Critical Residues for Cofactor Binding and Catalytic Activity in the Aminoglycoside Resistance Methyltransferase Sgm††

Miloje Savic,1 Tatjana Ilic-Tomic,2 Rachel Macmaster,1 Branka Vasiljevic,2 and Graeme L. Conn1*  

Manchester Interdisciplinary Biocentre, Faculty of Life Sciences, The University of Manchester, 131 Princess Street, Manchester M1 7DN, United Kingdom, and Institute of Molecular Genetics and Genetic Engineering (IMGGE), P.O. Box 23, 11010 Belgrade, Serbia2

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The 16S rRNA methyltransferase Sgm from “Micromonospora zionensis” confers resistance to aminoglycoside antibiotics by specific modification of the 30S ribosomal A site. Sgm is a member of the FmrO family, distant relatives of the S-adenosyl-L-methionine (SAM)-dependent RNA subfamily of methyltransferase enzymes. Using amino acid conservation across the FmrO family, seven putative key amino acids were selected for mutation to assess their role in forming the SAM cofactor binding pocket or in methyl group transfer. Each mutated residue was found to be essential for Sgm function, as no modified protein could effectively support bacterial growth in liquid media containing gentamicin or methylate 30S subunits in vitro. Using isothermal titration calorimetry, Sgm was found to bind SAM with a $K_D$ (binding constant) of 17.6 M, and comparable values were obtained for one functional mutant (N179A) and four proteins modified at amino acids predicted to be involved in catalysis in methyl group transfer. In contrast, none of the G135, D156, or D182 Sgm mutants bound the cofactor, confirming their role in creating the SAM binding pocket. These results represent the first functional characterization of any FmrO methyltransferase and may provide a basis for a further structure-function analysis of these aminoglycoside resistance determinants.

Antibiotic resistance by rRNA methylation is a frequently used mechanism among macrolide and aminoglycoside antibiotic-producing actinomycete strains (8). Members of the FmrO protein family, named after fortimicin A, the resistance methyltransferase (MT) from Micromonospora olivasterospora (25), methylate 16S rRNA and thus protect bacteria against the action of aminoglycoside antibiotics. Like the majority of MT enzymes, they are dependent upon the methyl donor S-adenosyl-L-methionine (SAM) as a cofactor.

Two 16S rRNA modifications are most commonly employed by aminoglycoside-producing actinomycetes, the guanine-N7 methylation of G1405 (m$^\text{N}$ G1405) and the adenine-N1 methylation of A1408 (m$^\text{N}$ A1408) (4, 8). To date, m$^\text{N}$ A1408 MTs were observed only among aminoglycoside producers, except for one instance of plasmid-mediated A1408 NpmA MT found in a clinically isolated Escherichia coli isolate that confers pan-aminoglycoside resistance among pathogenic bacteria (33). In contrast, a significantly increased spread of m$^\text{N}$ G1405 MTs among various gram-negative pathogens from both clinical and veterinary isolates has been observed recently (9).

Around 130 members of the SAM-dependent MT family have been classified (EC 2.1.1.X) based on their substrate specificity (small molecule, lipid, protein, or nucleic acid) and on the atom targeted for methylation. Despite sharing almost no sequence similarity with other enzymes, actinomycete MTs are remote relatives of the RNA MT subfamily of SAM-dependent MTs. However, the limited overall sequence similarity and “domain swapping” make inference about the structure of the FmrO family very difficult compared to other SAM-dependent MT families. Structural studies indicate that the SAM-dependent MTs represent a large structurally conserved superfamily, where the profound structural conservation is not reflected in a corresponding sequence conservation. As an alternative to using the extent of sequence conservation at analogous positions, MTs can be grouped based on the relative linear order of functional regions. Broadly speaking, SAM-dependent MTs comprise the following three functional domains: SAM binding, substrate recognition and binding, and catalysis of methyl transfer. The MTs differ in the relative linear order of these regions as defined by the presence of a conserved sequence (11). However, identification of putative key residues forming these conserved motifs may provide a platform to begin structure-function analyses of the actinomycete MT enzymes.

