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Cloning and Characterization of an Aminoglycoside Resistance Determinant from *Micromonospora zionensis*

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The sisomicin-gentamicin resistance methylase (*sgm*) gene was isolated from *Micromonospora zionensis* and cloned in *Streptomyces lividans*. The *sgm* gene was expressed in *Micromonospora melanosporea*, where its own promoter was active, and also in *Escherichia coli* under the control of the *lacZ* promoter. The complete nucleotide sequence of 1,122 bp and a transcription start point were determined. The sequence contains an open reading frame that encodes a polypeptide of 274 amino acids. The methylation of 30S ribosomal subunits by Sgm methylase accounts adequately for all known resistance characteristics of *M. zionensis*, but expression of high-level resistance to hygromycin B is background dependent. A comparison of the amino acid sequence of the predicted Sgm protein with the deduced amino acid sequences for the 16S rRNA methylases showed extensive similarity to Grm and significant similarity to KgmB but not to KamB methylase.

Antibiotic-producing organisms must be able to protect themselves against their own toxic products. Mechanisms of resistance variously employ inactivation of the products by antibiotic-modifying enzymes, alteration of the target sites to which the drugs normally bind, and the ability to exclude antibiotics from the cell (for a review, see reference 4). Much of the available data relate to inhibitors of protein synthesis that normally act against the ribosome but can be prevented from doing so in the respective producing organisms either by detoxification of the drugs or via ribosomal modification. Interestingly, whenever antibiotic-resistant ribosomes had previously been observed in producers of the respective drugs, the mechanism of resistance had invariably been shown to involve methylation of rRNA at a single site characteristic of each different phenotype (2, 24, 26).

In the case of *Micromonospora* strains which produce aminoglycosides, ribosomes are at least in part responsible for the resistance phenotype and it has been shown that the 30S subunit was responsible for the resistance (17). More specifically, it was ascertained that the state of 16S RNA in the 30S subunits from *Micromonospora purpurea*, the gentamicin producer, is responsible for resistance (20, 27). Concerning genes originated in *Micromonospora* spp., virtually no data are available. Thus, it is not known whether the genes responsible for antibiotic biosynthesis in *Micromonospora* strains are organized and controlled in the same manner as in *Streptomyces* strains. As a first step in studying the genetic organization of antibiotic biosynthetic genes in *Micromonospora* strains, it was decided to clone the resistance determinant from *Micromonospora zionensis*, the producer of G-52 (6′-*N*-methyl-sisomicin) (3, 29).

Cloning of the *sgm* gene in *Streptomyces lividans*. The *M. zionensis* resistance determinant was isolated by using the well-characterized expression system of the related actinomycete *S. lividans* (12). Total genomic DNA from *M. zionensis* was partially digested with *Sau*3AI or *Bam*HI and ligated with *Bgl*II-cleaved and terminally dephosphorylated vector pIJ702 (the vector was obtained from D. A. Hopwood, John Innes Institute, Norwich, United Kingdom) (13). The ligation mixture was used to transform protoplasts of *S. lividans* TK21 according to standard procedures (12). Among thiostrepton-resistant transformants containing *Bam*HI inserts, three colonies were resistant to 50 μg of gentamicin per ml. Plasmids were isolated from transformants and subjected to restriction analysis, which revealed the presence of the same 4.5-kb insert, in either orientation, which is depicted in Fig. 1. These plasmids conferred resistance to both gentamicin and thiostrepton when reintroduced into *S. lividans* TK21, and one such transformant, designated MK3, was chosen for further analysis. The *sgm* gene has been located within pMK3 by successive subcloning experiments in pIJ702 and also in pUC18/19 vectors. It was shown that a 1.12-kb *Sal*I-*Sal*I DNA fragment conferred resistance to gentamicin when ligated into pUC19 and introduced into *Escherichia coli* MN522 (8). However, the resistance gene could be expressed only when it was in the same orientation as the *lacZ* promoter, suggesting that expression of the *M. zionensis* gene was dependent on the *E. coli* transcriptional signal (data not shown).

