and transformants were selected on R2YE plates containing 25 μg of thiostepton per ml of media. Plasmid pIJ702 [12] was used as a vector for transformation of *S. lividans* and pBluescript SK(+) was used for subcloning in *E. coli.*

DNA Manipulation

Chromosomal DNA from *Stall. hindustanus* and plasmid DNA from *S. lividans* were isolated as described by Hopwood *et al.* [12]. General DNA manipulation was carried out following the methods of Sambrook *et al.* [31]. Plasmid DNA and bacteriophage RF DNA were isolated from *E. coli* by alkaline SDS extraction of cell lysate [31]. A large-scale DNA purification was performed using the QUIGEN resin (Chartworth, CA, U.S.A.). Single-stranded DNA was isolated by precipitating phage particles. Agarose gel electrophoresis was performed in Tris-acetate or Tris-borate buffer [31].

Nucleotide Sequencing and Sequence Analysis

Nested deletions were constructed with the Erase-a-Base system (Promega Biotech, Madison, WI, U.S.A.) according to the manufacturer's instructions. Derivatives of pBluescript SK(+) containing *Stall. hindustanus* DNA (*Apa*I-*Bcl*I fragment) were digested with *Bam*HI and *Xba*I (5'-protruding end of the *Xba*I site was protected with the α-phosphorothioate nucleotide and Klenow fragment) prior to being treated with exonuclease III. Single- or double-stranded templates were prepared by established methods [31] and sequenced with Sequenase 2.0 (U.S. Biochemical, Cleveland, Ohio, U.S.A.) according the manufacturer's instructions. ^{32}P-dATP was the labeled nucleotide and 7-deaza-dGTP was substituted for dGTP in the sequencing reactions [32]. Labeled DNA was separated on 6% polyacrylamide wedge gels. Comparison of the nucleotide and amino acid sequences with databases were performed by the BLAST network service [1]. Protein sequences were aligned with the Genetic Computer Group software package [8] or CLUSTAL V program [9].

RESULTS

Cloning of the *nbrB* Gene from *S. lividans*

The *Stall. hindustanus* resistance determinant was isolated by shotgun cloning. *Stall. hindustanus* total DNA (1 mg) was partially digested with *Pst*I. DNA fragments between 3-kb and 6-kb in size were isolated from a 0.8% agarose gel with the QUIGEN kit. Four hundred nanograms of size-selected DNA were ligated with one hundred nanograms of pIJ702 [12] that had previously been digested with *Pst*I and treated with alkaline phosphatase. Transformation of *S. lividans* TK24 protoplasts and selection of transformants with thiostrepton were carried out as described by Hopwood [12]. After regeneration, approximately 3,000 colonies were replica-plated onto R2YE media containing 50 μg/ml tobramycin. Plasmid was isolated from selected transformants and subjected to restriction analyses, which revealed the presence of a 5.8-kb insert containing the *nbrB* gene. The *nbrB* gene was futher located within a 2.6-kb *Apa*I fragment by successive subcloning experiments (Fig. 1). It was shown that a 1.2-kb *Apa*I-*Bcl*I DNA fragment conferred resistance to thiostrepton and tobramycin. MICs (minimal inhibitory concentrations) of several selected aminoglycoside antibiotics were determined for *S. lividans* harboring the *nbrB* gene in pIJ702. As can be seen in Table 1, our clone was markedly resistant to the aminoglycoside antibiotics gentamicin (>500 μg/ml), tobramycin (>500 μg/ml), kanamycin (>500 μg/ml), and sisomicin (>500 μg/ml) than to apramycin (50 μg/ml)

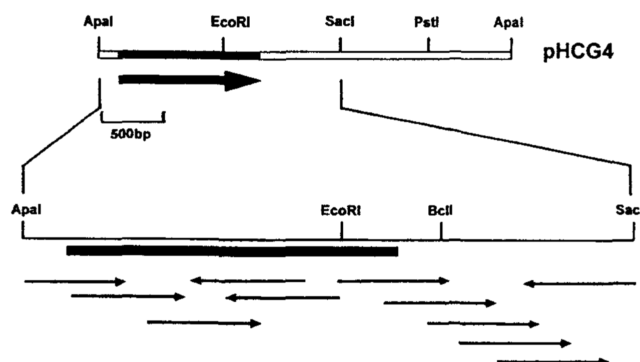

Fig. 1. Restriction map and sequencing strategy of the *nbrB* locus.