A sismicin-gentamicin aminoglycoside resistance MT gene, the sgm gene from the actinomycete “Micromonospora zionensis,” prevents self-intoxication by a mechanism that involves methylation of the intact 30S ribosomal subunit (20). Although the precise site of methylation has not been directly determined, it has been inferred to be the same site as that of another family member, KgmB, which methylates G1405 in 16S rRNA (18; T. Ilic-Tomic, I. Moric, G. L. Conn, and B. Vasiljevic, unpublished data). To date, no further biochemical characterization or structure-function analysis of Sgm or any other FmrO family member (Fig. 1A) has been conducted. Here, we have used amino acid conservation within resistance MTs across 13 aminoglycoside-producing actinomycete strains to direct a mutagenesis strategy to
To characterize the SAM cofactor binding and catalytic properties of Sgm using both in vivo and in vitro assays of enzyme function.

MATERIALS AND METHODS

Targeted site-directed mutagenesis of Sgm. Conserved amino acids were identified by alignment of 13 MT sequences from aminoglycoside-producing actinomycete strains using ClustalW (32) and further analysis using MUSCLE (10). Conserved residues were plotted on an Sgm homology model (Fig. 1 and see supplemental material) using the ConSurf algorithm (21) and amino acids (Table 1) selected for mutation to alanine by QuikChange II site-directed mutagenesis (Stratagene). The presence of each target mutation and the absence of further mutations or indels in either the coding sequence or promoter were confirmed by automated DNA sequencing. All mutant Sgm proteins were expressed in E. coli isolates in a soluble form at sufficient levels to allow extraction and purification for in vitro analysis.

Determination of aminoglycoside MICs. Gentamicin and kanamycin MICs were measured in triplicate in liquid culture for E. coli strains DH5α and BL21(DE3) harboring plasmid-encoded wild-type and mutant Sgm proteins. Initial cultures were grown from a single colony in LB medium supplemented with 100 μg/ml ampicillin to an optical density at 600 nm (OD600) of 0.7 to 0.8. A total of 100 μl of this culture was used to inoculate 10 ml of LB medium with 100 μg/ml of ampicillin and various concentrations of gentamicin or kanamycin in the range of 0 to 1,500 μg/ml. These cultures were grown at 37°C for a further 16 h, at which time a final OD600 measurement was taken and the MIC was recorded.

<table>
<thead>
<tr>
<th>Proposed function</th>
<th>Protein (mutation)</th>
<th>Gentamicin MIC (μg/ml)</th>
<th>SAM K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Empty vector</td>
<td>&lt;10</td>
<td>17.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Sgm</td>
<td>&gt;1,500</td>
<td>14.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Sgm (N179A)</td>
<td>&gt;1,500</td>
<td>14.1 ± 1.4</td>
</tr>
<tr>
<td>SAM binding</td>
<td>Sgm (G135A)</td>
<td>15</td>
<td>No binding</td>
</tr>
<tr>
<td></td>
<td>Sgm (D156A)</td>
<td>&lt;10</td>
<td>No binding</td>
</tr>
<tr>
<td></td>
<td>Sgm (D182A)</td>
<td>15</td>
<td>No binding</td>
</tr>
<tr>
<td>Catalytic activity</td>
<td>Sgm (K199A)</td>
<td>30</td>
<td>5.3 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sgm (E205A)</td>
<td>50</td>
<td>7.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Sgm (R236A)</td>
<td>60</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Sgm (E267A)</td>
<td>30</td>
<td>5.9 ± 1.7</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>Sgm (P191S)</td>
<td>&gt;1,500</td>
<td>5.9 ± 1.7</td>
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<tr>
<td></td>
<td>Sgm (D193Y)</td>
<td>&gt;1,500</td>
<td>5.9 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values from fit with fixed stoichiometry (N = 1).
determined as the minimal concentration of gentamicin or kanamycin that pre-
vents bacterial growth. Bacteria lacking a plasmid or those transformed with an
empty vector were used as negative controls. Measurements were made with and
without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction of protein
expression with no effect on the MIC observed.

