5420–5431 *Nucleic Acids Research, 2009, Vol. 37, No. 16* *Published online 9 July 2009*
doi:10.1093/nar/gkp575

Determination of the target nucleosides for members of two families of 16S rRNA methyltransferases that confer resistance to partially overlapping groups of aminoglycoside antibiotics

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Received April 7, 2009; Revised June 19, 2009; Accepted June 22, 2009

ABSTRACT

The 16S ribosomal RNA methyltransferase enzymes that modify nucleosides in the drug binding site to provide self-resistance in aminoglycoside-producing micro-organisms have been proposed to comprise two distinct groups of *S*-adenosyl-L-methionine (SAM)-dependent RNA enzymes, namely the Kgm and Kam families. Here, the nucleoside methylation sites for three Kgm family methyltransferases, Sgm from *Micromonospora zionensis*, GrmA from *Micromonospora echinospora* and Krm from *Frankia* sp. Ccl3, were experimentally determined as G1405 by MALDI-ToF mass spectrometry. These results significantly extend the list of securely characterized G1405 modifying enzymes and experimentally validate their grouping into a single enzyme family. Heterologous expression of the KamB methyltransferase from *Streptoalloteichus tenebrarius* experimentally confirmed the requirement for an additional 60 amino acids on the deduced KamB N-terminus to produce an active methyltransferase acting at A1408, as previously suggested by an *in silico* analysis. Finally, the modifications at G1405 and A1408, were shown to confer partially overlapping but distinct resistance profiles in *Escherichia coli*. Collectively, these data provide a more secure and systematic basis for classification of new aminoglycoside resistance methyltransferases from producers and pathogenic bacteria on the basis of their sequences and resistance profiles.

INTRODUCTION

The aminoglycoside antibiotic streptomycin was the first drug discovered through systematic screening of natural products active against *Mycobacterium tuberculosis* in 1944 (1). In the following decades, many aminoglycosides were isolated from soil bacteria, primarily the Gram-positive actinomycetes of the genera *Streptomyces* and *Micromonospora* (2,3). These antibiotics comprise a structurally varied family of poly-cationic compounds with a central aminocyclitol ring, most frequently 2-deoxystreptamine or streptamine, connected via glycosidic bonds to amino sugars. The numerous hydroxyl and primary amine groups of these substituents give aminoglycosides their overall positive charge and, based on their position, define three distinct structural classes of drug. The 4,6-disubstituted 2-deoxystreptamines (4,6-DOS) include kanamycin and most clinically useful aminoglycosides, such as gentamicin, tobramycin and amikacin. The same core may alternatively be 4,5-disubstituted (4,5-DOS) as in the aminoglycosides neomycin and paromomycin, while the final group of compounds consists of those that do not fit into either of these groups, such as apramycin, streptomycin, hygromycin B and spectinomycin.

Various strategies have evolved in aminoglycoside antibiotic-producing micro-organisms to prevent self-intoxication, including mechanisms to decrease intracellular

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drug concentration, or modify either the target site or the drug itself, and multiple mechanisms can commonly be found operating simultaneously in the cell (4). Resistance by 16S rRNA methylation, accomplished by *S*-adenosyl-L-methionine (SAM)-dependent rRNA resistance methyltransferases (MTs), is a frequently occurring mechanism alongside drug modification in the aminoglycoside-producing actinomycetes (5,6). Modifications at two sites, G1405 and A1408, are thought to be incorporated by two different methyltransferase protein families (6–9). However, these sites of modification have only been experimentally determined as G1405 for KgmB from *Streptoalloteichus tenebrarius* (10), formerly classified as *Streptomyces tenebrarius* (11), and A1408 for KamA (also known as IrmA) and KamC from *Streptomyces tenjimariensis* and *Saccharopolyspora hirsuta* respectively (5,9). Methylation sites have also been identified for functionally equivalent methyltransferases from isolates of bacterial pathogens, as G1405 for ArmA and RmtB, and A1408 for NpmA (12–14). Typically, activity for other MTs has been inferred indirectly by their inability to further methylate ribosome subunits already protected by one of these enzymes (15). Furthermore, although it is clear that these base methylations can confer high-level resistance to specific combinations of aminoglycoside antibiotics (5), despite their close proximity the action spectra of each does not entirely overlap and few systematic studies have been performed to date. The emergence in the last decade of several plasmid-mediated G1405 MTs among pathogenic Gram-negative rods from both clinical and veterinary settings (16,17) and one identification of a novel A1408 resistance MT from pathogens (14), make thorough analysis of these resistance MT enzymes, methylation targets and their conferred action spectra essential.

Recently, the limited biochemical data on actinomycetes' G1405 MTs were enhanced by functional probing of Sgm, the sisomicin-gentamicin aminoglycoside resistance MT from *Micromonospora zionensis*. These studies demonstrated the existence of two structural domains within Sgm (18), and three functional classes of amino acids responsible for SAM binding, target recognition and methyl group transfer (19). Here, we describe experimental identification using mass spectrometry (MS) of the methylation site on 16S rRNA for Sgm and two further enzymes from other actinomycetes genera, GrmA from *Micromonospora echinospora* (formerly known as *Micromonospora purpurea*), and the MT from *Frankia* sp. CcI3, for which we will use the gene abbreviation *krm* (kanamycin resistance MT) (Figure 1). Comparison of antibiotic resistance patterns between Kgm and Kam family MTs unambiguously identifies functional differences correlating with modification at G1405 and A1408 in 16S rRNA.

MATERIALS AND METHODS

Phylogenetic analysis of different methyltransferase families

Unique open reading frames (ORFs) of resistance MTs were used to infer phylogenetic relationships within MT groups proposed to modify G1405 and A1408. Amino acid sequences were aligned using MUSCLE (20). Maximum likelihood (ML) phylogenetic trees were calculated using PHYML (21,22), and the consensus tree was calculated from 1000 ML trees by the bootstrap method of Felsenstein (23).

Over expression and purification of resistance methyltransferases

Construction of expression vectors for Sgm (24) and KgmB (25) was described previously. DNA for other enzymes were ligated into pQE-30 (Qiagen) following either PCR amplification of genomic DNA (*grmA*) or total gene synthesis (*krm* and *kamB*; GeneArt). All MTs were expressed as 6×His-tagged proteins in *Escherichia coli* BL21(DE3) and natively purified by Ni^{2+} affinity chromatography (Ni^{2+}-NTA Agarose; Qiagen) as previously described for Sgm (19). The identity of each MT protein was confirmed by MS following in-gel trypsin digestion of the excised SDS–PAGE bands (data not shown).

Measurement of aminoglycoside MIC values in liquid culture

Aminoglycoside minimal inhibitory concentrations (MIC) were measured in triplicate in liquid culture for *E. coli* strain BL21(DE3) harboring plasmid-encoded resistance methyltransferase proteins as previously described (19). Concentrations of gentamicin, kanamycin, neomycin, paromomycin, apramycin, hygromycin B and streptomycin were varied in the range of 0 to 1000 μg/ml. MIC values are defined as the minimal concentration of antibiotic that prevents bacterial growth. Bacteria lacking a plasmid or those transformed with an empty vector were used as negative controls.