The black bar represents the coding region of the *nbrB* gene and the arrow at the top shows the direction of the open reading frame. pHCG4 is pBluescript SK (+) with a 2.6-kb *Apa*I fragment encompassing *nbrB* gene. A, *Apa*I; E, *Eco*RI; S, *Sac*I; P, *Pst*I; B, *Bcl*I.

Table 1. MICs of aminoglycoside antibiotics to the strains harboring the *nbrB* gene.

Antibiotics	MIC (μg/ml)			
	S. lividans	*S. lividans* (pIJ702)	*S. lividans* (pTMR31)	*Stall. hindustanus*
Apramycin	<20	<20	50	>1000
Gentamicin	<20	<20	>500	>1000
Kanamycin	<20	<20	>500	>1000
Neomycin	<20	<20	50	>1000
Sisomicin	<20	<20	>500	>1000
Tobramycin	<20	<20	>500	>1000

pTMR31 is a derivative of pIJ702 with a 5.8-kb DNA fragment containing the *nbrB* gene.

and neomycin (50 µg/ml). However, the resistance gene could not be expressed in *E. coli* with its own promoter (data not shown).

Sequence Analysis of the *nbrB* Gene

The complete DNA nucleotide sequence of a 1.2-kb *Apa*I-*Bcl*I DNA fragment, containing the *nbrB* gene, was determined. The employed sequencing strategy is shown in Fig. 1. In total, 1,217-bp of sequence were determined, as indicated in Fig. 2. The DNA sequence has been deposited in GenBank under the accession number AF 038408. The positions of potential coding regions were mapped using the CODONPREFERENCE program, taking advantage of the very high vias in favor of G or C in the third position of the codons of streptomycetes genes [3].

Sequence analysis of the 1.2-kb *Apa*I-*Bcl*I DNA fragment revealed one complete open reading frame. The open reading frame was named *nbrB* and predicted to begin with the ATG codon at position 116 and end with the TGA codon at position 959 (Fig. 2), encoding 281 amino acids with a predicted molecular weight of 30,992. A potential Shine-Dalgarno sequence, which exhibits varying degrees of complementarity to the 3' end of 16S rRNA of *S. coelicolor* and *Stall. hindustanus* (5'-GCGGCUGGAUCACCUCCUU-3'), was found to be separated by 7 nucleotides from the predicted translational start codon [2, 20]. Codon usage in the *Streptoalloteichus nbrB* gene was analyzed. The overall G+C content of the coding sequence is 71.7%. The high G+C content is reflected in the third position (97.9%); this result of an extremely biased usage of synonymous codons as have been reported previously for *Streptomyces* genes [3].

Comparison of the predicted amino acid sequences with known sequences in the database revealed strong similarities between the NbrB from *Stall. hindustanus* and KgmB from *S. tenebrarius* (nebramycin producer), Sgm from *M. zionensis* (6'-*N*-methyl-sisomicin producer), and Grm from *M. rosea* (sisomicin producer), which could have been expected since these genes attribute to very similar phenotypes. The alignment of these amino acid sequences is shown in Fig. 3. The deduced amino acid sequence of *nbrB* showed identities of 85.1%, 59.6%, and 57.7% to the sequences of *S. tenebrarius kgmB, M. zionensis sgm*, and *M. rosea grm*, respectively, suggesting that *nbrB* encodes a 16S rRNA methylase.

