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Expansion of the aminoglycoside-resistance 16S rRNA (m¹A1408) methyltransferase family: expression and functional characterization of four hypothetical enzymes of diverse bacterial origin

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Abstract

The global dissemination, potential activity in diverse species and broad resistance spectrum conferred by the aminoglycoside-resistance ribosomal RNA methyltransferases make them a significant potential new threat to the efficacy of aminoglycoside antibiotics in the treatment of serious bacterial infections. The N1 methylation of adenosine 1408 (m¹A1408) confers resistance to structurally diverse aminoglycosides, including kanamycin, neomycin and apramycin. The limited analyses to date of the enzymes responsible have identified common features but also potential differences in their molecular details of action. Therefore, with the goal of expanding the known 16S rRNA (m¹A1408) methyltransferase family as a platform for developing a more complete mechanistic understanding, we report here the cloning, expression and functional analyses of four hypothetical aminoglycoside-resistance rRNA methyltransferases from recent genome sequences of diverse bacterial species. Each of the genes produced a soluble, folded protein with a secondary structure, as determined from circular dichroism (CD) spectra, consistent with enzymes for which high-resolution structures are available. For each enzyme, antibiotic minimum inhibitory concentration (MIC) assays revealed a resistance spectrum characteristic of the known 16S rRNA (m¹A1408) methyltransferases and the modified nucleotide was confirmed by reverse transcription as A1408. In common with other family members, higher binding affinity for the methylation reaction by-product S-adenosylhomocysteine (SAH) than the cosubstrate S-adenosyl-L-methionine (SAM) was observed for three methyltransferases, while one unexpectedly showed no measurable affinity for SAH. Collectively, these results confirm each hypothetical enzyme is a functional 16S rRNA (m¹A1408) methyltransferase but also point to further potential mechanistic variation within this enzyme family.

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Keywords

antibiotic resistance; bacteria; ribosomal RNA; methylation; S-adenosyl-L-methionine; S-adenosylhomocysteine

1. Introduction

Bacterial antibiotic resistance has been a significant problem almost since the first introduction of these important drugs to clinical practice. However, the continuing emergence of serious human pathogens resistant to many or all available antibiotic regimens may now constitute possibly the greatest threat to modern healthcare [1,2]. Studies revealing the molecular details of resistance determinant function are essential to underpin novel strategies that could extend the effectiveness of current antibiotics and to minimize the potential for resistance to future drugs.

The bacterial ribosome is the major target for multiple classes of antibiotics which, collectively, can disrupt almost every aspect of normal ribosome function [2]. Aminoglycosides, for example, constitute a large, structurally diverse group of poly-cationic compounds, with a central aminocyclitol ring typically (2-deoxystreptamine or streptamine) linked to amino sugars, that primarily bind to the A site of the small ribosomal subunit and disrupt the fidelity of decoding [3]. Members of this drug class have historically played important roles in the treatment of serious infections but the emergence of other broad-spectrum antibiotics with fewer toxicity issues has led to a decline in their application. Aminoglycosides nonetheless remain in use today and, with retained activity, may yet prove essential in the battle against serious Gram-negative pathogens resistant to other antibiotics [4]. Current clinical resistance to aminoglycosides arises predominantly through the action of broadly disseminated aminoglycoside-modifying enzymes which confer resistance to one or several related aminoglycosides [5,6]. However, the emergence of 16S rRNA methyltransferases that modify the aminoglycoside binding site among pathogenic bacterial populations poses a major new threat as this activity potentially confers resistance to all clinically useful aminoglycosides including the latest generation [7,8].