In vitro methylation of ribosomal subunits

Small ribosomal subunits were purified as previously described (26). Methylation reaction mixtures contained 10 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM 2-mercaptoethanol, 1 mM SAM, 100 pmol 30S ribosomal subunits and 100 ng of MT proteins. Incubation was carried out at 37°C for 60 min. The reactions were stopped by adding phenol–chloroform to extract ribosomal proteins and MT enzyme, and 16S rRNA was recovered by ethanol precipitation.

Matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) MS analysis of methylated 16S rRNA

16S rRNA was isolated from 30S particles previously *in vitro* methylated (as described above), or from *E. coli* BL21(DE3) cells expressing recombinant Sgm protein (i.e. *in vivo* methylated). A defined 16S rRNA sequence, C1378-G1432, was isolated by hybridization to a complementary deoxyoligonucleotide (27). 16S rRNA (100 pmol) was incubated with deoxyoligonucleotide (1000 pmol) at 95°C for 5 min in 200 μl hybridization buffer (250 mM HEPES pH 7.5, 500 mM KCl), followed by slow cooling to 30°C over 3 h. Unhybridized regions

of the 16S rRNA were digested with 40 U mung bean nuclease (NEB) and 0.5 μg RNase A (Sigma), for 60 min at 37°C. The resulting DNA–RNA hybrid was recovered by phenol–chloroform extraction and ethanol precipitation. The target RNA sequence was excised from a 13% polyacrylamide gel containing 7 M urea, after visualization with ethidium bromide, and purified using an RNaid Spin Kit (MP Biochemicals). 3-hydroxypicolinic acid (3-HPA; 0.5 M, 1 μl) was added to 2 μl of the purified 55-mer RNA (30–40 pmol), and this digested with 40 U of RNase T1 for 16 h at 37°C. The resulting 3′-cyclic phosphates were hydrolysed by adding 0.25 vol of 0.5 M HCl and incubating samples for 30 min at room temperature. Samples were air dried and redissolved in H_2O. In some cases, the RNA fragments were further purified using Zip Tip^{TM} C18 according to the manufacturer's instructions (Millipore). Between 10 and 30 pmol of the RNase T1 digest in 0.7 μl H_2O was mixed with 0.7 μl saturated 3-HPA in 50% acetonitrile/0.03 M di-amonium citrate and spotted on MALDI target plates. Mass spectra were recorded in positive ion and either linear or reflectron mode on a Kratos Shimadzu CFR MALDI-ToF MS as previously described (28).

RESULTS

Phylogenetic relatedness of aminoglycoside resistance 16S rRNA methyltransferases

Aminoglycoside resistance MTs form a distinct group within the SAM-dependent RNA methyltransferase superfamily. Based on sequence similarity, all MTs proposed to methylate G1405 on 16S rRNA are grouped in the Pfam database as 16S rRNA MT with FmrO domain (29). Within this group, MTs originating from both G+C-rich and G+C-low bacteria (Gram-positive and Gram-negative) are clustered together irrespective of their phylogenetic relationships. To date, in addition to nine distinct sequences from the actinomycetes, five unique enzymes have been identified in *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp (30), and together are classified as the Arm MTs (*rmtA, rmtB, rmtC, rmtD* and *armA*). We analysed amino acid sequence relatedness across this group of enzymes and identified a general division between G1405 MTs from the actinomycetes producers and those found in pathogens (Figure 1A). However, two enzymes from the actinomycetes *Micromonospora* and *Frankia* genera, FmrO and Krm, respectively, were found to cluster with MTs originating in pathogenic Gram-negative species. This observation supports the previous suggestion that pathogens may have acquired their resistance MTs from unidentified Gram-positive micro-organisms via horizontal gene transfer (31,32).

In contrast, only a single example of a plasmid-mediated A1408 MT resistance enzyme (NpmA) from clinically isolated *E. coli* (14) has been identified in addition to the three distinct Kam MTs from the actinomycetes (5,9) (Figure 1B), although it is highly likely that further examples of this group will be identified in the future. Since the evolutionary relationship of MT enzymes from producers and pathogens in both groups is not clear, we previously proposed classification into four distinct subfamilies based on their origin and target (31): 'Kgm' family (kanamycin–gentamicin MTs) in producers and 'Arm' family (aminoglycoside resistance MTs) in pathogens methylating G1405, and 'Kam' family (kanamycin–apramycin MTs) in producers and 'Pam' family (pan-aminoglycoside resistance MTs) in pathogens methylating A1408 residue. This classification is supported by our maximum likelihood analysis of sequence phylogenies (Figure 1A and B). Two Kam MTs, CmnU and Kmr, belong to the same clade as NpmA originating from the pathogenic bacteria (Figure 1B), suggesting a possible acquisition route for Pam MTs in pathogens. However, for these two Kam MTs methylation sites are currently unknown (33,34).

Identification of the Sgm target nucleoside in 16S rRNA

Unmethylated and *in vivo* methylated 16S rRNA was isolated from *E. coli* 30S ribosomal subunits and analysed by MALDI-ToF MS to detect changes in methylation patterns. MALDI-ToF MS can measure masses of small RNAs with accuracy well below 150 p.p.m., and thus the presence or absence of a methyl group can be readily identified by a mass difference of 14.02 Da. The preferred RNA size for analysis is in the trinucleotide to dodecanucleotide range. Therefore, molecules as large as 16S and 23S rRNAs require prior digestion with specific RNases to yield fragments of suitable sizes (28). Fragmentation was achieved by hybridization of a complementary deoxyoligonucleotide to the 55 nt sequence from C1378 to G1432 in 16S rRNA, digestion with nucleases and denaturing PAGE isolation of the resulting 55-nt RNA. This was followed by digestion of the purified RNA fragment with RNase T1 to generate the final short fragments for analysis.

Comparison of RNase T1 fragment m/z signals from unmethylated and *in vivo* Sgm methylated 16S rRNAs reveals that the spectra are very similar, with only two specific differences. Peaks at m/z 1307.22 and 3197.41, that correspond to fragments 1402-C^{2m}CCG-1405 and 1406-U^{m}CACACCAUG-1415, respectively, are observed in the unmethylated RNA spectrum but are completely absent for the methylated sample (Figure 2A and B). In contrast, the methylated RNA spectrum contains a peak at m/z 4487.57 that is not observed in the unmethylated RNA (Figures 2 and 3). These observations are entirely consistent with methylation of G1405 by Sgm. The introduction of the methyl group on G1405 would render this position resistant to RNase T1 cleavage (35), resulting in a composite fragment for nts 1402–1415. This sequence from 16S rRNA has three methyl groups originating from 'house-keeping' MTs that are located on C1402 (dimethylated) and C1407 (36) (Figure 1C). These methylations are also observed in the RNA from *E. coli* not transformed with Sgm-encoding plasmid, and no RNA unmethylated at these positions is observed (see peak 3′, 3 and 8 in Figure 2A). Thus, expression of Sgm results in the sequence 1402-C^{2m}CCG$^{(m)}$UC^{m}ACACCA

