Aminoglycoside-producing strains utilize ribosomal modification as a means of self-defense, regardless of whether they also employ drug-modifying enzymes. Some of the genes cloned from actinomycetes that produce aminoglycoside antibiotics include the grm gene from Micromonospora purpurea (Kelemen et al., 1991; Vasiljevic and Cundliffe, 1990), sgm from Micromonospora zionensis (Kojic et al., 1992) and two genes (kamB and kgmB) from Streptomyces tenebrarius (Holmes and Cundliffe, 1991; Skeggs et al., 1987). In each case, resistance is due to methylation of ribosomal RNA at positions which are crucial for their action. The sgm sisomicin-gentamicin resistance gene from Micromonospora zionensis methylates G1405 positioned in the A site of 16S rRNA, which includes a CCGCCC hexanucleotide. The same hexanucleotide is also present 14 nucleotides in front of the ribosome binding site of sgm mRNA. The model proposed for translational regulation of sgm assumes that Sgm binds to this motif, both on 16S RNA and on the 5' untranslated region (UTR) of its own mRNA. The 5' UTR mRNA sequence was overexpressed on 3'-truncated sgm mRNA, and the effect on gentamicin resistance conferred by Sgm was tested in Escherichia coli and in Micromonospora melanosporea. Overexpression of the sgm mRNA regulatory region decreases the resistance to gentamicin in both E. coli and M. melanosporea. This effect is likely to be due to titration of Sgm molecules by the overexpressed 5' UTR.

Key words: Sgm, 16S rRNA methylase, gentamicin resistance, regulation

OVEREXPRESSION OF SGM 5' UTR MRNA REDUCES GENTAMICIN RESISTANCE IN BOTH ESCHERICHIA COLI AND MICROMONOSPORA MELANOSPOREA CELLS

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Institute of Molecular Genetics and Genetic Engineering, 11010 Belgrade, Serbia

Abstract — The 16S rRNA methylases are expressed by most of the antibiotic producing bacteria in order to protect themselves against antibiotics by methylation of 16S rRNA at positions which are crucial for their action. The sgm sisomicin-gentamicin resistance gene from Micromonospora zionensis methylates G1405 positioned in the A site of 16S rRNA, which includes a CCGCCC hexanucleotide. The same hexanucleotide is also present 14 nucleotides in front of the ribosome binding site of sgm mRNA. The model proposed for translational regulation of sgm assumes that Sgm binds to this motif, both on 16S RNA and on the 5' untranslated region (UTR) of its own mRNA. The 5' UTR mRNA sequence was overexpressed on 3'-truncated sgm mRNA, and the effect on gentamicin resistance conferred by Sgm was tested in Escherichia coli and in Micromonospora melanosporea. Overexpression of the sgm mRNA regulatory region decreases the resistance to gentamicin in both E. coli and M. melanosporea. This effect is likely to be due to titration of Sgm molecules by the overexpressed 5' UTR.

Key words: Sgm, 16S rRNA methylase, gentamicin resistance, regulation

INTRODUCTION

Aminoglycoside-producing strains utilize ribosomal modification as a means of self-defense, regardless of whether they also employ drug-modifying enzymes. Some of the genes cloned from actinomycetes that produce aminoglycoside antibiotics include the grm gene from Micromonospora purpurea (Kelemen et al., 1991; Vasiljevic and Cundliffe, 1990), sgm from Micromonospora zionensis (Kojic et al., 1992) and two genes (kamB and kgmB) from Streptomyces tenebrarius (Holmes and Cundliffe, 1991; Skeggs et al., 1987). In each case, resistance is due to methylation of ribosomal RNA at a single site characteristic of a given phenotype, i.e. resistance to a particular group of aminoglycoside antibiotics. For example, the KgmB enzyme methylates residue G1405 in 16S rRNA using S-adenosylmethionine (SAM) as a co-factor (Beauclerk and Cundliffe, 1987). It was recently shown in vitro methylation assays that Sgm and KgmB methylases act at the same residue, that is at G1405 within 16S rRNA (manuscript in preparation).