Resistance profiles of gentamicin-resistant clones. MICs of several selected aminoglycoside antibiotics were determined for *S. lividans* MK33 (the *sgm* strain, containing pMK33) and TSK1 (control strain containing pIJ702). *M. zionensis* was used in this experiment as well, in order to compare resistance patterns. As can be seen in Table 1, our clone is cross resistant to kanamycin as well as to the other antibiotics of the gentamicin family. In this respect, the MIC profile for the *S. lividans sgm* strain closely resembled that for *M. zionensis*, with the exception of the hygromycin B MIC. Surprisingly, when the *sgm* gene was introduced into *Micromonospora melanosporea* protoplasts (16), transformants were resistant to hygromycin B, whereas *S. lividans* transformants were hygromycin sensitive (Table 1). It was observed previously that several (if not all) producers of gentamicin-like compounds are highly resistant to hygromycin B (17, 19). It may well be, therefore, that those organisms all share a common mode of hygromycin B resistance. We do not have enough data to account for these observations, but it is clear that expression of hygromycin B resistance determined by the *sgm* gene is background dependent. A

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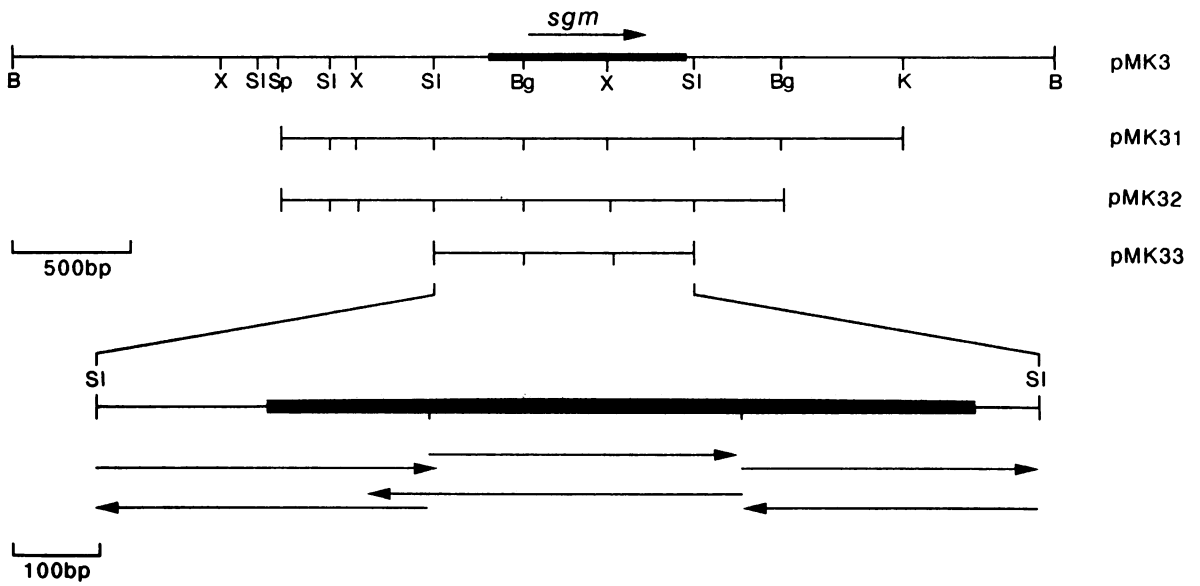

FIG. 1. Restriction map and subcloning and nucleotide sequencing strategy of the *sgm* gene. The black bar represents coding region of the *sgm* gene, and the arrow at the top shows the direction of transcription. The set of arrows below the *SalI-SalI* fragment shows the sequencing strategy. The restriction enzymes have been abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; Sl, *Sal*I; Sp, *Sph*I; X, *Xho*I.

mutation affecting residue U-1495 gives resistance to hygromycin B in *Tetrahymena thermophila* (25), so it is tempting to conclude that the U-1495 region in *M. melanosporea* differs from the same region of 16S rRNA in *S. lividans*. In other words, methylation of one residue within 16S rRNA of *S. lividans* confers resistance to gentamicin but not to hygromycin B, while in *M. melanosporea*, because of the altered context of the U-1495 region, the same gene is responsible for resistance to both gentamicin and hygromycin B. It is necessary to take into consideration the fact that various residues are all brought together in models of the secondary structure of 16S rRNA. In any event, the mapping of modified residues and sequencing of the relevant region of 16S rRNA of *M. melanosporea* may give an answer.