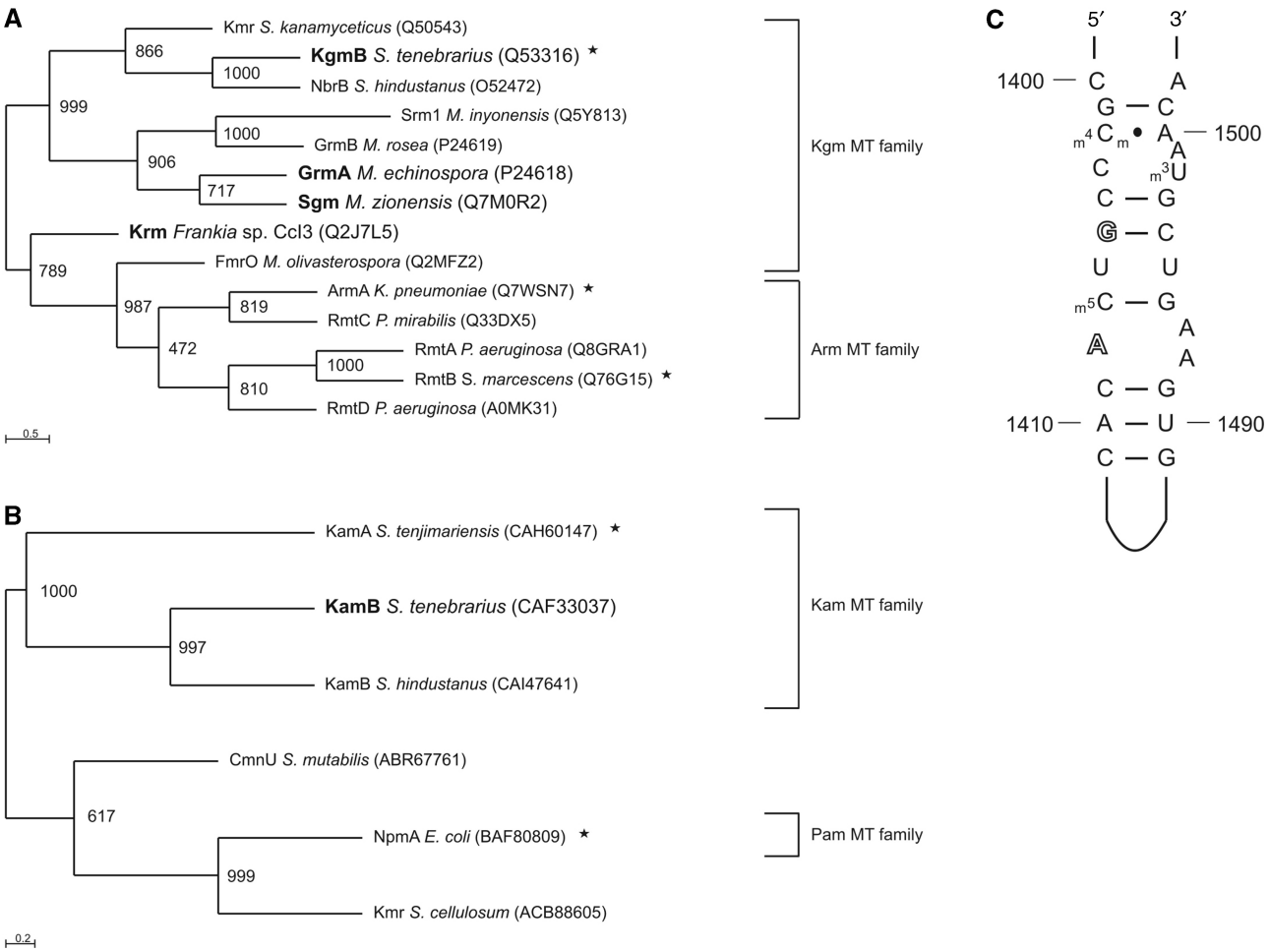
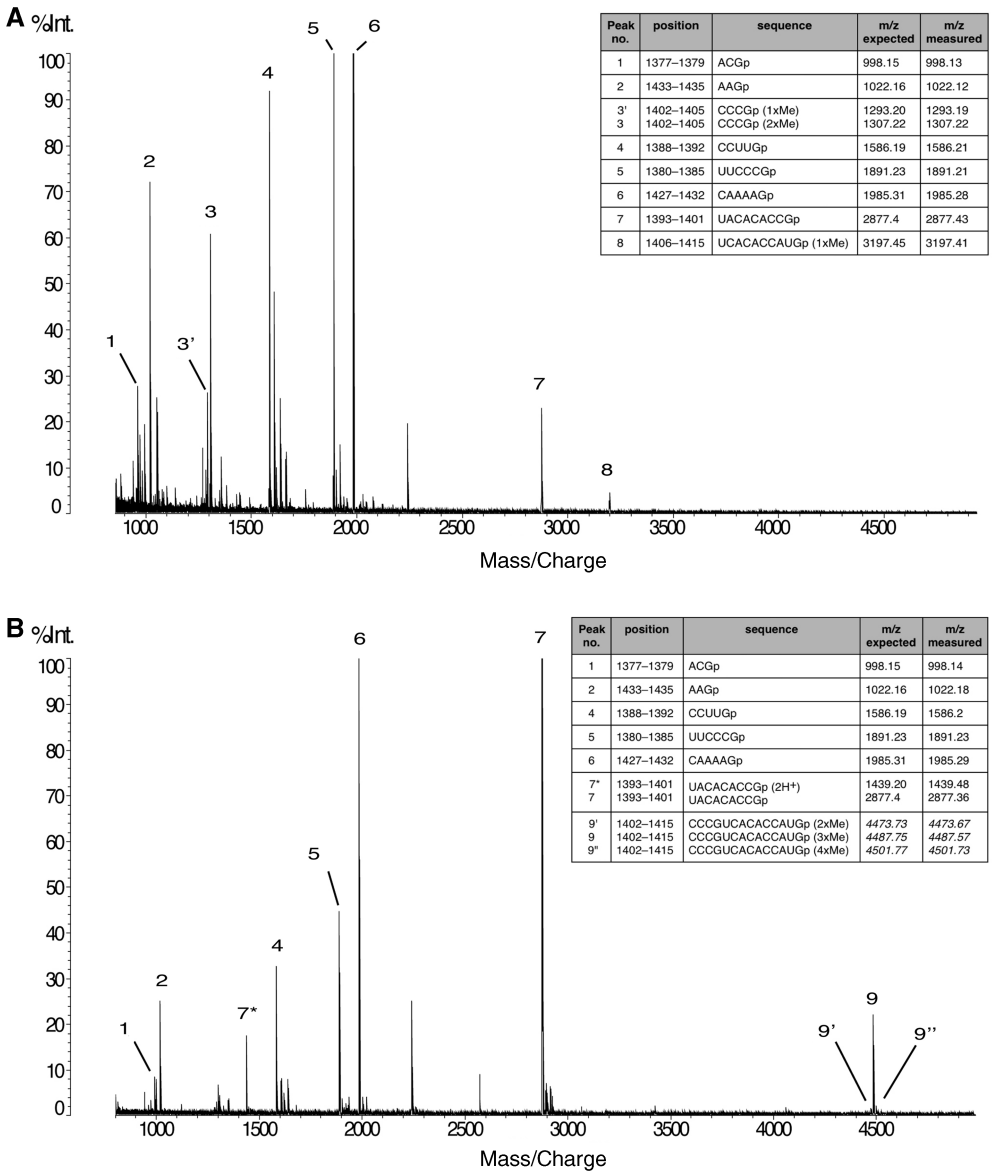


Figure 1. Phylogenetic relationship of 16S rRNA aminoglycoside resistance methyltransferase families. Consensus maximum likelihood phylogenetic trees for proposed and confirmed (denoted asterisk) (**A**) G1405 methyltransferases (Kgm and Arm families), and (**B**) A1408 methyltransferases (Kam and Pam families). Bootstrap support is noted for each node, the bar represents amino acids substitutions per position. Methyltransferases analysed in this study are shown in bold with all protein sequence accession numbers in the Pfam database (29) given in parenthesis after the strain from which it was isolated. (**C**) Sequence and secondary structure of the 16S rRNA A-site surrounding the G1405 and A1408 target sites (shown in outline font) for aminoglycoside antibiotic resistance methyltransferases.