Both sgm and kgmB appear to be down-regulated at the posttranscriptional level by a mechanism of translational autoregulation (Kojic et al., 1996; Vajic et al., 2004). According to the model proposed, expression of the resistance genes ensures that enough methylase molecules modify 16S rRNA (the primary target), and when all ribosomes are protected unnecessary translation is prevented by binding to their own mRNA (the secondary target). A CCGCCC hexanucleotide has been identified 14 nucleotides upstream of the ribosome binding site of the sgm, and the same hexanucleotide is also present in the A site of 16S rRNA, i.e., the region where most of the aminoglycoside resistance methylases act (Fourmy et al., 1996, Vicens and Westhof, 2003). Moreover, Sgm methylase can down-regulate kgmB:lacZ fusions, presumably by binding to a CCGCCC motif present in the 5' UTR region of kgmB (Vajic et al., 2004).

In the course of elucidating the Sgm's translational autoregulation by employing the E. coli lacZ gene and operon fusion systems, it has been noticed
that overexpression of the secondary target, i.e., the sgm mRNA, could alter the resistance of *E. coli* to gentamicin. The aim of the present work was to confirm this observation. Accordingly, further experiments were designed to test this possibility, both in a heterologous host such as *E. coli* and in a homologous host, *M. melanosporea*.

MATERIALS AND METHODS

*Strains, growth conditions, and plasmids used in this study*

*E. coli* strain NM522, *Micromonospora melanosporea* DSM43126 and *Streptomyces lividans* TK21 were used. Growth media were prepared as described in *Sambrook et al.* (1989) and *Hopwood et al.* (1985). In short, the *E. coli* strain was grown in LB (Luria-Bertani) medium. The *Micromonospora* strain was grown on mM plates, and tryptophan soy broth (TSB) supplemented with manitol (3% final concentration) was used for growth in liquid medium. The *Streptomyces lividans* strain was grown on NE plates and YEME liquid medium. R2YE supplemented with MRS liquid medium was used for regeneration of *Micromonospora* protoplasts. Ampicillin at concentrations of 50 μg/ml and 30 μg/ml gentamicin were used for selection of transformants in *E. coli*. In *Micromonospora*, plasmids were selected at 30 μg/ml gentamicin and 40 μg/ml nosileptide. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used for transcriptional induction, its concentrations being as cited in the text. All plasmids used in this work, with relevant characteristics and details of their constructions, are listed in Table 1.

**Table 1.** Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genes and characteristics of interest</th>
<th>Source or reference</th>
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<tr>
<td>pUC19</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, high copy number plasmid</td>
<td>Yanisch-Perron et al., 1985</td>
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<tr>
<td>pUF1</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, pUC19, <em>sgm-lacZ</em> fusion preceded by the regulatory sequence under control of inducible <em>P&lt;sub&gt;tl&lt;/sub&gt;</em> promoter.</td>
<td>Kojic et al., 1996</td>
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<tr>
<td>pFAK</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, pUC19, plasmid carries <em>sgm-lacZ</em> fusion without the regulatory sequence under control of <em>P&lt;sub&gt;tl&lt;/sub&gt;</em>.</td>
<td>This study</td>
</tr>
<tr>
<td>pULL</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, pUC19, <em>PvuII-EcoRI</em> α-fragment of β-galactosidase under control of <em>P&lt;sub&gt;tl&lt;/sub&gt;</em>.</td>
<td>This study</td>
</tr>
<tr>
<td>pUF6KS2</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, Gm&lt;sup&gt;r&lt;/sup&gt;, pUC19, <em>sgm-lacZ</em> fusion preceded by the regulatory sequence under control of the inducible <em>P&lt;sub&gt;tl&lt;/sub&gt;</em> and <em>sgm</em> gene under control of constitutive <em>P&lt;sub&gt;kan&lt;/sub&gt;</em>.</td>
<td>This study</td>
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<tr>
<td>pUFARKS2</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, Gm&lt;sup&gt;r&lt;/sup&gt;, pUC19, plasmid carries <em>sgm-lacZ</em> fusion without the regulatory sequence under control of <em>P&lt;sub&gt;tl&lt;/sub&gt;</em> and <em>sgm</em> gene under control of constitutive <em>P&lt;sub&gt;kan&lt;/sub&gt;</em>.</td>
<td>This study</td>
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<tr>
<td>pMZ1</td>
<td>Cryptic plasmid from <em>Micromonospora zionensis</em></td>
<td>Oshida et al., 1986</td>
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<tr>
<td>pBMZ13</td>
<td>Gm&lt;sup&gt;r&lt;/sup&gt;, pMZ1, <em>sgm</em> gene under control of its own promoters.</td>
<td>Vukov and Vasiljevic, 1998</td>
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<tr>
<td>pMRSP1</td>
<td>Nh&lt;sup&gt;r&lt;/sup&gt;, pIJ486, N-terminal region of <em>sgm</em> under control of P1 promoter from pMZ1.</td>
<td>This study</td>
</tr>
<tr>
<td>pMRSP29</td>
<td>Nh&lt;sup&gt;r&lt;/sup&gt;, pIJ486, N-terminal region of <em>sgm</em> under control of P29 promoter from pMZ1.</td>
<td>This study</td>
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according to standard protocols (Sambrook et al., 1989; Hopwood et al., 1985). The *Micromonospora melanosporea* strain was transformed using transformation procedures described previously (Kojic et al., 1991).