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1     G GGC CCG GCT CGC TAG GCT GGG ACG ACA GTG GAG CGG CGC CCG GAC   46
47   GAG CCC GGA CGC GCC CGC GCC GTG GCC GTC CCG GCC GGC GTG CCG TCA   94
95   CCG AGC GGA GAG GAC CCC GTG ATG CCG CAC CCG GCC CCC GGA CCG GCC  142
1                                 M   P   H   P   A   P   G   P   A    9

143  GAC GCC GAG GAC CCC CGG CTG GCG GAG GTC ATG GCG GCG GTG CGG TCG  190
10    D   A   E   D   P   R   L   A   E   V   M   A   A   V   R   S   25

191  AGC CGG CGC TAC CAG AGC GTC GCG CCC GAG ACC GTG CGC CGG CTC GCC  238
26    S   R   R   Y   Q   S   V   A   P   E   T   V   R   R   L   A   41

239  GCG AAC GCC CTC GTG GCC AGC CGG GGC GAC CTC GCG GAG GCG GTC AAG  286
42    A   N   A   L   V   A   S   R   G   D   L   A   E   A   V   K   57

287  CGC ACC AAG CGC AGC CTG CAC GAG GTC TTC GGC GCC TAC CTG CCC AGC  334
58    R   T   K   R   S   L   H   E   V   F   G   A   Y   L   P   S   73

335  CCG CCC AAG TAC GAC GCG CTG CTG CGC CAG CTC CGG GAC GCG GTC GAC  382
74    P   P   K   Y   D   A   L   L   R   Q   L   R   D   A   V   D   89

383  GCC GGC GAC GAC GAG GCC GTC CGG GCC GTG CTG CAC CGC GCG ATG TCC  430
90    A   G   D   D   E   A   V   R   A   V   L   H   R   A   M   S  105

431  ACG CAC GCC TCC ACC CGC GAG CGG CTG CCC ATC CTG GAG GAG TTC TAC  478
106   T   H   A   S   T   R   E   R   L   P   I   L   E   E   F   Y  121

479  CGG GAG GTC TTC GCC CGG CTC GAC GCC CCG ACC AGC GTG CGC GAC CTG  526
122   R   E   V   F   A   R   L   D   A   P   T   S   V   R   D   L  137

527  GCG TGC GGG ATG AAC CCG TTG GCC GCG CCC TGG ATG CCC GGC TCG GAC  574
138   A   C   G   M   N   P   L   A   A   P   W   M   P   G   S   D  153

575  GCG TTC ACC TAC CAC GCC TCG GAC ATC GAC ACC CGG CTC ACG GAG TTC  622
154   A   F   T   Y   H   A   S   D   I   D   T   R   L   T   E   F  169

623  CTC GCC GCG GCC CTG GAG ACG CTC GGC GTC GCC CAC GAC GTG CGG GTG  670
170   L   A   A   A   L   E   T   L   G   V   A   H   D   V   R   V  185

671  CGC GAC CTG ATG ACC GGG GTG GGC GAG GTG GCC ACC GAC GTC ACC CTG  718
186   R   D   L   M   T   G   V   G   E   V   A   T   D   V   T   L  201

719  CTG CTC AAG ACC CTG CCC TGC ATC GAG GCG CAG GGC AGG GGG CAG GGG  766
202   L   L   K   T   L   P   C   I   E   A   Q   G   R   G   Q   G  217

767  TGG GAC CTG ATC GAC GCG ATC CGG TCG CCG GTG GTC GTG GTG AGT TTC  814
218   W   D   L   I   D   A   I   R   S   P   V   V   V   V   S   F  233

815  CCG ACG AAG TCC CTC GGC CAG CGT TCC AAG GGG ATG TTC AAC ACC TAC  862
234   P   T   K   S   L   G   Q   R   S   K   G   M   F   N   T   Y  249

863  TCG GCG AAT TTC GGC GCC TGG TTG GAG AAC CGG CCG CAC GAC GTC GAG  910
250   S   A   N   F   G   A   W   L   E   N   R   P   H   D   V   E  265

911  CAG GTC GAA TTC AGG AAC GAA CTC GTC TAT TTC GTG CGG AAG AAC GCC  958
266   Q   V   E   F   R   N   E   L   V   Y   F   V   R   K   N   A  281

959  TGA CAA TCC CGG GGC ACC CGT CGG CCG GCG CGC CGC CGG GCG GGC CGG 1006
282   *                                                              282