UG-1415, where the additional methylation in parenthesis is the confirmed modification by Sgm. The measured m/z of 4501.73 for the largest ion correlates very well with the theoretical m/z of 4501.80 for a tetramethylated sequence (Figures 2A and 3C). Our data therefore confirm both these *E. coli* modifications and that Sgm methylates G1405.

The major peak in the spectrum does not, however, correspond to this most heavily modified fragment but has an m/z of 4487.57, corresponding to a trimethylated RNA (theoretical m/z 4487.78). This ion could result from the loss of any one of the four methyl groups during the MALDI process and is typical for measurements in the reflectron ToF mode (36). Indeed, in addition to the minor peak corresponding to four methylations at m/z 4501.73, the major peak is accompanied by an additional minor peak (Figure 3C) that corresponds to a dimethylated fragment (m/z 4473.67), i.e. the loss of two methyl groups (theoretical m/z 4473.76). To examine whether the triply and doubly methylated RNAs might be formed during the measurement from a 4-fold methylated precursor, we examined the loss of methyl groups from the fully methylated composite 1402-1415 RNA fragment (m/z 4501.73) in a post-source decay (PSD) experiment (Figure 4). The ion gate was set to allow only ions from m/z 4500–4560 (shaded region in the spectra of Figure 4) into the field-free drift region of the ToF tube with the laser intensity increased to cause a higher degree of laser-induced dissociation (LID). As expected, the parent ion was measured at m/z 4501.46 and, in this mode of operation, all smaller ions must be fragments derived from this ion. A strong fragment measured at m/z 4445.55, resulting from the loss of all four methyl groups from the composite 1402–1415 RNA fragment was observed (Figure 4B). This supports our hypothesis that the heterogeneity in the data for the composite RNA fragment results from the loss of methyl group(s) during the measurement and that the fragment with the largest mass is methylated at four different sites, including by Sgm at G1405 (Figures 2B and 3C). We do not observe any signals for the RNA



Peak no.	position	sequence	m/z expected	m/z measured
1	1377–1379	ACGp	998.15	998.13
2	1433–1435	AAGp	1022.16	1022.12
3' 3	1402–1405 1402–1405	CCCGp (1xMe) CCCGp (2xMe)	1293.20 1307.22	1293.19 1307.22
4	1388–1392	CCUUGp	1586.19	1586.21
5	1380–1385	UUCCCGp	1891.23	1891.21
6	1427–1432	CAAAAGp	1985.31	1985.28
7	1393–1401	UACACACCGp	2877.4	2877.43
8	1406–1415	UCACACCAUGp (1xMe)	3197.45	3197.41

Peak no.	position	sequence	m/z expected	m/z measured
1	1377–1379	ACGp	998.15	998.14
2	1433–1435	AAGp	1022.16	1022.18
4	1388–1392	CCUUGp	1586.19	1586.2
5	1380–1385	UUCCCGp	1891.23	1891.23
6	1427–1432	CAAAAGp	1985.31	1985.29
7* 7	1393–1401 1393–1401	UACACACCGp ($2H^+$) UACACACCGp	1439.20 2877.4	1439.48 2877.36
9' 9 9''	1402–1415 1402–1415 1402–1415	CCCGUCACACCAUGp (2xMe) CCCGUCACACCAUGp (3xMe) CCCGUCACACCAUGp (4xMe)	*4473.73* *4487.75* *4501.77*	*4473.67* *4487.57* *4501.73*

Figure 2. MALDI-reflectron ToF MS analysis of Sgm *in vivo* methylated 16S rRNA. Spectra of fragments of 16S rRNA nucleotides 1378–1432 generated by digestion with RNase T1 for 30S subunits isolated from (**A**) *E. coli* transformed with empty pQE-30 vector ('unmethylated' 30S subunits), and (**B**) *E. coli* expressing Sgm (*in vivo* methylated 30S ribosomal subunits). Theoretical and measured monoisotopic masses for expected 16S rRNA fragments are given in the tables. The average mass of the large composite fragment is shown (highlighted in italics).

fragments resulting from RNase T1 cleavage at nt 1405 in *E. coli* expressing Sgm and no signal for the composite 1402–1415 RNase T1 fragment in untransformed *E. coli* (Figure 3). Taken together with the PSD data, we conclude therefore that the methylation of G1405 in *E. coli* expressing Sgm is stoichiometric.

Analysis of *in vitro* methylation of 16S rRNA by other members of the Kgm family and KamB

Our analysis of 16S rRNA fragments methylated *in vivo* by Sgm confirmed that this enzyme, like Kgm (5,7), methylates G1405. To extend this analysis across the Kgm family we selected two further G1405 MTs, including the previously uncharacterized 'Krm' from *Frankia* sp. CcI3. Although originating in a producer strain, this MT aligns more closely with the group of G1405 MTs from pathogenic bacteria in our sequence phylogeny analysis (Figure 1A).

Purified small ribosomal subunits were incubated *in vitro* with recombinant Sgm, GrmA and Krm MTs in the presence of SAM, and isolated 16S rRNA fragments were subjected to MALDI-ToF MS analysis as before. Again, a composite fragment of nts 1402–1415 was detected due to the inability of RNase T1 to cut at m^7G1405 producing peaks at m/z ~4487 and/or ~4501 that correspond to tri- and tetramethylated fragments respectively (Figure 5). We note that for Sgm the trimethylated composite fragment is again predominant, while for GrmA and Krm the tetramethylated RNA is observed.