Sgm protein expression and purification. N-terminal His6-tagged wild-type
and mutant Sgm proteins were expressed in E. coli BL21(DE3) as previously
described (19). Protein expression was induced with 1 mM IPTG at an OD600 of
0.6 to 0.8, and growth continued for 3 to 4 h at 37°C. Cell pellets were resus-
pended and incubated for 1 h in lysis buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM MgCl2, 10% glycerol, and 10 mM imidazole) containing 1 mg/ml
lysozyme, protease inhibitor cocktail (Sigma), and 0.1 mM phenylmethylsulfonyl
fluoride before being fully broken by sonication. Sgm proteins were purified by
metal ion affinity chromatography under native conditions (Ni2+-nitrilotriacetic
acid agarose; Qiagen) in a batch, followed by cation-exchange and gel filtration
chromatography using HiPrep 16/10 SP FF and HiPrep 26/60 Sephacryl S-100
columns (GE Healthcare) on an AKTApurifier system. Cation-exchange chro-
matography was performed in 20 mM HEPES, pH 7.5, 10% glycerol buffer, eluting Sgm by using a linear gradient of 1 M NaCl in the same buffer. Gel filtration chromatography was performed using 20 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM MgCl2, 10% glycerol, 5 mM 2-mercaptoethanol. Purified proteins
were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
and visualization with Coomassie blue staining. The identity of wild-type Sgm
was confirmed by mass spectrometry following in-gel trypsin digestion of the excised
sodium dodecyl sulfate-polyacrylamide gel electrophoresis band.

CD spectroscopy. Proteins (6 to 15 μM) were dialyzed into a solution con-
taining 20 mM sodium phosphate buffer, pH 7.5, and 150 mM NaF. Circular
dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter
equipped with a Peltier element temperature controller, using a 0.01-cm light
path quartz cell (Hellma). Each protein CD spectrum represents the average of
10 accumulations recorded between 190- and 260-nm wavelengths, with 0.2-nm
resolution, 0.5-nm bandwidth, 4-s response time, 100-millidegree sensitivity, and
a scan speed of 10 nm/min. Sgm unfolding experiments were collected over the temperature range of 5 to 85°C in increments of 5°C. Background reference
spectra were recorded before and after sample spectrum recording.

The background-corrected spectrum of the wild-type Sgm at 20°C was ana-
yzed using the K2D (16) and SELCON3 (30) algorithms via Dichroweb (http:
//www.cryst.bbk.ac.uk/cdweb/html/home.html). For a comparison to the experi-
mental estimates of Sgm secondary structure content, the predicted secondary
structure was also calculated from the Sgm protein sequence using the method of
Garnier et al. (15) and Jpred (7) and from the Sgm homology model with the pro-
gram STRIDE (13).

In vitro MT activity assays. Methylation reaction mixtures contained 10 mM
HEPES-KOH, pH 7.5, 10 mM MgCl2, 50 mM NH4Cl, 5 mM 2-mercaptoethanol,
1 mM [3H]SAM (15 Ci/mM; Amersham-Pharmacia), 500 units/ml RNasin
(Ambion), 20 pmol 30S ribosomal subunits, and 5 μg of Sgm proteins (or the equivalent volume of cell extract from cells with an empty vector as the negative control). Incubation was carried out at 35°C for 40 min with aliquots removed at
0, 10, 20, 30, and 40 min. The reaction was stopped by adding ice-cold 5%
trichloroacetic acid. The samples were allowed to precipitate at 0°C for 10 min
and were collected on GF/C filters (Whatman). The filters were suspended in
Ultima Gold F scintillation fluid (Perkin Elmer), and the incorporated radioac-
tivity was measured using a scintillation counter (1219 RackBeta liquid scintil-
lation counter; LKB Wallac). Each assay was performed at least twice using
independent preparations of protein.