Prior to the present work, it had been already shown that ribosomes from *M. zionensis* were resistant in vitro to a range of aminoglycosides, which reflected the in vivo resistance phenotypes (17). This finding suggested that the *sgm* allele was responsible for the ribosomal modification resulting in aminoglycoside resistance. To test this hypothesis, the *S. lividans* strain containing *sgm* on plasmid pMK3 was examined for methylase activity. Figure 2 shows that extracts from *S. lividans* MK3 readily methylated 30S ribosomal subunits isolated from *S. lividans* TSK1(pIJ702) in the presence of the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine (SAM) as a cosubstrate. It was also observed that 30S ribosomal subunits from *S. lividans* BV1 (*grm* from *M. purpurea* [14, 28]) were not a substrate for the methylase from the *sgm* strain. These data suggest that the Sgm and Grm methylases might act at a common site within 16S rRNA of the 30S ribosomal subunit. It is known that specific sites within rRNA (at which methylation or base changes resulting from mutations occur) can confer resistance to antibiotics (for a review, see reference 5). These sites are closely related to those at which ribosome-bound antibiotics give protection against chemical modification (18). For example, methylation of residue A-1408 by the Kam enzyme from *Streptomyces tenjimariensis* confers resistance to kanamycin and apramycin, whereas Kgm methylase of *Streptomyces tenebrarius* modifies residue G-1405 and gives resistance to kanamycin plus gentamicin (2). It is therefore reasonable to suppose that Sgm and Grm methylases might act at nearby sites of action of the phenotypically related Kgm enzyme.

Nucleotide sequence of the *sgm* gene. The nucleotide sequence of about 1.12 kb of *M. zionensis* DNA (*SalI-SalI*

TABLE 1. MICs of aminoglycoside antibiotics for the *sgm* strains

Antibiotic	MIC (μg/ml) for:				
	S. lividans TSK1[a]	*S. lividans* MK33[b]	*M. melanosporea* MTS1[a]	*M. melanosporea* MMK33[b]	*M. zionensis*
Sisomicin	<1	>300	5	>300	>300
Gentamicin	2	>500	3	>500	>500
Kanamycin	<1	>500	5	>500	>500
Tobramycin	<1	100	5	>500	>500
Apramycin	<1	<1	<1	<2	<1
Neomycin	<1	<1	<1	<2	<1
Hygromycin B	2	10	5	>500	>500

[a] *S. lividans* TSK1 and *M. melanosporea* MTS1 are the control strains containing pIJ702.
[b] *S. lividans* MK33 and *M. melanosporea* MMK33 are the strains containing pMK33.

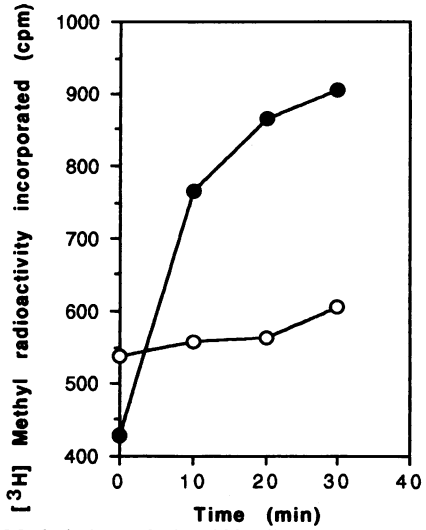

FIG. 2. Methylation of the 30S ribosomal subunits from *S. lividans* TSK1 (sensitive strain; ●) and *S. lividans* BV1 (the *grm* strain; ○) (28). The S30 extract from *S. lividans* MK3 (the *sgm* strain containing pMK3) was used as the source of methylase together with SAM as the cosubstrate.

DNA fragment containing *sgm*) was determined by the dideoxy chain termination method (22), using either double-stranded plasmid DNA (32) or single-stranded M13 phage DNA (31). The sequencing strategy is shown in Fig. 1. In total, a sequence of 1,122 bp was determined, as indicated in Fig. 3. A computer-aided search for open reading frames revealed only one, reading left to right in the orientation of Fig. 1, starting with an ATG codon (nucleotide 215) and terminating at TAG (nucleotide 1037). A potential ribosome-binding site is separated by 5 nucleotides from the predicted translational start codon. The open reading frame comprised 274 sense codons that could specify a 33-kDa protein. For the *sgm* coding sequence, the usage of G and C in codon positions 1, 2, and 3 was 69, 45, and 81%, respectively. The overall G+C content of 64% is slightly lower than the 71 to 73% average for micromonospora DNA or 68 to 78% for streptomyces DNA (7).