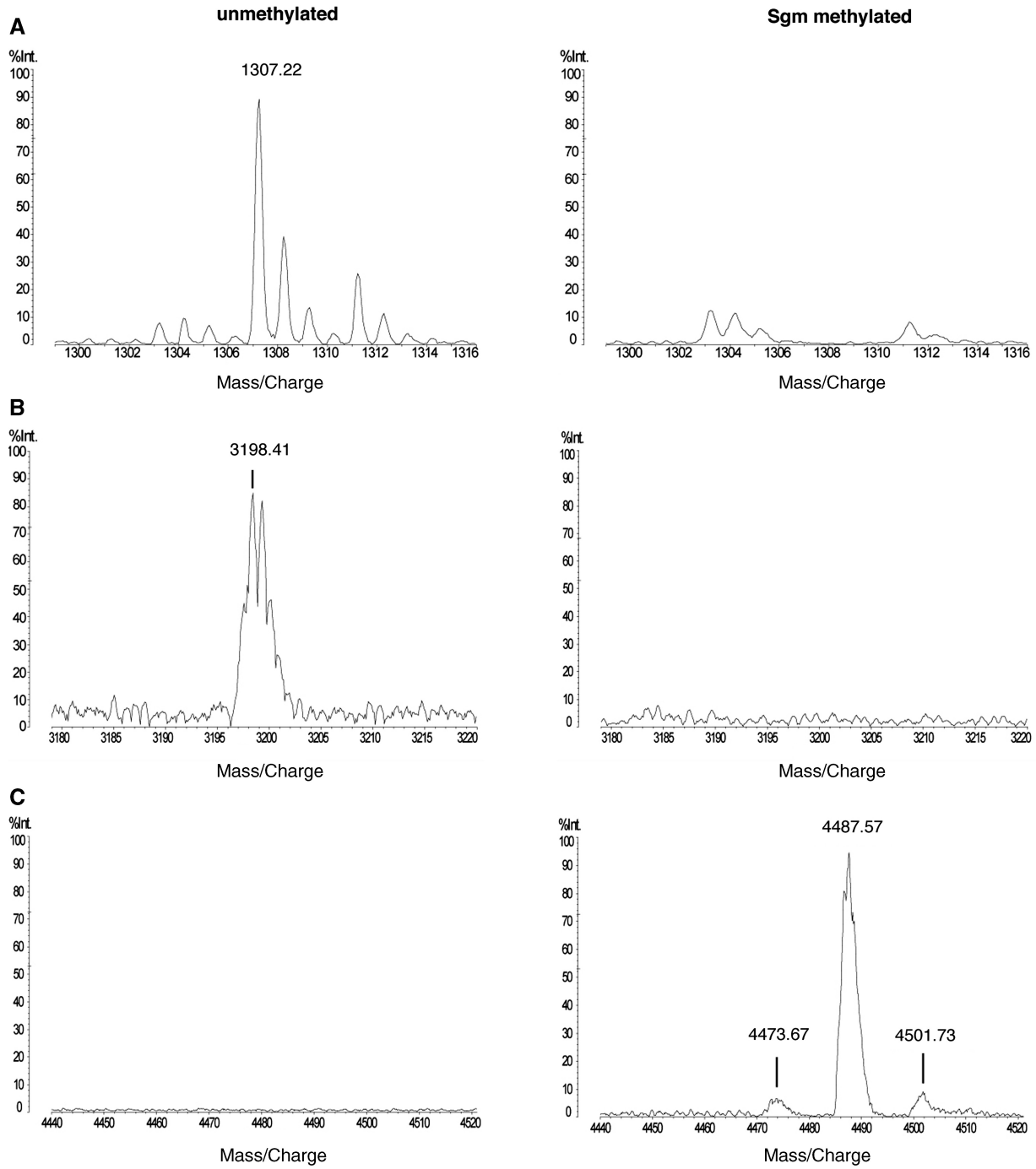


Figure 3. Details of spectral regions for fragments containing the G1405 target residue from control and Sgm *in vivo* methylated fragments. Spectra of 'unmethylated' and *in vivo* Sgm methylated 16S rRNA in regions for (**A**) 1402–1405, (**B**) 1406–1415 and (**C**) the composite fragment 1402–1415 resistant to RNase T1 cleavage. Values indicated are monoisotopic, monoisotopic +1, and average masses in panels (A) to (C) respectively.

Since we would expect all samples to be similarly affected by loss of methyl groups in the instrument, this casts some doubt on the idea that this mechanism leads to the trimethylated fragments for Sgm. An alternative explanation that Sgm can somehow alter the methylation pattern in this region other than at G1405, while GrmA and Krm cannot, would require further investigation. Most importantly, however, for each enzyme the composite fragment can only be observed following methylation at G1405 making the site resistant to RNase T1. Thus, we present experimental confirmation that each of these MTs, together with KgmB, originating from different actinomycetes genera, constitute a family of 16S rRNA G1405 MTs. Signals at m/z 1307 (fragment 1402–1405) and m/z 3198 (fragment 1406–1415) corresponding to the individual fragments without G1405 modification

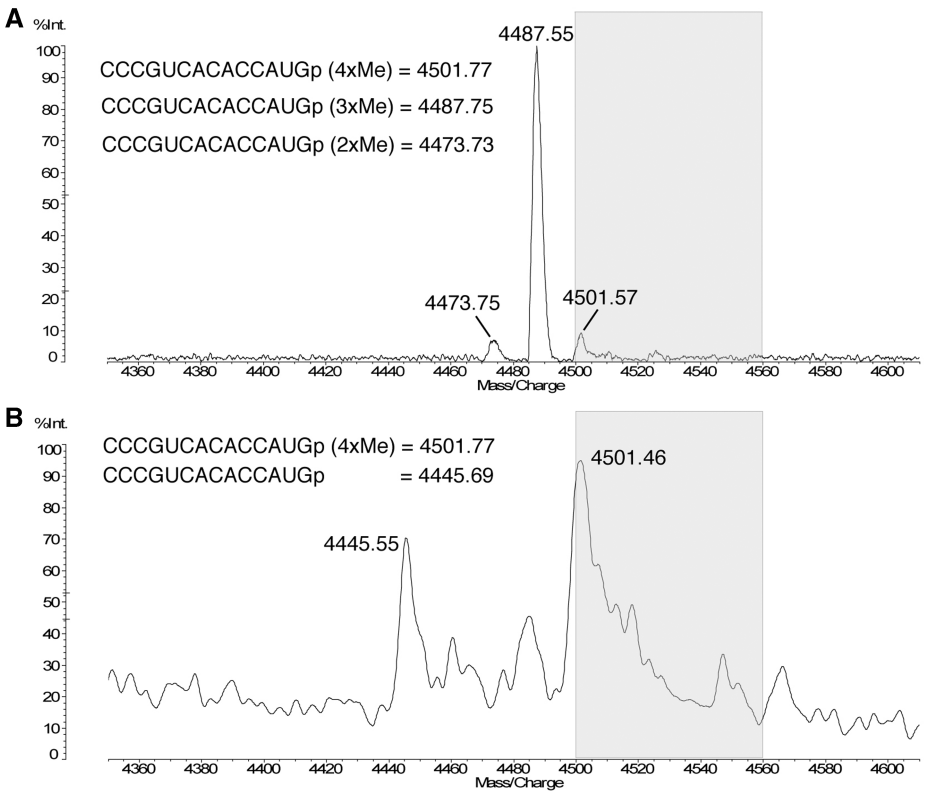


Figure 4. Post-source decay (PSD) analysis of composite RNA fragment 1402–1415 after Sgm *in vivo* methylation. (**A**) Three ions are observed for the Sgm *in vivo* methylated composite 1402–1415 fragment, the largest of which is selected for PSD by setting the ion gate over the mass range shaded grey. (**B**) PSD analysis of ion with m/z 4501.46. In both panels the theoretical average masses for the observed ions are shown with Me denoting the number of attached methyl groups.

were also observed in all *in vitro* (Figure 5) but not *in vivo* (Figures 2 and 4) methylated samples. This indicates that 30S subunits were only partially modified at the target residue under the *in vitro* conditions used.