RESULTS

Identification of putative key residues for cofactor binding and methyl group transfer. Conserved residues in Sgm were identified based on a sequence alignment of all complete an-
notated open reading frames of FmrO family members and mapped onto an Sgm homology model using ConSurf (21) (Fig. 1). Within the C-terminal domain, a conserved cluster of residues was identified that might form a pocket for binding SAM cofactor. Putative key residues for methyl group transfer (and/or target site recognition) were also identified in the C-
terminal domain. Finally, two further clusters of conserved basic/charged residues were identified within the N-terminal domain that we hypothesize are important for target site rec-
nognition and binding (Fig. 1B and C). To test these functional predictions, we chose to focus on residues identified as poten-
tially being involved in cofactor binding or methyl group trans-
fer (see Discussion for comments on the likely complexity of target recognition). Each of these residues (Table 1) was muta-
ted to alanine by site-directed mutagenesis, and Sgm mutants
were initially selected by streaking colonies in parallel on LB
agar containing either ampicillin or gentamicin. A lack of
growth on gentamicin was observed for all targeted mutants,
defecting a lack in MT activity. During this process, a fur-
ther two spontaneous mutants defective in growth on LB
agar containing gentamicin were identified (Table 1). Curiously,
however, these mutants could support growth in liquid medium
at levels similar to the wild-type protein and were therefore not
analyzed further.

Analysis of Sgm protein expression and folding. Wild-type Sgm and mutant proteins were successfully expressed and puri-
fied according to procedures previously established for the wild-type enzyme (19). CD spectroscopy was first used to as-
sess the secondary structure and thermostability of the wild-
type Sgm protein. CD spectra can be deconvoluted, by com-
parison to large data sets of protein CD spectra, in order to
obtain an experimental estimate of the protein secondary
structure content (16, 30). The Sgm CD spectrum (Fig. 2) was

FIG. 2. CD spectroscopy analysis of Sgm. CD spectrum of purified
wild-type Sgm collected at 20°C and used for analysis of protein sec-
secondary structure content via Dichroweb (Table 2), deg., degrees.
thus used to determine the protein secondary structure content using Dichroweb, giving an estimated α-helical content of 40 to 46%. The values obtained agree well with both the predicted secondary structure from the Sgm amino acid sequence and the homology model structure (Table 2). The CD spectrum obtained for Sgm therefore indicates the protein is folded and that it has a structure consistent with our model.

Sgm thermostability was examined by collecting CD spectra over the range of 5 to 85°C in 5°C increments. Sgm is fully stable up to 45 to 50°C, after which point the spectra become progressively more like those of an unstructured random coil (data not shown). No further change is observed beyond 65°C, where all secondary structure appears to be lost. The temperature-induced melting of α-helical secondary structures, monitored at 220 nm, showed an increase in molar ellipticity beyond 45°C, indicating a rapid disorder in the molecular structure after this point.

Before beginning a detailed analysis of the function of mutant Sgm proteins, we wished to eliminate the possibility that any of the mutations introduced might cause major misfolding and/or degradation of Sgm. Each mutated protein was successfully expressed in E. coli BL21(DE3) and purified under conditions identical to those of the wild-type Sgm. CD spectra were recorded for each mutant protein (see Fig. S1 in the supplemental material). Although some small differences were observed, the CD spectrum for each mutant indicated it was a folded protein with a structure similar to that of the wild-type Sgm. None of the mutants displayed gross changes in the CD spectrum similar to those obtained in the thermally induced unfolding experiments with the wild-type protein.

**Measurement of MIC in liquid culture.** The function of wild-type and mutant Sgm proteins was assessed in vivo by determination of MICs in liquid cultures of E. coli transformants containing an sgm-carrying plasmid (Table 1). The wild-type Sgm conferred resistance to both of the 4,6-disubstituted 2-deoxystreptamines tested (gentamicin and kanamycin) with a MIC of >1,500 μg/ml. Each protein containing an alanine mutation at one target residue within the Sgm C-terminal domain was classified into one of the following two groups according to the observed phenotype: mutations that had no apparent phenotype and mutations that had a dramatic influence on cell survival in the presence of gentamicin (similar results were obtained with kanamycin). The mutation of all residues that were implicated in either cofactor binding or methyl group transfer resulted in a severely gentamicin-sensitive phenotype (Table 1). Only one mutation to alanine of a nonconserved amino acid (N179) near the proposed SAM binding pocket produced a protein with a wild-type phenotype (Table 1).