Two distinct groups of S-adenosyl-L-methionine (SAM)-dependent aminoglycoside-resistance methyltransferases have been identified which modify 16S rRNA at one of two sites: guanine-N7 methylation of G1405 (m^7G1405 ; EC 2.1.1.179) or adenine-N1 methylation of A1408 (m^1A1408 ; EC 2.1.1.180) [9–12]. These two nucleotide modifications within the A site of the small (30S) bacterial ribosome subunit result in distinct aminoglycoside resistance profiles [13,14]. The m^7G1405 modification confers resistance to the 4,6-disubstituted 2-deoxystreptamines, including both kanamycin and gentamicin, but not other structural classes of aminoglycosides. In contrast, the m^1A1408 modification confers resistance to specific aminoglycosides within each structural class including kanamycin, neomycin and apramycin. Both enzymes were first identified in the aminoglycoside-producing *Actinomycetales* where they serve as a mechanism of self-protection [9]. Although little direct evidence exists, it is assumed that the functionally orthologous enzymes now increasingly found in pathogenic bacteria were, like the majority

of such acquired antibiotic resistance determinants, obtained via horizontal gene transfer [15] with likely environmental origins [16–18].

To date, eleven unique members of the 16S rRNA (m¹A1408) methyltransferase family have been identified, including four hypothetical enzymes for which methylation activity has not been experimentally determined (Fig. 1A). Among the functionally verified m¹A1408 rRNA methyltransferases the protein sequence identity can be low, e.g. 28% identity between KamB, from the tobramycin producer *Streptoalloteichus tenebrarius*, and NpmA, from a clinical isolate of *E. coli* strain ARS3. However, X-ray crystallographic structures of KamB and NpmA show these enzymes possess a highly conserved structural fold [19,20]. Such structural similarity immediately suggested that these enzymes should act via conserved mechanisms of substrate recognition and catalysis of rRNA modification. However, these studies and the structure of NpmA bound to the 30S substrate [21] also revealed surprising differences in the likely molecular details of their function. For example, many residues found to be critical for KamB activity are either not conserved in NpmA or their mutation has no effect [19,20]. Two key residues identified in NpmA, R207 responsible for A1408 base flipping and E146 which supports this action, are not present in KamB [21]. Furthermore, another A1408 methyltransferase, Kmr from the cellulose degrading bacterium, *Sorangium cellulosum*, appears to be uniquely dependent on the 30S to bind SAM and thus form a catalytically competent structure (Savic *et al.*, manuscript in preparation). More broadly, the SAM binding fold is also preserved in some proteins without methyltransferase activity [22]. Therefore, classification and inference of functional mechanisms for the 16S rRNA (m¹A1408) methyltransferases should be made with caution when based on either sequence or structural homology alone. Detailed experimental analyses of further enzymes of this family are thus urgently needed to extend our functional understanding of antibiotic resistance conferred by the 16S rRNA (m¹A1408) methyltransferases.

We report here the cloning, expression, and purification of four putative 16S rRNA (m¹A1408) methyltransferases of diverse bacterial origins, which are also distantly related to previously characterized family members (Table 1, Fig. 1). We assessed the solution secondary structure, binding of SAM cosubstrate and the reaction by-product S-adenosylhomocysteine (SAH), and activity *in vivo* and *in vitro* of each enzyme confirming its status as a functional aminoglycoside-resistance 16S rRNA methyltransferase acting at nucleotide A1408. However, our results also highlight further unexpected potential structural and mechanistic functional diversity among the members of this enzyme family.

2. Materials and methods

2.1. Cloning, protein expression and purification

Genes encoding four predicted 16S rRNA (m¹A1408) methyltransferases (Table 1) were synthesized with codon optimization for expression in *E. coli* (GeneArt). All genes were inserted between 5' flanking *Nde*I or *Bam*HI and 3' *Hind*III restriction sites within a modified pET44a vector which we have used previously for expression of other aminoglycoside-resistance methyltransferases [23]. *Nde*I/*Hind*III ("pET") and *Bam*HI/*Hind*III ("pET-HT") inserts respectively encoded proteins expressed with an authentic N-

terminus or with a N-terminal 6 × His tag (H) followed by a thrombin cleavage site (T) to allow the removal of the tag from the expressed fusion protein if needed (Fig. 1B). Each of the expression constructs was verified by automated DNA sequencing (Genewiz).

In the absence of a suitable systematic naming convention for the intrinsic and acquired aminoglycoside-resistance 16S rRNA (m¹A1408) methyltransferases, for brevity we refer to the expressed products of the four genes using a three letter species abbreviation (or *Unc*, for uncultured bacterium) prepended to the enzyme name “Kam”, for kanamycin-apramycin resistance methyltransferase (Table 1, *final column*).