Finally, we examined one member of the Kam family, KamB from *S. tenebrarius*, for which a modification to the originally suggested open reading frame was recently proposed based upon *in silico* protein fold-recognition analysis and modelling (37). The model indicated that the *Streptoalloteichus hindustanus* KamB (CAI47641) lacked a significant part of the SAM-binding domain and therefore could not be a functional methyltransferase enzyme. Subsequent DNA sequence analysis of all *kam* genes revealed a conserved region upstream of the start codon originally suggested by Holmes *et al.* (9). Choosing an alternative upstream translation initiation site within the published gene sequence added 60 amino acids to the deduced protein N-terminus and completed the expected SAM-binding fold in the model (37). The KamB protein expressed here contained this additional sequence at its N-terminus. MALDI-ToF MS analysis of RNA fragments from KamB methylated 16S rRNA identified an additional fragment of m/z 3211.49 (Figure 6). This corresponds well with the predicted m/z 3211.47 for the dimethylated RNA fragment that results from an additional methylation of 1406-U^{m}CACACCAUG-1415 (theoretical m/z 3197.45) at A1408 by KamB. This site of methylation is further supported by the complete absence of any signal for the monomethylated form of this prominent RNA fragment (compare Figures 3A, 3B and 6B). Although the precise methylation site was not determined here, a study on the closely related KamA from *S. tenjimariensis* (Figure 1B) showed that methylation occurs at the N1 position of A1408 (5). Strikingly, in contrast to the incomplete *in vitro* methylation by Kgm family MTs, close to 100% efficiency was achieved under the same conditions by KamB. We can only speculate on the differences between the Kgm and Kam family MTs tested that might cause this disparity though differing enzyme processivity or activity of purified enzymes are the most obvious candidates since the 30S subunits used in both experiments were from single preparation and therefore of identical quality.

Resistance profiles conferred by the Kgm and Kam family MTs

The minimum inhibitory concentrations (MICs) of aminoglycosides were measured using *E. coli* BL21(DE3) transformants containing Kgm family and KamB MT-encoding plasmids (Table 1). In liquid culture, all Kgm family MTs conferred high-level resistance to 4,6-DOS aminoglycosides tested (kanamycin and gentamicin), with a MIC exceeding 1000 μg/ml. A 3-fold increase in resistance to hygromycin B was also observed (MIC

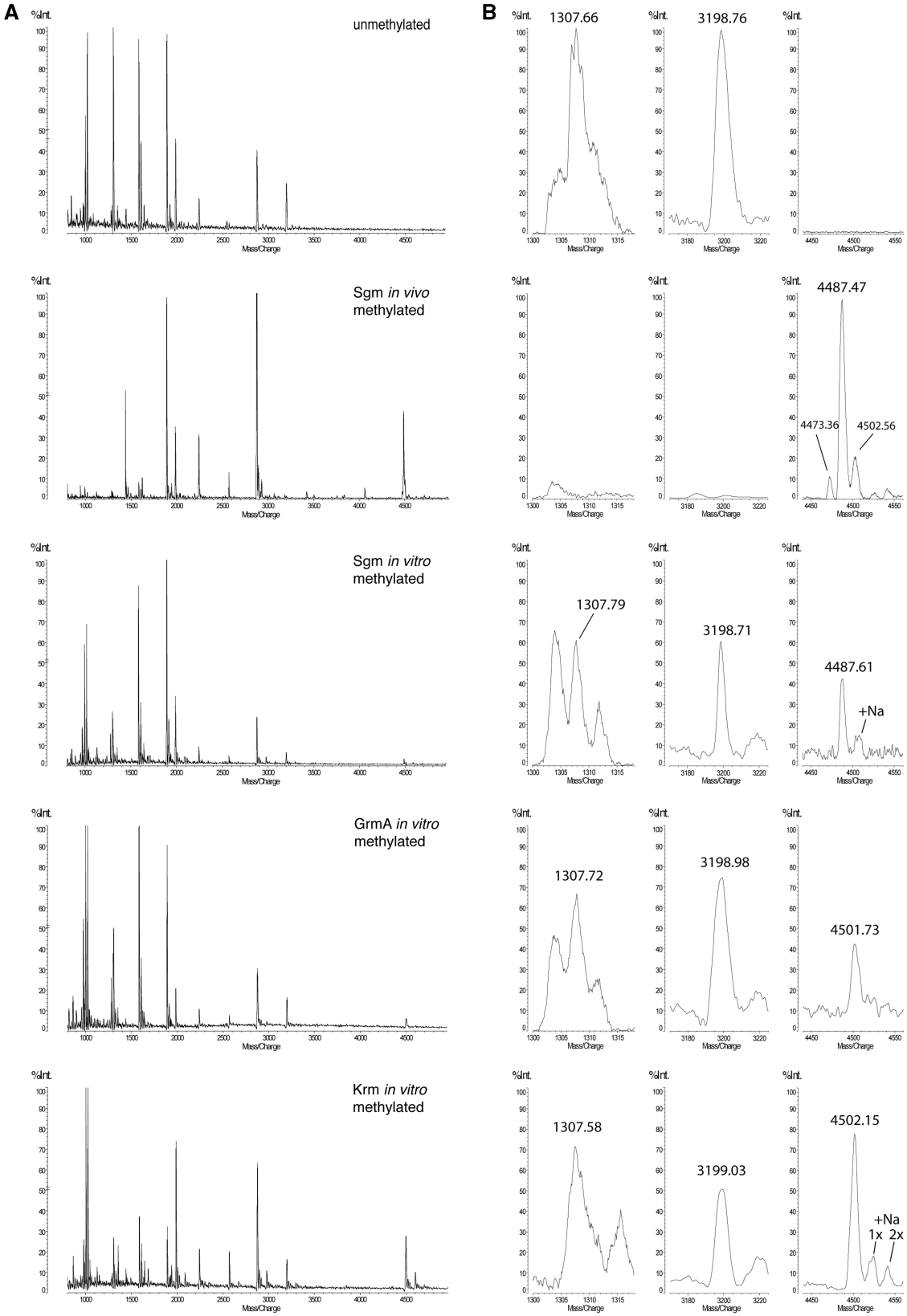
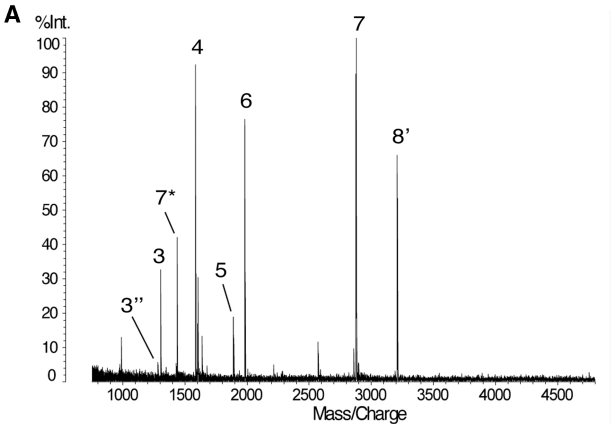


Figure 5. Analysis of methylation target site for Kgm family MTs. Comparative analysis of RNA fragments after *in vitro* methylation of 30S ribosomal subunits by different Kgm MTs. (**A**) Full MALDI-ToF MS spectra and (**B**) spectral details of potential RNA fragments 1402–1405, 1406–1415 and 1402–1415 (composite), for 'unmethylated' rRNA, Sgm *in vivo* methylated, and *in vitro* methylated with Sgm, GrmA and Krm as noted on the spectra in panel (A). The measured average mass is indicated for each peak.