Transcript analysis of the *sgm* gene. The transcriptional start point (TSP) was determined by primer extension with avian myeloblastosis virus reverse transcriptase on total RNA isolated from *S. lividans* MK33 carrying the *SalI-SalI* DNA fragment of *M. zionensis*. Priming with an oligonucleotide which is complementary to a region approximately 40 bp downstream of the ATG codon (as indicated in Fig. 3) revealed a single extension product corresponding to a G at position 143, which is 72 nucleotides upstream from the *sgm* coding region (Fig. 4). The sequence in the region upstream from the TSP is not closely related to the *E. coli* promoter consensus sequence, which is consistent with the observation that the *sgm* gene cannot be expressed in *E. coli* under control of its own promoter. Moreover, there is an apparent difference between transcription signals of the *sgm* and the *grm* genes. The TSP of the *grm* gene is located 11 nucleotides upstream of the translational start codon, while the *tsp* of the *sgm* gene is 72 nucleotides in front of ATG codon. Also, the promoter region of the *grm* gene shows significant

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                                                                                                    TCGACGTGCAAAAAGCGGCCAAGACGGGCTTGCAGGACAGGCCGACCAATATGGACGTGACTGTCGTGTGGTGTGCGGCGCGCCCCCTGCCCGC  94
                                                                                                                                                                      *
                                                                                                                                                                     RBS
TCGTAATACCCCTGTAACAACTCTTGGTAGACGAAGCAGCAACTCTTGGTGTGCGATCCGGTGGGCCGGCGAGCGGAATTACCCGGTTCGCCGCCCATTGATGACCTTTTGGAGGGACCG  214

ATGACGGCACCTGCGGCCGACGACCGTATCGACGAGATTGAGCGGGCCATCACCAAGAGCAGGCGTTACCAGACGGTGGCGCCGGCCACCGTGCGCCGCCTGGCCCGCGCTGCTCTCGTC  334
M  T  A  P  A  A  D  D  R  I  D  E  I  E  R  A  I  T  K  S  R  R  Y  Q  T  V  A  P  A  T  V  R  R  L  A  R  A  A  L  V   40

GCCGCGCGGGGTGACGTGCCCGACGCGGTGAAGCGCACCAAGCGGGGTCTGCACGAGATCTACGGCGCCTTCCTGCCGCCCAGCCCTCCCAACTACGCAGCGTTGCTGCGGCACCTGGAC  454
A  A  R  G  D  V  P  D  A  V  K  R  T  K  R  G  L  H  E  I  Y  G  A  F  L  P  P  S  P  P  N  Y  A  A  L  L  R  H  L  D   80

TCGGCAGTGGACGCCGGTGACGACGAGGCGGTTCGAGCGGCCCTACTTCGCGCTATGTCCGTACATATCTCCACCCGCGAGCGATTGCCGCACCTCGACGAGTTCTACCGGGAACTCTTC  574
S  A  V  D  A  G  D  D  E  A  V  R  A  A  L  L  R  A  M  S  V  H  I  S  T  R  E  R  L  P  H  L  D  E  F  Y  R  E  L  F  120

CGGCACCTCCCCCGACCGAACACGCTGCGTGACCTCGCCTGTGGTCTCAACCCCCTGGCCGCGCCCTGGATGGGCCTGCCCGCCGAGACCGTCTACATCGCCTCGGACATCGACGCCCGC  694
R  H  L  P  R  P  N  T  L  R  D  L  A  C  G  L  N  P  L  A  A  P  W  M  G  L  P  A  E  T  V  Y  I  A  S  D  I  D  A  R  160

CTGGTCGGCTTCGTGGACGAGGCCCTGACCCGACTCAATGTTCCACATCGGACGAACGTGGCCGACCTGCTCGAGGACCGTCTTGACGAGCCGGCCGACGTCACGCTATTGCTGAAGACG  814
L  V  G  F  V  D  E  A  L  T  R  L  N  V  P  H  R  T  N  V  A  D  L  L  E  D  R  L  D  E  P  A  D  V  T  L  L  L  K  T  200