For protein expression, all media was supplemented with ampicillin (0.1 mg/ ml) and cultures grown at 37 °C with vigorous shaking, unless otherwise stated. Proteins were expressed in *E. coli* BL21 (DE3) beginning from a single colony used to inoculate 5 ml Lysogeny Broth (LB) medium and grown to saturation overnight. These starting cultures were used to inoculate 500 ml Terrific Broth (TB) medium (for *CacKam* and *TcuKam* expression) or LB medium (for *CarKam* and *UncKam* expression) and these cultures grown to an OD₆₀₀ of ~0.6. Cultures for expression of *CacKam* and *TcuKam* were then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM) and growth continued for 3 hours post-induction. Cultures for expression of *CarKam* and *UncKam* were transferred to 20 °C and, after being allowed to equilibrate to the lower temperature, induced with IPTG (1 mM) and grown for additional 20 hours post-induction. Cells were harvested by centrifugation at 3,400 × g for 30 minutes. Cell pellets were resuspended in 5 ml of lysis buffer per 1 g of wet cells. The lysis buffer contained 50 mM NaH₂PO₄ at pH 8.0, 300 mM NaCl, and 10 mM imidazole for *CacKam* and *TcuKam*, or 50 mM Tris at pH 8.0, 500 mM NaCl, 1 mg/ml lysozyme, 10% glycerol and 1% Triton-X100 for *UncKam* and *CarKam*. For *CacKam*, *TcuKam* and *UncKam*, the resuspended cells were lysed by sonication for 15 min (cycling 0.9 s on, 0.6 s off, level 5; Misonix S3000). Resuspended cells for *CarKam* expression were lysed by two passages through a French Press. For all lysates, insoluble debris was removed by centrifugation at 13,000 × g for 25 min, and the supernatant filtered through 0.45 μm filters (Millipore) prior to chromatographic purification.

Filtered protein samples were loaded onto a 5 ml HisTrap HP (Ni²⁺-affinity) column attached to an ÄKTApurifier10 system (both GE Healthcare) and pre-equilibrated with the appropriate lysis buffer (but lacking lysozyme and Triton-X100 in the case of *UncKam* and *CarKam*). Unbound proteins were washed out with five column volumes of the same buffer before protein elution using a linear gradient of 10–250 mM imidazole (all proteins eluted between 50–75 mM imidazole). Fractions containing the target protein, as determined by SDS-PAGE analysis, were combined and concentrated using Millipore Ultrafree-15 devices (MWCO 10,000 Da). Concentrated proteins were further purified on a Superdex 75 16/60 gel filtration column (GE Healthcare), equilibrated with a running buffer containing 20 mM Tris pH 8.0 and 150 mM NaCl for *CacKam* and *TcuKam*, or 20 mM Tris pH 7.5, 10 mM magnesium acetate, 250 mM NH₄Cl, 10 % glycerol and 6 mM β-mercaptoethanol for *UncKam* and *CarKam*. Fractions corresponding to purified proteins were identified, pooled and concentrated as before to approximately 3 mg/ml. Proteins were flash frozen dropwise in liquid nitrogen and stored at –80 °C.

2.2. Antibiotic minimum inhibitory concentration (MIC) measurements

Antibiotic minimum inhibitory concentration (MIC) measurements were made in liquid cultures of *E. coli* BL21 (DE3) transformed with an empty pET44a vector or expression construct encoding one of the four putative methyltransferases. Both the pET and pET-HT constructs were tested for each enzyme. Initial cultures were grown overnight to saturation in LB medium supplemented with 0.1 mg/ml ampicillin. Fresh LB (4 ml) was inoculated with overnight culture at 1:100 dilution, and grown to OD₆₀₀ of ~0.1 (~1 × 10⁶ cfu/ml). MIC assays were conducted in a 96-well plate format with each well containing 200 µl LB medium supplemented with 5 µM IPTG, 1 × 10⁵ cfu/ml cells, and antibiotic ranging from 0–1024 µg/ml (apramycin, gentamicin, kanamycin or neomycin). Plates were incubated at 37 °C with shaking for 24 hours. Absorbance measurements at 600 nm were recorded on a Synergy4 plate reader (BioTek) and the MIC defined as the lowest concentration of antibiotic that inhibited growth (OD₆₀₀ < 0.05).