**Determination of minimal inhibitory concentrations (MIC)**

Equal masses of micelial fragments were resuspended in 1 ml of TSB medium. 100 µl from each suspension was streaked on mM medium plates containing different concentrations of antibiotics. Lists of antibiotics and concentrations used are given in Table 2. Cell growth in liquid media was monitored by reading OD at 550 nm.

**RESULTS**

*Overexpression of 3’-truncated sgm mRNA lowers resistance to gentamicin conferred by Sgm in E. coli*

To determine whether overexpression of Sgm’s secondary target might effect Sgm-conferred gentamicin resistance in *E. coli*, three vectors were constructed, each harboring *sgm* under control of the *P*<sub>kan</sub> promoter, while the 5’ UTR of *sgm* was expressed from the inducible *P*<sub>L</sub> promoter (Fig. 1). pUF6KS2 expresses the *sgm*-lacZ fusion, which contains the regulatory hexanucleotide preceding the RBS of *sgm*, while pUFΔRKS2 is characterized by deletion of the hexanucleotide. The pULLKS2 plasmid was used as a negative control; it contains only the α-fragment of β-galactosidase without 5’ UTR sequences and the N-terminal region of *sgm*. The growth rate of *E. coli* NM522 cells containing plasmids pUF6KS2, pUFΔRKS2, and pULLKS2 was measured under conditions of increasing concentrations of gentamicin and induction of transcription from *P*<sub>L</sub> with IPTG. *Cells were grown in overnight cultures* in the presence of 50 µg/ml ampicillin. Batches of liquid cultures with increasing concentrations of gentamicin and in the absence or presence of 1 mM IPTG were inoculated with 1% of overnight cultures and cell growth was monitored after 16 h of growth (Fig. 2). It was observed that cells with the pUF6KS2 plasmid, which overexpress the *sgm* secondary target, show a marked reduction of growth rate in the presence of gentamicin. Cells carrying pUFΔRKS2, which do not express the *sgm* secondary target, showed the same growth rate in the presence of gentamicin as observed for the control strain with pULLKS2.

**Correlation between induction of secondary target transcription and sensitivity to gentamicin**

Correlation between induction of transcription of secondary target (5’ UTR plus) and sensitivity to gentamicin in *E. coli* cells was further tested by measuring growth rates of cells with pUF6KS2, pUFΔRKS2, and pULLKS2 throughout the course of the experiment. Cells were grown in LB medium

<table>
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<th>Table 2. Resistance of <em>Micromonospora melanosporea</em> clones to gentamicin and nosiheptid.</th>
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<tr>
<td><strong>M. melanosporea</strong></td>
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<tr>
<td>+pBMZ13 +pMRSP1</td>
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<tr>
<td>+pBMZ13 +pMRSP29</td>
</tr>
<tr>
<td><strong>M. melanosporea</strong> + pBMZ13</td>
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**Fig. 1.** Plasmid system for analysis of the influence of Sgm secondary target overexpression on E. coli gentamicin resistance. Only relevant plasmid regions for this study are shown. Shaded boxes indicate sgm and lacZ gene regions, arrows the direction of transcription, triangle the RBS region of sgm, and circles the hexanucleotide on the mRNA target.