Peak no.	position	sequence	m/z expected	m/z measured
3"	1402–1405	CCCGp	1279.18	1279.24
3	1402–1405	CCCGp (2xMe)	1307.22	1307.23
4	1388–1392	CCUUGp	1586.19	1586.2
5	1380–1385	UUCCCGp	1891.23	1891.26
6	1427–1432	CAAAAGp	1985.31	1985.36
7*	1393–1401	UACACACCGp ($2H^+$)	1439.2	1439.19
7	1393–1401	UACACACCGp	2877.4	2877.42
8'	1406–1415	UCACACCAUGp (2xMe)	3211.47	3211.49

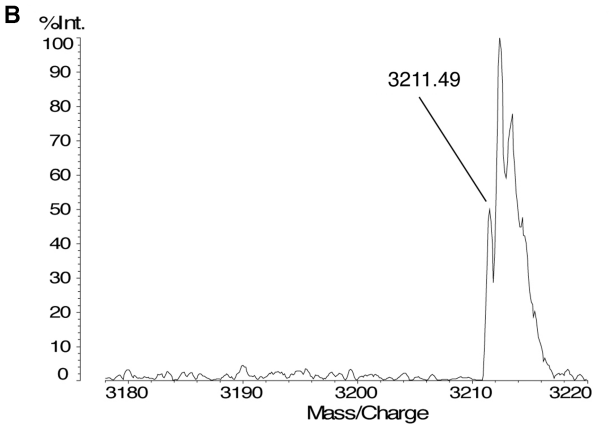


Figure 6. KamB *in vitro* methylation of 30S ribosomal subunits. (**A**) Full MALDI-ToF MS spectrum of RNA fragments after KamB *in vitro* methylation. (**B**) Spectral region around fragment 1406–1415 (the same range as in Figure 3B) with the expected m/z of 3197.45 shifted to 3211.49 corresponding to methylation within this sequence. Expected and measured monoisotopic masses are given in the table.

150 μg/ml) (Table 1). However, susceptibilities to the 4,5-DOS aminoglycosides neomycin and paromomycin, and also to apramycin and streptomycin were unaffected. In contrast, *E. coli* BL21(DE3) containing KamB-expressing plasmid had ~40 times increased resistance to neomycin in addition to high-level resistance to kanamycin and apramycin (Table 1). Unlike the Kgm family MTs, KamB MT did not confer resistance to gentamicin. Growth on hygromycin B was comparable and yielded a MIC value similar to that observed for Kgm MTs (Table 1).

DISCUSSION

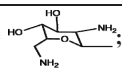
It is generally accepted that two 16S rRNA methylations, m^7G1405 and m^1A1408, confer resistance to overlapping sets of aminoglycoside antibiotics (4,31). However, very little direct experimental evidence for the target site for modification previously existed for the majority of the enzymes proposed to catalyse incorporation of these modifications and systematic analyses of the resistance profiles conferred are lacking. This is a particular concern since resistance profiles often form a significant part of the basis for classification of new 16S rRNA MT enzymes from producer strains and pathogenic bacterial isolates (34,38,39). Furthermore, confusion in the literature on the origin and nature of certain aminoglycoside resistance enzymes has compounded this problem. For example, it was originally thought that the methylation site for GrmA from *M. echinospora* (synonym *M. purpurea*) was successfully experimentally identified as G1405 (5), but this was later shown to be KgmB from *S. tenebrarius* (7). Our study has now experimentally demonstrated that three further methyltransferases originating from different actinomycetes genera (Sgm, GrmA and Krm) methylate the 16S rRNA residue G1405, as was shown for KgmB (5,7).

A potential resolution of a second historical point of confusion with the Kam family of A1408 MTs was recently suggested by Koscinski and colleagues (37) on the basis of protein structure prediction and homology searches. Initial studies on these enzymes used genomic libraries from encoding strains to express the resistance MTs from randomly cloned DNA fragments in *Streptomyces lividans* and *E. coli* and observe resistance phenotypes (9). However, the deposited ORF sequence (NCBI accession number CAF33037) was shortened by 180 nt encoding the Kam protein N-terminus that forms the SAM-binding domain (37). Any attempt to express KamB from the originally suggested start codon failed and recombinant protein did not provide resistance to any aminoglycoside antibiotics (Vasiljevic,B., unpublished data). Therefore, although methylation of A1408 was explicitly shown for *kamA* (*imrA*) from *S. tenjimariensis* and *kamC* from *S. hirsuta*, their originally deduced protein sequences of 156 amino acids are unlikely to confer resistance due to the N-terminal truncation (37). Our analysis of KamB from *S. tenebrarius*, thus experimentally confirms that these 60 additional N-terminal amino acids are essential for a functional Kam family enzyme and that it methylates at A1408 as predicted.

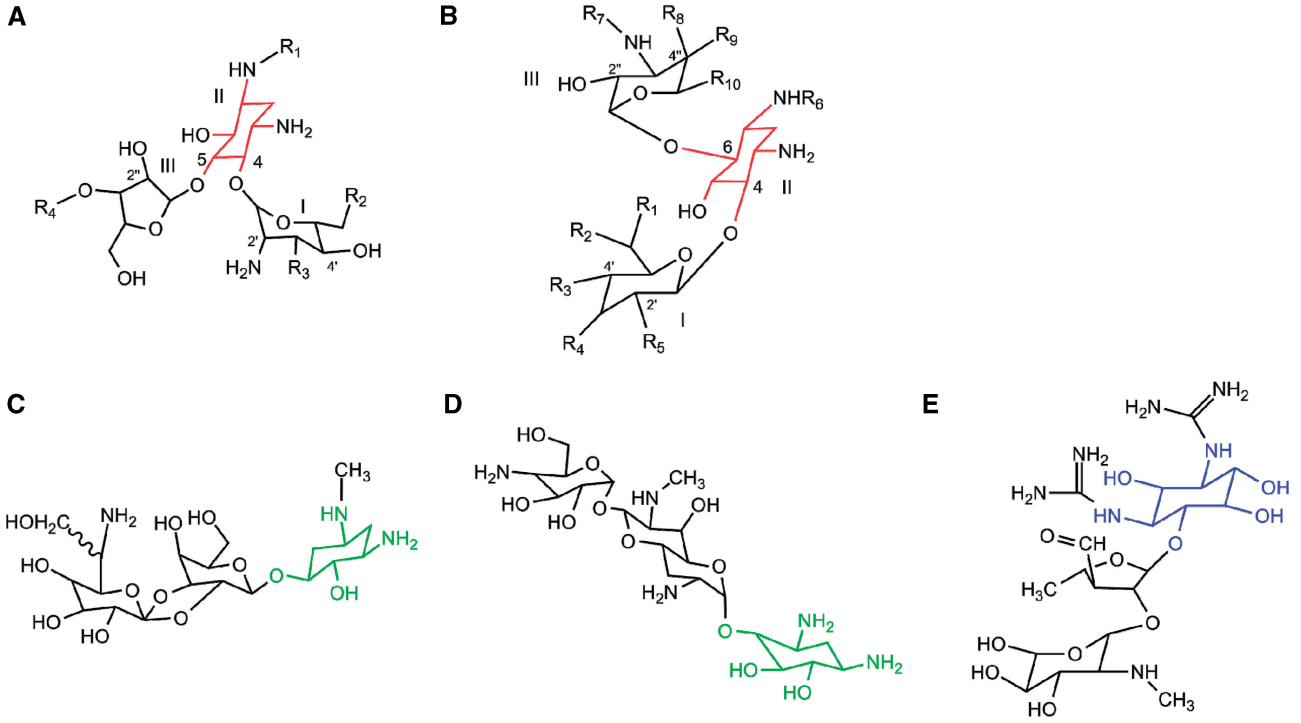
The Kgm and Kam enzymes share the Rossmann-like fold that forms the SAM-binding and catalytic domain of these MTs and, given the close proximity of their target nucleosides, they must recognize a very similar molecular surface of the 30S subunit. A key question is therefore how the observed specificity of these enzymes is achieved. Our data, together with recent structural probing of Sgm, and *in silico* modelling studies of Kgm and Kam MTs indicate that enzymes of each protein family adopt similar