2.3. 30S ribosome subunit methylation assays

E. coli 30S ribosomal subunits were purified as previously described [24]. Methylation reactions (125 µl) contained 100 pmol of 30S ribosomal subunits, 200 pmol of purified recombinant methyltransferase (*CacKam*, *TcuKam*, *UncKam* or *CarKam*), 1 mM SAM, 10 mM HEPES-KOH pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, and 5 mM β-mercaptoethanol. Before addition of ribosomal subunits, the reaction mixture was incubated at 37 °C for 10 minutes. Methylation reactions were initiated by addition of 30S subunits and performed at 37 °C for 60 min. Reactions were quenched and rRNA recovered by phenol/ chloroform extraction and followed by ethanol precipitation. Reverse transcriptase (RT) primer extension was carried out using a 5'-end ³²P-labeled DNA primer complementary to *E. coli* 16S rRNA nucleotides 1457–1473 (5'-CAAAGTGGTAAGCGCCC) and AMV reverse transcriptase (NEB). Extension products were resolved on 10% polyacrylamide urea denaturing sequencing gels and visualized using a Typhoon Trio phosphor imaging system (GE Healthcare).

2.4. Circular dichroism (CD) spectroscopy

CD spectra were recorded at 20 °C on a J-810 spectropolarimeter (Jasco) using a 0.1 cm path-length cell and protein (5 µM) in 5 mM phosphate buffer at pH 7.4 containing 150 mM NaF. Three spectra were recorded for each protein from 260 to 190 nm, with 0.2 nm resolution and scan speed of 100 nm/min, and averaged and background corrected using the Spectra Manager software provided with the instrument. Analysis of protein secondary structure was performed using the CDSSTR deconvolution algorithm via Dichroweb [25], which previously provided good quality fits for other methyltransferase enzymes [23]. Experimental data were fit over the wavelength range 190–240 nm and fit quality assessed by the resulting normalized root mean square deviation (NRSMD) [26].

2.5. Isothermal titration calorimetry (ITC)

ITC measurements were performed at 25 °C using an Auto-iTC₂₀₀ microcalorimeter (GE Healthcare/ MicroCal). Purified proteins were exhaustively dialyzed against 20 mM Tris pH 8.0 and 150 mM NaCl for *CacKam* and *TcuKam*, or in 20 mM Tris pH 7.5, 10 mM

magnesium acetate, 250 mM NH₄Cl, 10 % glycerol and 6 mM β -mercaptoethanol for *CarKam* and *UncKam*. The final dialysis buffer for each protein was used to prepare SAM and SAH solutions at 1.5 and 0.5 mM concentration, respectively. Titration experiments comprised 16 $\times$ 2.5 μ l injections of SAM or SAH into the sample cell containing methyltransferase protein (50–70 μ M). The titration isotherm was fit using the single binding site model in Origin 7 software (MicroCal). Titrations were performed in at least duplicate for each protein-ligand pair. To further ensure validity of comparisons between the four enzymes, each protein preparation was titrated with SAM or SAH in parallel and at least two different proteins were included in each set of experiments using the same ligand preparations.

3. Results

3.1. Cloning, expression and purification of putative 16S rRNA (m¹A1408) methyltransferases

The aminoglycoside-resistance 16S rRNA (m¹A1408) methyltransferases were first identified in several related aminoglycoside-producing bacteria [10,27]. Subsequently, orthologous resistance enzymes were identified in the capreomycin producer *Streptomyces capreolus* (CmnU), a clinical *E. coli* isolate with the pARS3 plasmid (NpmA), and the cellulose degrading myxobacterium *Sorangium cellulosum* (Kmr) [28–30]. Most recently (since 2009), genome sequencing studies have added further hypothetical proteins to the database with putative methyltransferase function (typically, tRNA m⁷G) inferred from the electronic annotation. These include two unique sequences, one each from *Catenulispora acidiphila* and *Thermomonospora curvata*, and multiple closely related sequences from two other sources, *Candidatus Arthromitus* and uncultured bacterium from ground water samples (Table 1).