**Fig. 2.** Growth curve of E. coli NM522 cells transformed with pUF6KS2, pUFΔRKS2, and pULLKS2 measured under noninducing conditions (- IPTG) or in the presence of 1 mM IPTG (+ IPTG).

containing 5 µg/ml of gentamicin and induced with 0, 0.01 mM, 0.1 mM and 1 mM IPTG. The results clearly demonstrate that only overexpression of the regulatory sequence (construct pUF6KS2) causes dose-dependent reduction in the growth rate (Fig. 3). Correlation between induction of the secondary target and sensitivity to gentamicin was confirmed in this way. These results therefore suggest that the 5' UTR sequence, when overexpressed along with the sgm gene, presumably exerts its negative effect on the establishment of Sgm conferred gentamicin resistance in E. coli cells by titrating the Sgm molecules. These observations also raised the question as to whether the same is true in the homologous background. To learn the answer, we next introduced a secondary target into *Micromonospora melanosporea* and examined its effect on Sgm-conferred gentamicin resistance in this bacterium.

**Influence of sgm secondary target overexpression on gentamicin resistance in *Micromonospora melanosporea***

*Micromonospora melanosporea* does not produce any antibiotic; it is sensitive to aminoglycosides including gentamicin and thus suitable for testing the effect of overexpression of the sgm regulatory region on mRNA in a homologous background. *M. melanosporea* was transformed with pBMZ13, a low copy plasmid that contains sgm under control of its own promoter (Vukov and Vasiljevic, 1998).

The sgm secondary target was expressed in trans from plasmids expressing this regulatory region under the control of two different promoters: either P1, which confers resistance to 30 µg/ml kanamycin when cloned into pIJ486, the *Streptomyces* promoter-probe plasmid; or the P29 promoter isolated from the pMZ1 plasmid, which confers resistance to 400 µg/ml kanamycin when tested in the same vector (Kojic et al., 1994). The constructed plasmids containing the sgm secondary target under control of the P1 and P29 promoters were named pMRSP1 and pMRSP29, respectively. Resistance to gentamicin was measured in *M. melanosporea* cells cotransformed with either pBMZ13 plus pMRSP1 or pBMZ13 plus pMRSP29. As a control, a strain con-
taining the pBMZ13 plasmid was used. Growth was monitored on mM plates with increasing concentrations of gentamicin (Table 2). Nosileptid was added to the media to ensure the presence of pMRSP1 and pMRSP29, plasmids that have gene conferring resistance to this antibiotic, as a selective marker. Perhaps surprisingly, the results showed that expression of 5' UTR under the weak P1 promoter was sufficient to cause a dramatic decrease of gentamicin resistance, and that the effect was further potentiated by expression under the strong P29 promoter, when reduction of gentamicin resistance was almost complete. Thus, the presence of sgm regulatory sequence expressed under the P1 promoter reduces the level of resistance to 20 µg/ml gentamicin, whereas expression from the stronger P29 promoter reduces resistance even more (5-10 µg/ml). Hence, the results clearly indicate that resistance to gentamicin is reduced when the secondary target is overexpressed in M. melanosporea, as was observed in E. coli.

DISCUSSION

Our interest in regulation of the sgm gene derives from the fact that we were unable to detect Sgm protein synthesis in an E. coli minicell system,
despite the fact that E. coli cells containing the cloned gene were gentamicin resistant (Ko j ć et al., 1992). The Sgm protein has a limited number of intracellular target sites, and it is anticipated that relatively few enzyme molecules are sufficient for complete modification of the target (i.e., 16S rRNA). Sgm, like many other components involved in translation, is autoregulated at the post-transcriptional level (for a review, see Koz ăk, 2005, and references therein). Autoregulation of the sgm has been proved in vitro using gene and operon sgm-lac Z fusions (Koj ć et al., 1996). The results presented in this paper show a relationship between the expression of sgm mRNA (secondary target) and gentamicin resistance in E. coli and M. melanospora strains. It is known that the resistance level conferred by RNA-modifying methylases is not gene dosage dependent, so a small number of molecules is enough to render ribosomes resistant to antibiotics. However, the results presented in this paper clearly demonstrate that an excess of the secondary target can titrate down the amount of Sgm methylase and thus reduce the number of available molecules necessary to obtain a high level of resistance. Experiments with the E. coli system show a direct correlation of sensitivity to gentamicin with the level of induction of the P₁ promoter by IPTG (Fig. 3). This correlation was not so obvious in M. melanospora cells, where two constitutive promoters of different strength were used – P₁, a relatively weak promoter, and P₂₉, a relatively strong one. Despite the fact that expression of the secondary target could not be controlled by induction of transcription (as it is in E. coli), experiments with the Micromonospora system also show that the stronger promoter, i.e., one that results in more of the secondary target in the cell, lowers resistance more than the weaker promoter. Moreover, the MIC of gentamicin with the P₂₉ promoter was diminished almost to the MIC value of the M. melanospora wild-type strain (3 µg/ml, as reported in Ko j ć et al., 1999). Such a strong effect in the Micromonospora strain can be attributed to structural specificity of Micromonospora 16S rRNA. The 16S rDNA sequences of numerous Micromonospora strains have been determined (Ko c h et al., 1996), and it was shown that all key nucleotides in the A site are conserved, although one interesting feature is discernible in sequences of M. melanospora and M. purpurea. Namely, these two strains have C-1409 changed to G, so that the stem beginning with the 1409-1491 base pair is disrupted. The importance of this basepair for paromomycin resistance in E. coli was demonstrated by mutagenesis of 16S rRNA and by chemical probing (De Staio et al., 1989). However, M. melanospora and M. purpurea are sensitive to paromomycin, like all other tested Micromonospora strains (Mat kovi c et al., 1984).