![FIG. 3. In vitro methylation activities of wild-type and mutant Sgm proteins.](image_url)

**TABLE 2. Prediction and experimental estimation of Sgm secondary structure**

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>α-Helices</th>
<th>β-Sheets</th>
<th>Loops and coils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garnier</td>
<td>43.8</td>
<td>20.5</td>
<td>24</td>
</tr>
<tr>
<td>Jpred</td>
<td>45</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Stride</td>
<td>40.5</td>
<td>27.7</td>
<td>31.8</td>
</tr>
<tr>
<td>K2D</td>
<td>46</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>SELCON3</td>
<td>40.2</td>
<td>13.6</td>
<td>18.6</td>
</tr>
</tbody>
</table>

a Garnier et al. (15) and Jpred provide secondary structure content predictions based on protein sequence. Stride assigns secondary structure content to known structures or models, and K2D and SELCON3 allow deconvolution of protein CD spectra for experimental estimation of secondary content (see Materials and Methods for details).
Methylation assay of 30S ribosomal subunits. The MT activity of wild-type and mutant Sgm proteins was assessed by an in vitro assay of 30S ribosomal subunit methylation with \[^{3}H\]SAM cofactor (Fig. 3). The reaction resulted in the transfer of the tritiated methyl group to the 16S rRNA component of the 30S subunit isolated from the *E. coli* BL21(DE3) strain ("sensitive 30S"). In vivo-methylated 30S subunits, isolated from bacteria harboring a plasmid encoding wild-type Sgm ("resistant 30S") were not further in vitro methylated by Sgm (Fig. 3A). A further negative control, the addition of extract from *E. coli* BL21(DE3) without Sgm-encoding vector, provided a measure of the background incorporation for all experiments. The wild-type Sgm was able to efficiently methylate sensitive 30S subunits (Fig. 3A), but none of the mutant proteins showed activity above the background. For mutations targeted to both putative SAM binding (Fig. 3B) and catalytic (Fig. 3C) residues, the rate of tritiated methyl group incorporation was the same as that for the background (negative control).

Measurement of SAM binding by ITC. To test whether the functional defect observed in the putative cofactor pocket mu-
tants (G135A, D156A, and D182A) was indeed due to a deficiency in SAM binding, we used ITC to measure the thermodynamics of binding (Table 1; Fig. 4). The wild-type Sgm and a protein mutated at a variable residue near the proposed binding site (N179A) showed clear binding to SAM, with an affinity in the low micromolar range. The fits for both titrations produced reasonable stoichiometries, with $N$ equal to 1.07 $\pm$ 0.7 and 0.89 $\pm$ 0.3 for the wild-type and N179A Sgm, respectively, indicating one SAM molecule bound per protein. Each of the proteins mutated at potential catalytic residues (K199A, E205A, R236A, and E267A) also retained SAM-binding affinity (Table 1). In contrast, none of the proteins mutated at putative key residues for cofactor binding (G135A, D156A, and D182A) showed detectable binding of SAM, confirming their role in creating the cofactor binding pocket.

**DISCUSSION**

Aminoglycoside antibiotics comprise a structurally varied family of polycationic bactericidal agents that interfere with protein translation by mimicking the conformational change in 16S rRNA induced by correct codon-anticodon pairing (24). Mutations or modifications, such as methylation, that perturb hydrogen bonding between aminoglycosides and target residues or that generate steric clashes within the A site result in a lowered affinity for the antibiotic for the A site and can lead to bacterial resistance.

To provide a structural platform for functional characterization of the sisomicin-gentamicin resistance MT Sgm, we employed a range of computational structure modeling and sequence analysis methods. The model shows a clear division of Sgm into two major structural domains separating the N- and C-terminal amino acids. The 164 C-terminal amino acids form a Rossmann-like MT fold, as expected for an MT enzyme, and the structure closely resembles a previous model of this domain constructed for a homologous MT from *Streptomyces kanamyceticus* (5). The complete Sgm protein model was experimentally evaluated by analysis of the protein CD spectrum, which showed good correspondence between predicted and observed secondary structure content. We next incorporated amino acid conservation across all FmrO family members by mapping these onto the homology model. An initial classification was made for three clusters of conserved residues as potential key residues for SAM binding, catalysis, or target recognition.