An inferred phylogenetic tree was derived from the primary sequences of these confirmed and hypothetical methyltransferases (Fig. 1A). As might be expected, the aminoglycoside-producer ‘Kam’ proteins form a tight cluster of closely related sequences, whereas the other enzymes constitute a more distantly related and diverse group in which the sequence identity ranges from 17–56 % (Table 2). From this analysis, we selected four hypothetical enzymes (shaded in Fig. 1A) for functional analyses. DNA encoding each putative enzyme was chemically synthesized with suitable flanking restriction enzyme sites for cloning into plasmids for expression of either N-terminally 6 $\times$ His tagged or tag-free recombinant protein (Fig. 1B) [23]. Constructs with the 6 $\times$ His tag additionally encoded a thrombin recognition site for tag removal. As described below, proteins with and without the tag displayed similar activity in the antibiotic minimum inhibitory concentration (MIC) assays. Therefore, with their simpler purification procedure, tagged constructs were used for subsequent protein expression for *in vitro* studies of enzyme function.

Initial protein expression for each construct was tested using TB medium at 37 °C. Under these conditions, *CacKam* and *TcuKam* were readily produced as predominantly soluble proteins (ultimately yielding ~20 mg pure protein/ L culture). In contrast, the majority of *UncKam* and *CarKam* protein was present in the insoluble cell fraction (~5% soluble). Medium type, growth and induction conditions, method of protein release from cells, and

composition of buffers used for lysis and purification, were therefore tested as variables in order to maximize the final yield of *UncKam* and *CarKam* proteins suitable for *in vitro* study. Conditions for optimal expression of soluble protein were identified as 20 °C growth temperature, induction of expression at 1 mM final IPTG concentration, and duration of expression of 20 hours. Furthermore, optimal cell lysis and protein solubility was achieved in Tris buffer at pH 8.0 (50 mM) with increased salt concentration (500 mM) and addition of 1% Triton-X100, 1 mg/ml lysozyme and 10% glycerol. The use of a French Press, rather than sonication, was the method of choice for lysing cells used in *CarKam* expression. These optimized procedures produced final yields of *UncKam* and *CarKam* of ~4 mg pure protein /L culture.

All four 6×His proteins were purified using the same two-step procedure involving Ni²⁺-affinity and gel filtration chromatographies, but with protein-specific running buffer modifications analogous to those used for cell lysis. SDS-PAGE analysis of an example of expression and purification (for *CacKam*) and final quality for each protein is shown in Figure 1C.

3.2. Proteins are folded with secondary structures consistent with known 16S rRNA (m¹A1408) methyltransferases

Circular dichroism (CD) spectroscopy is a valuable low-resolution technique for examining the folding and overall secondary structural features of proteins in solution [26]. The far-UV CD spectrum (260-190 nm) for each purified putative 16S rRNA (m¹A1408) methyltransferase was recorded and in each case indicated the protein was well-folded and of mixed α/β structure (Fig. 2). CD spectra can be quantitatively deconvoluted by comparison to large data sets of protein CD spectra to obtain an experimental estimate of the protein secondary structure content in solution [31]. This process was accomplished for the spectrum of each new protein using the CDSSTR algorithm via the Dichroweb server [25]. The secondary structural estimates obtained and the NRSMD between theoretical and calculated CD spectra are shown in Table 3. The NRMSD was less than 0.05 in each case indicating a high quality fit. The results indicate that each of the four putative enzymes has a similar secondary structural content with significant β -strand structure, as expected for a methyltransferase SAM-binding fold protein.

The secondary structure values are also remarkably similar to those similarly derived from CD spectra for two confirmed 16S rRNA (m¹A1408) methyltransferases, KamB and NpmA (Table 3), for which high-resolution structures subsequently became available [20,23]. For KamB and NpmA, the estimated values from CD for α -helical and “other” content were lower and higher, respectively, than those crystallographically determined. This is most likely due to the weaker signal at the far end of the spectrum (200-190 nm) which is dominated by and therefore important for accurate α -helical structure content. Nonetheless, the congruence of secondary structure values derived from CD spectra for KamB, NpmA and the four new enzymes strongly suggests that each possesses the same overall protein fold corresponding to a 16S rRNA (m¹A1408) methyltransferase. Furthermore, with the caveat of the likely underestimated α -helical content, CD spectroscopy appears to be a

reliable experimental approach to support inclusion of a new enzyme within the A1408 methyltransferase family.