The obtained results should also be discussed in the light of specific binding of methylase to the 30S subunit and its own mRNA. Strong decrease of gentamicin resistance upon overexpression of 5’ UTR in E. coli might not be surprising, but the similar effect in M. melanospora was certainly unexpected. In the latter case, it would hardly be anticipated that the situation is similar to that in E. coli, since it would suggest that the binding of methylase is, even in the homologous system, stronger to its own mRNA than to 30S ribosomal subunits. Unfortunately, specificity of binding could not be confirmed in electrophoretic mobility shift assays with purified Sgm and either the mRNA or the 16S rRNA oligonucleotide model (Ilić-Tomic and Vasiljevic, unpublished results), leading to the conclusion that some other components of translational machinery are involved in recognition of both targets.

In any case, the results presented here raise the interesting question as to whether the expression of 5’ UTR in the producer Micromonospora zionensis affects resistance of this organism to gentamicin. If so, this would seem to be at odds with the fact that 16S rRNA is the primary target of Sgm; hence, the enzyme should bind to it with much higher affinity than to its own mRNA. In M. zionensis, sgm is transcribed from two promoters (Koj ć et al., 1992), so it is formally possible that M. zionensis regulates the establishment of its resistance by changing the availability of 5’ UTR via activation of the P2 promoter. Conceivably, by activating the P2 promoter (located 350 nt upstream of the start codon), M. zionensis might trap the 5’ UTR responsible for autoregulation in a secondary structure of the longer mRNA, thereby rendering it less available for Sgm.
In summary, the findings presented here clearly indicate the need for a more precise understanding of the regulatory interplay between Sgm methylase and its primary and secondary targets.

Acknowledgments — This work was supported by Grant 143056 from the Ministry of Science of the Republic of Serbia. We would like to thank Dr. Graeme Conn for carefully reading and correcting the manuscript.

REFERENCES


Vasiljevic, B., and E. Cundliffe (1990). Cloning of grm, a genta-


ОВЕРЕКСПРЕСИЈА 5′ UTR MRNK SGM ГЕНА СМАЊУЈЕ РЕЗИСТЕНЦИЈУ НА ГЕНТАМИЦИН У ЋЕЛИЈАМА ESCHERICHIA COLI И MICROMONOSPORA MELANOSPorea

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16S rRNK метилазе су експримиране у већини бактерија које произведе антибиотике да би се заштитиле од дејства антибиотиката путем метиљације 16S rRNK на позицијама које су битне за њихово дејство. Ген *sgm* који је одговоран за резистенцију на сисомицин и гентамицин у соју *Micromonospora zionensis*, метилује G1405 у оквиру А места 16S rRNA где се налази и CCGCCC хексануклеотид. Исти хексануклеотид се налази и 14 нуклеотида испред места везивања рибозома на *sgm* информационој RNK. Предложени модел транслационе регулације *sgm* гена претпоставља да се Sgm протеин везује за овај мотив како на 16S rRNK, тако и на 5′ нетранслирајућем регионалином генетичком региону (UTR) сопствене информационе RNK. 5′ UTR секвенца је оверекспримирана на *sgm* информационој RNK са скраћеним 3′ крајем и тестиран је ефекат на гентамицинску резистенцију у ћелијама *E. coli* и *Micromonospora melanosporea*. Оверекспресија ове регулаторне секвенце доводи до смањења резистенције у оба тестирана соја највероватније због титрације Sgm молекула од стране 5′ UTR-а.