Table 1. MIC values against three groups of aminoglycoside antibiotics for *E.coli* expressing Kgm and Kam family MTs

Aminoglycoside antibiotic	Minimum inhibitory concentration (MIC) (μg/ml)						
	No vector	pQE-30	pQE-Sgm	pQE-GrmA	pQE-Krm	pQE-KgmB	pQE-KamB
4,6-DOS							
Kanamycin	<5	<5	>1000	>1000	>1000	>1000	1000
Gentamicin	<5	<5	>1000	>1000	>1000	>1000	10
4,5-DOS							
Neomycin	<5	<5	15	15	15	15	200
Paromomycin	<5	<5	20	20	25	25	30
Others							
Apramycin	15	15	30	20	20	15	1000
Streptomycin	10	10	15	15	15	15	15
Hygromycin B	50	50	150	150	150	150	150

Chemical structures of aminoglycosides tested: (**A**) 4,5-DOS: R_1 = H, R_2 = NH_2/OH (neomycin/paromomycin), R_3 = OH, R_4 =  ;

(**B**) 4,6-DOS: R_1 = H, R_2 = NH_2, R_3 = OH/H (Kanamycin A/Gentamicin C1A), R_4 = OH/H (K/G), R_5 = OH/NH_2 (K/G), R_6 = H, R_7 = H/CH_3 (K/G), R_8 = H/OH (K/G), R_9 = OH/CH_3 (K/G), R_{10} = CH_2OH/H (K/G); (**C**) Hygromycin B; (**D**) Apramycin; (**E**) Streptomycin. In (C) and (D) the aminocyclitol ring is shown green and in E the streptamine core is coloured blue.



topologies but differ primarily in the order of the domains (18,19,37). The co-enzyme/catalytic function is located in the C-terminal domain of Kgm MTs but in the N-terminal domain of Kam enzymes. The position of the additional domain that is presumed to play the major role in target recognition (18,19) is thus reversed in each family. We would speculate that the domain order is the critical factor contributing to the observed specificity of these two enzymes families.

To effectively provide resistance, modification of G1405 and A1408 in the aminoglycoside binding pocket of the 30S A-site must perturb interaction between the drug and its target residues in order to sufficiently lower its affinity and thus efficacy. Our results systematically establish the link between the 16S rRNA modification type and aminoglycoside antibiotic resistance pattern conferred by these modifications. Both groups of 2-DOS antibiotics bind to 16S rRNA so that their Ring I substituents (see chemical structures associated with Table 1) are placed in close proximity to A1408. The methylated nucleotide (m^1A1408) is positively charged at neutral pH, and can therefore affect drug binding not only by steric hindrance but also by charge repulsion. This modification confers resistance to apramycin and the kanamycin group of 4,6-DOS aminoglycosides other than gentamicin. The modification does not, however, confer significant resistance to paromomycin (5) despite the observation of two direct hydrogen bonds to A1408 in the crystal structure

of the antibiotic-30S complex (40). It is possible that additional contacts made by other parts of these drugs sufficiently compensate for those lost near A1408. Alternatively, secondary binding sites on the 70S ribosome might account for the unaltered antibiotic susceptibility as shown recently for paromomycin and gentamicin which also bind to 23S rRNA helix 69 in the vicinity of the P-site tRNA to inhibit ribosome recycling (41). Paromomycin is also known to inhibit 30S subunit assembly with approximately equal efficiency as it inhibits translation (42,43). Thus, simple protection of the primary target site by methylation may not be sufficient to provide resistance against these antibiotics.

In high-resolution structures of the 4,6-DOS aminoglycosides gentamicin C1a (44) and tobramycin (45) in complex with A-site model RNAs, both antibiotics make direct contacts to G1405 via their Ring III substituents. Methylation of this nucleotide would thus directly interfere with antibiotic binding, by inducing a steric clash between the modified base and Ring III, in addition to possible electrostatic repulsion by the positive charge on modified nucleobase. In contrast, 4,5-DOS aminoglycosides, such as paromomycin and neomycin, project their substituent at position 5 at different angle, directing it away from G1405, so that methylation at this site does not interfere with their binding. The m^7G1405 modification by Kgm MTs is thus only effective against 4,6-DOS aminoglycosides, but does confer high-level resistance to both kanamycin and gentamicin antibiotic groups as measured in our MIC analysis with several Kgm family MTs (Table 1).

To date, aminoglycoside resistance spectra for Kgm MTs were primarily analysed only in homologous host strains, i.e. high G+C Gram-positive bacteria such as *S. lividans* TK21 and *Micromonospora melanosporea* (10,46). Our study therefore provides new functional insight into the activity of these enzymes in the Gram-negative heterologous host *E. coli*. We have shown that all resistance MTs analysed, Sgm, GrmA, KgmB and Krm, can confer high level resistance to 4,6-DOS antibiotics (Table 1) via modification of G1405 of 16S rRNA in *E. coli*. Each of these resistance MTs from three different actinomycetes genera is equally effective in providing high-level aminoglycoside resistance at a level comparable to the original Gram-positive hosts. This observation firmly establishes resistance MTs acquisition by Gram-negative micro-organisms from surrounding Gram-positive bacteria as a potential source of these enzymes in pathogens.

Genes from Gram-positive organisms are known to express in Gram-negative bacteria and recent data have showed that although horizontal gene transfer is extensive it is polarized in direction, from Gram-positive to Gram-negative organisms (47). Therefore, it is possible for resistance MT genes to transfer from antibiotic producing Gram-positive bacteria to Gram-negative pathogens, supporting the notion that Arm family MTs originated in actinomycetes or related antibiotic-producing organisms (48,49). This idea recently found an anchor point with the realization of the tremendous potential of soil microbial communities to overcome any antibiotic pressure and even use antibiotics as sole carbon source to support growth (50,51). This soil antibiotic resistome forms an immense and diverse pool of resistance genes ready to be mobilized into any micro-organism, including pathogenic bacteria. This is undoubtedly a major factor in the ability of pathogenic bacteria to rapidly develop resistance to antibiotics and contribute to the increasing frequency of multi-drug resistant isolates (52). As this global antibiotic resistance problem has escalated, aminoglycosides have assumed an increasing importance in clinical practice due to their broad antimicrobial spectrum, rapid bactericidal action and ability to act synergistically with other antibiotics, such as beta-lactams. A detailed understanding of all resistance mechanisms for aminoglycosides will therefore be an essential component of combating this threat.

FUNDING

Grant 078374 from the Wellcome Trust; and the Ministry of Science of the Republic of Serbia (Grant no. 143056 to B.V.). Funding for open access charge: The Wellcome Trust.

Conflict of interest statement. None declared.

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