1007 TGC CGG CGA CCG CAC TAG ACT CCT GGC GTG GGT TCC CCT GAC GAT CCG 1054
1055 GGC CGG TTG GTC CTC GCC GCC ACG CCC CTC GGT GAC TTC GGG GAC GCC 1102
1103 TCG CCC CGC CTG GTC GAG ATG CTG GCC ACC GCC GAC GTG ATC GCG GCC 1150
1151 GAG GAC ACC CGG CAC CTG CGC ACC CTG ACC GGT GTG CTG GGG GTG AAC 1198
1199 CCG ACC GGG CGC GTG ATC A                                       1217
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Fig. 2. Nucleotide sequence of a 1.2-kb *Apa*I-*Bcl*I DNA fragment, encompassing the *nbrB* gene.

The deduced amino acid sequence of *nbrB* is indicated below the DNA sequence by single letter designations. The ribosomal binding site is underlined.

DISCUSSION

Structurally similar antibiotics are often produced by taxonomically distant microorganisms [13]. Such an intergenetic distribution of antibiotic biosynthetic pathways is of great interest from the point of view of the evolution and distribution of secondary metabolism [30].

The nebramycin-factor, which consists of apramycin, kanamycin B, tobramycin, and their derivatives, is produced by two strains, *S. tenebrarius* and *Stall. hindustanus* [34, 35]. These antibiotics are believed to be synthesized by similar biosynthetic pathways. Previously, we reported that the kanamycin-apramycin resistance-

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NbrB  MPHPAPGPADAEDPRLAEVMAAVRSSRRYQSVAPETVRRLAANALVASRGDLAEAVKRTK
Grm   MTTSTG------DDRIDQLQQAITKSRRYQTVAPATVRRLARAALVASRGDVPDAVKRTK
KgmB  MPHPAPGPGDPEDPRLAEVVDAVRSSRRYQSVAPETVRRLATSALVASRGDLAEAVKRTK
Sgm   MTAPAA------DDRIDEIERAITKSRRYQTVAPATVRRLARAALVAARGDVPDAVKRTK
      *   .       * *. ..  *.  *****.*** ******  ****.***. .******

NbrB  RSLHEVFGAYLP-SPPKYDALLRQLRDAVDAGDDEAVRAVLHRAMSTHASTRERLPILEE
Grm   RGLHEIYGAFLPPSAPNYTALLRHLDSAVEAGDDEAVVRWDRRAMSVHMSTRERVPHLDE
KgmB  RGLHEIFGAYLP-SPPKYDALLRQLRGAVDAATTRPCGHPAPRHVHARLHPRA-LPILDE
Sgm   RGLHEIYGAFLPPSPPNYAALLRHLDSAVDAGDDEAVRAALLRAMSVHISTRERLPHLDE
      * ***..**.** * * * ****.*  **.*           * .  .   *  .* *.*

NbrB  FYREVFARLDAPTSVRDLACGMNPLAAPWMPGSDAFTYHASDIDTRLTEFLAAALETLGV
Grm   FYREIFRHVPRPNTLRDLACGLNPLAVPWMGLSDETVYVASDIDARLMDFVGAALTRLGV
KgmB  FYREVFARCADPASVRDLACGMNPLAAPWMPGSDAFTYHASDIDTRLMEFLDAALETLGV
Sgm   FYRELFRHLPRPNTLRDLACGLNPLAAPWMGLPAETVYIASDIDARLVGFVDEALTRLNV
      ****.* .   * ..******.**** ***       * *****.**  *.  **  *.*

NbrB  AHDVRVRDLMTGVGEVATDVTLLLKTLPCIEAQGRGQGWDLIDAIRSPVVVVSFPTKSLG
Grm   AHRTSVVDLLEARLDEPADVTLLLKTLPCLETQQRGSGWEVIDIVNSPIIVVTFPTKSLG
KgmB  AHDVRVRDLMTGVGEVETDVTLLLKTVPCIEAQGRGQGWDLIDAIRSPLVVVSFPTKSLG
Sgm   PHRTNVADLLEDRLDEPADVTLLLKTLPCLETQQRGSGWEVIDIVNSPNIVVTFPTKSLG
       *   * **.    .  .********.**.*.* ** **..** . ** .**.*******