Amino acids G135, D156, and D182 were identified as potentially forming the SAM binding pocket and were mutated to alanine. All three mutations abrogated Sgm activity as measured in vivo by growth of *E. coli* in liquid media and by in vitro methylation assays of 30S substrate. To confirm the role of these residues in SAM cofactor binding, the affinity for each protein was assessed by ITC. The wild-type Sgm bound SAM with a dissociation constant, $K_{sp}$, of 17.5 $\mu$M, and a similar value of 14.2 $\mu$M was obtained for the functional N179A mutant. This lies within the range of known affinities for SAM-dependent MTs (0.1 to 19 $\mu$M) (22, 23, 26, 31) and is comparable to that of another RNA MT, RsmC (SAM $K_{sp}$, 4.8 $\mu$M), which methylates G1207 in 16S rRNA (31). It is also similar to the affinity for the cofactor measured by other methods for RsmD and RlmE (SAM $K_{m}$, $\sim$26 $\mu$M), both endogenous MTs from *E. coli* that also recognize highly structured substrates in 30S and 50S ribosomal subunits, respectively (1, 3). In contrast, no binding of SAM could be detected for the G135, D156, and D182 mutants. Based on a survey of various MTs of known structure and the roles of equivalent residues in these proteins (11, 12, 17, 28, 31), we speculate that D156 and D182 may position the adenine component of SAM via contacts with the ribose hydroxyl groups and adenine base, respectively, and G135 may directly influence the positioning of the methyl group. As each position is extremely highly conserved, whatever their precise roles, we infer that these positions and those around them in the Sgm model will fulfill the same functions across the entire FmrO family of enzymes.

We further hypothesized that residues K199, E205, R236, and E267 should have a role in methyl group transfer. Each residue was again mutated to alanine, and the function of the mutated protein was tested in vivo and in vitro. As before, MICs for gentamicin were dramatically reduced (20- to 50-fold) and MT activity was abolished for each mutant enzyme. These proteins were, however, still competent in SAM cofactor binding. Together, these results support the hypothesis that this group of amino acids contributes directly to Sgm catalytic function, though it is not possible to eliminate a potential additional role in substrate recognition.

The precise site of methylation has not been determined but has been inferred indirectly to be G1405 in 16S rRNA by virtue of the protection against further methylation of 30S subunits by Sgm following in vivo protection by the methylated 1405 MT KgmB (18; Ilic-Tomic et al., unpublished). The methylation reaction is highly substrate specific and requires intact 30S ribosomal subunit (20), as has been observed for two endogenous *E. coli* MTs, RsmE (3) and YebU (2). This implies that recognition of the target site is at least partially related to the subunit structure around the target nucleotide. It is also possible that docking to ribosomal proteins, such as the nearby S12, in addition to RNA is also necessary. Our model and analysis of conserved amino acids indicate that the N-terminal part of Sgm is likely to be involved in target site recognition. However, in addition to these uncertainties with regard to recognition mechanism, there is also a precedence for modifications of MT residues involved in target recognition having only mild or moderate phenotypes (22, 31), making detailed analysis of their roles difficult.

The aminoglycoside resistance spectrum for Sgm was previously assessed only in homologous host strains, i.e., in high-G+C bacteria such as *Streptomyces lividans* and *Micromonospora melanospora*. The present study therefore provides a first demonstration that a high level of resistance can be achieved by expression of a gene from an aminoglycoside producer in a heterologous host, the gram-negative bacterium *E. coli*. Aminoglycoside resistance determinants such as Sgm can be easily transferred to pathogenic strains by means of mobile genetic elements (14), making a complete understanding of their structures, activities, and target recognition essential. We have demonstrated that homology-based analysis coupled with mutational analysis of Sgm can provide a platform for a detailed structure-function analysis of these enzymes prior to the determination of the high-resolution X-ray crystallographic structure(s) of FmrO family members. These approaches could ultimately provide a deeper understanding of how FmrO MT enzymes recognize their rRNA/ribosomal
protein binding site(s) on the 30S subunit with such exquisite specificity.

ACKNOWLEDGMENTS

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