CTGCCCTGTCTGGAGACTCAGCAACGAGGATCGGGCTGGGAAGTGATTGACATTGTCAACTCGCCGAATATCGTGGTAACCTTCCCGACCAAGTCTCTCGGTCAGCGATCGAAGGGGATG  934
L  P  C  L  E  T  Q  Q  R  G  S  G  W  E  V  I  D  I  V  N  S  P  N  I  V  V  T  F  P  T  K  S  L  G  Q  R  S  K  G  M  240

TTTCAGAACTATTCACAGAGTTTTGAGTCCCAGGCCAGAGAGCGGTCATGCCGTATTCAGCGACTGGAGATTGGCAACGAGCTGATTTACGTCATTCAGAAATAGCTTTTCGCCACTCCT 1054
F  Q  N  Y  S  Q  S  F  E  S  Q  A  R  E  R  S  C  R  I  Q  R  L  E  I  G  N  E  L  I  Y  V  I  Q  K  <                   274

CGGGAGAATCGGACGGAGTCGTGCGTGTTCTTCGGCTAACCCCTTTTTTCCACCACGACTACGTCGAC                                                    1122
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FIG. 3. Nucleotide sequence of the *SalI-SalI* region of the cloned DNA and predicted amino acid sequence for the Sgm gene product. The TSP determined by primer extension (experiment shown in Fig. 4) is marked by an asterisk above the corresponding nucleotide. The position of the putative ribosomal binding site (RBS) is indicated, and the region complementary to the 24-mer oligonucleotide that was used for identification of the TSP is underlined. The upper and the lower numbers on the right indicate the nucleotide and amino acid positions, respectively.

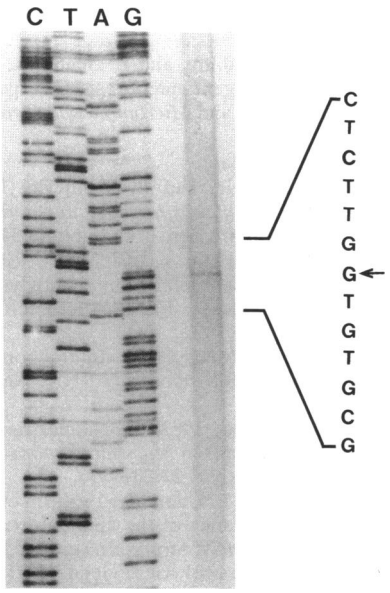

FIG. 4. Mapping the *sgm* gene TSP by primer extension. An arrow within the nucleotide sequence on the right indicates the TSP. Lanes C, T, A, and G contain the products of dideoxy sequencing reactions carried out with the same oligonucleotide primer.

homology with conserved sequences of *E. coli*-like *Streptomyces* consensus promoters (30). On the other hand, the promoter region of *sgm* differs from that known for *Streptomyces* or *Micromonospora* species (1, 21). Furthermore, there is an indication that the *sgm* gene in *M. zionensis* is transcribed from tandem promoters: one characterized by primer extension, as shown in Fig. 4, and the other, more upstream (~350 nucleotides), which was determined by using total RNA from *M. zionensis* (15). This could enable a differential gene expression of the *sgm* gene in the producing organism. Currently, we are undertaking more-detailed studies to determine precisely the TSP of the second promoter and the mode of its expression. To conclude at this point, the sequences used as transcriptional and translational signals must have evolved independently from the coding sequences of the *sgm* and *grm* genes.

Sequence comparisons between methylases. Comparison of the nucleotide and predicted amino acid sequences with the various data bases revealed strong similarities between the *sgm* gene from *M. zionensis* and *grm* genes from *M. purpurea* and *Micromonospora rosea*, a gentamicin producer and a sisomicin producer, respectively (14), which could be expected since these genes have very similar phenotypes. Extensive nucleotide similarities are noted in the intragenic regions of resistance genes of three antibiotic-producing strains, but nucleotide sequences 5′ to the genes showed considerable divergences. One would also expect similarities with the other 16S rRNA methylases which confer resistance to aminoglycoside antibiotics, such as *kgmB* and *kamB* products (10, 11). Indeed, comparison of the deduced amino acid sequence revealed that there is 90, 54, and 23% identity between Sgm and the Grm, KgmB, and KamB proteins, respectively. Alignment of these amino acid sequences is shown in Fig. 5. Since these enzymes act in a similar way and also utilize SAM as a cofactor, it would be interesting to see whether some of the regions with higher similarity are involved in coenzyme binding, as proposed for ATP sites in APH enzymes (6). Recently, comparison of a number of very diverse SAM-dependent methyltransferases has shown that a glycine-rich motif could be involved in binding of SAM (9). In contrast, the 16S rRNA methylases taken into consideration in this work did not show any significant sequence similarities to the proposed consensus sequence, i.e., the glycine-rich motif. On the basis of the present data, one of