NbrB  QRSKGMFNTYANFGAWLENRPHDVEQVEFRNELVYFVRKNA
Grm   QRSKGMFQNYSQSFEQASERSCRIQRLEIGNELIYVIHK--
KgmB  QRSKGMFNTYSNFDAWLENRPHDVEQLEFRNELVYFVRKNA
Sgm   QRSKGMFQNYSQSFEQARERSCRIQRLEIGNELIYVIQK--
      *******..*..       *   ....*  ***.* ..*
```

Fig. 3. Alignment of the amino acid sequences of NbrB with those of other 16S rRNA methylases.

The amino acid sequences of Kgm (10), Sgm (19), and Grm (16) were initially matched with the NbrB protein using the multiple sequence alignment CLUSTAL V program, after which the resulting comparisons were refined manually (9). Those amino acids that are identical or similar in all four proteins are indicated by asterisks or dots, respectively.

encoding gene (*kamB*) from *S. tenebrarius* shares a high degree of similarity with the aminoglycoside multiple-resistance gene from *Stall. hindustanus* [17]. Subsequent studies revealed that aminoglycoside producers generally have multiple aminoglycoside resistances whose patterns correlate with the type of antibiotics produced. Characterization of the biochemical basis for these resistances have revealed that inactivating enzymes, such as phosphotransferases, acetyltransferases, and ribosomal resistance, are involved [37]. The similarity of ***nbrB*** and the *kgmB* suggests that *nbrB* encodes a rRNA modification similar to that encoded by *S. tenebrarius*.

Studies on the inactivation of antibiotics by the microorganisms that produce them are important for understanding the self-defense mechanisms against antibiotics and for providing clues for locating the gene clusters necessary for antibiotic biosynthesis [26]. As a first step toward cloning the genes responsible for the biosynthesis of nebramycin-factors in *Stall. hindustanus*, the aminoglycoside multiple resistance-encoding gene (*nbrB*) was cloned in *S. lividans*. *Stall. hindustanus* has at least two genetic loci for multiple-resistance to aminoglycoside antibiotics. One of them, *amr*, was previously cloned and its product (Amr) was suggested to be involved in methylation of 16S rRNA [17].

We have recently developed a method of constructing modified streptomycin (MSM). MSM was made by transforming a streptomycin producer, *Streptomyces griseus*, with cloned 4"-isovaleryltransferase and *amr* genes from carbomycin and nebramycin factor producing strains, respectively. The transformant produced a substance showing inhibitory activity against *Serratia* AG4410 which is able to endure various aminoglycoside antibiotics. Interestingly, we found that this transformant also inhibits the wild-type *S. griseus*. Therefore, introduction of cloned aminoglycoside multidrug-resistance genes enabled us the production of novel hybrid antibiotics.

The overwhelming majority of antibiotics from bacterial sources are produced by *Streptomyces* or other organisms of the actinomycete line. Some of these, including macrolide, anthracyclines, and aminoglycoside, have deoxyhexose or dideoxyhexose moieties [29]. Particularly notable are the 2,6- and 4,6-dideoxy hexoses found in a broad range of bioactive compounds, which are synthesized through biosynthetic pathways that have common early biosynthetic steps [22]. In 6-deoxyhexose (6-DOH) biosynthesis, glucose-1-phosphate is converted into TDP-D-glucose by the action of a glucose-1-phosphate thymidylyl transferase. Recently, we have developed oligonucleotide primers to amplify DNA fragments of the genes for glucose-1-phosphate thymidylyl transferase from actinomycetes which produce a large variety of medically important metabolites [15]. This gene is located within a gene cluster that encodes enzymes involved in deoxy- or dideoxy-hexose metabolism and is flanked by the gene for TDP-D-glucose 4, 6-dehydratase [36]. Therefore, we are close to obtaining the biosynthetic genes for the nebramycin complex from *Stall. hindustanus* using the resistance gene reported in this paper together with the described PCR method.

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