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Sgm    MTAPAA......DDRIDEIERAITKSRRVQTVAPATVRRLARAALVAARGDVPDAVKRTK   54
Grm    MTTSAP......EDRIDQVEQAITKSRRYQTVAPATVRRLARAALVAARGDVPDAVKRTK   54
KgmB   MPHPAPGPGDPEDPRLAEVVDAVRSSRRYQSVAPETVRRLATSALVASRGDLAEAVKRTK   60
KamB                    MEKIS.SKAAAKPAKAL.PNLLYLWQTAERLPPLSGVGE......   37

Sgm    RGLHEIYGAFLPPSPPNYAALLRHLDSAVDAGDDEAVRAALIRAMSVHISTRERLPHLDE  114
Grm    RGLHKIYGAFLPPSPPNYAALLRQLDSAVDAGDDEAVRAALRRAMSVHVSTRERLPHLAE  114
KgmB   RGLHEIFGAYLP.SPPKYDALLRQLRGAVDAATTRPCGHPAPRHVHARLHPRA.LPILDE  118
KamB   ..LH....VLMP.....WGSLLNGVLGS.........SPEMLAGMAAVCRPGA......S   71

Sgm    EYRELFRHLPRPNTLRDLACGLNPLAAPWMGLPAETVYIASDIDARLVGFVDEALTRLNV  174
Grm    FYQEIFRHVPQPNTLRDLACGLNPLAAPWMGLSDQTVYVASDIDARLIGFVDAALTRLGV  174
KgmB   FYREVFARCADPASVRDLACGMNPLAAPWMPGSDAFTYHASDIDTRLMEFLPAPLETLGV  178
KamB   FLVECNRSCRQE.PVPEV..GEHPEPTP.....................DSADEWLAP  105

Sgm    PHRTNVADLLEDRLDEPADVTILLKTLPCLETQQRGSGWEVIDIVNSPNIVVTFPPKSLG  234
Grm    AHRTSVVDLLEDRLDEPTDVTILLKTLPCLETQRRGSGWEVIDIVNSPIIVVTFPPKSLG  234
KgmB   AHDVRVRDLMTGVGEVETDVTILLKTVPCIEAQGRGQGWDLIDAIRSPLVVVSFPPKSLG  238
KamB   RYAEAGWKLADCRYLEPEEVAGL.............ETSW.................DRRL.  136

Sgm    QRSKGMFQNYSQSFESQARERSCRIQRLEIGNELIYVIQK     274
Grm    QRSKGMFQNYSQSFESQARERSCRIQRLEIGNELIYVIQK     274
KgmB   QRSKGMFNTYSANFDAWLENRPHDVEQLEFRNELVYFVRKNA   280
KamB   HSSRDREDV.................LALTGTISP          154
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FIG. 5. Comparison of amino acid sequences within the deduced products of genes conferring aminoglycoside resistance: *sgm*, *grm* (14), *kgmB* (10), and *kamB* (11). Only positions of identity between Sgm and other proteins are shaded. Those amino acids that are identical in all four proteins are enclosed by darker boxes. Gaps, indicated by dots, were introduced to maximize homology.

the possible explanations for that difference between 16S rRNA methylases and the other two classes of methylases (23S rRNA and DNA) is that later enzymes use naked nucleic acids as substrates, while 16S rRNA methylases could recognize only 30S ribosomal subunits and neither 16S rRNA nor 70S ribosomes. Thus, since recognition of substrates by these enzymes differs, they may also require different organization of the cofactor binding site.

Nucleotide sequence accession number. The sequence of the *sgm* gene has been deposited in the GenBank data base under accession number M87057.

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