3.3. All enzymes are functional aminoglycoside-resistance methyltransferases that modify A1408

The known 16S rRNA (m¹A1408) methyltransferases confer a characteristic resistance spectrum in bacteria, including high level resistance to apramycin, neomycin and the 4,6-DOS aminoglycoside kanamycin but not the structurally related gentamicin [14,23]. MIC measurements were made for each of these aminoglycosides in liquid cultures of *E. coli* BL21(DE3) harboring pET-methyltransferase or pET-HT-methyltransferase plasmids encoding *CacKam*, *TcuKam*, *CarKam* or *UncKam* (Table 4). All four enzymes conferred a resistance spectrum characteristic of the 16S rRNA (m¹A1408) methyltransferases. In each case, high-level resistance to kanamycin, neomycin and apramycin was observed, with negligible resistance to gentamicin. The kanamycin and apramycin MICs were slightly lower (512–1024 compared to >1024 µg/ml) for *CarKam* and *UncKam*. Similarly, a lower MIC was observed for these two enzymes against neomycin (64–128 µg/ml) compared to *CacKam*, *TcuKam* and *KamB* (256–512 µg/ml) but still substantially higher than in the absence of enzyme (empty pET44a vector). In summary, although minor differences were observed in the absolute level of resistance conferred to some antibiotics, the resistance spectrum of each new enzyme corresponds to that of a 16S rRNA (m¹A1408) methyltransferase.

The crystal structure of NpmA bound to the 30S subunit showed that the enzyme recognizes an extended tertiary surface of the 16S rRNA [21]. As this surface is only present in the mature subunit, this observation provided a clear structural rationale for the requirement of the intact 30S subunit as substrate and the inability of the 16S rRNA (m¹A1408) methyltransferases to modify naked 16S rRNA. To identify the substrate requirements and confirm the site of 16S rRNA modification for each of the new putative 16S rRNA (m¹A1408) methyltransferases, *in vitro* methyltransferase activity was assessed using reverse transcription (RT) analysis of *in vitro* methylated 30S subunits and naked 16S rRNA (Fig. 3). Each of the four new recombinant enzymes, along with *KamB* as a positive control, was incubated in the presence of SAM with either purified *E. coli* 30S subunits or protein-extracted, naked 16S rRNA from the same source. In each case, 16S rRNA was recovered and analyzed by RT using a 5'-end ³²P-labeled DNA primer complementary to helix 44 approximately 50 nucleotides 3' of A1408. Methylation of A1408 at N1 (m¹A1408) produces a strong stop in the RT reaction, resulting in an intense band on the sequencing gel. For each of the four enzymes acting on 30S subunit substrate, an intense new band was observed corresponding to modification at A1408 (Fig. 3A). The same band was present for *KamB* but absent in the unmodified 16S rRNA control. In contrast, none of the enzymes tested showed any methylation activity against the naked 16S rRNA (Fig. 3B).

These data conclusively demonstrate that all four recombinantly expressed proteins efficiently methylate 16S rRNA at nucleotide A1408 but only with the intact 30S subunit as substrate, and that this methylation confers an aminoglycoside resistance spectrum typical of the m¹A1408 modification. Further, these results provide experimental confirmation of the

placement of each of the four putative aminoglycoside-resistance enzymes in the 16S rRNA (m¹A1408) methyltransferase family.

3.4. Binding of cosubstrate SAM and by-product SAH

The 16S rRNA (m¹A1408) methyltransferases use SAM as the source of the methyl group in their modification reaction. From the studies to date on NpmA [19], KamB and Kmr (Savic *et al.*, manuscript in preparation), a potential emerging hallmark of this enzyme family is their remarkably low relative affinity for SAM compared to the methylation reaction by-product SAH. To test whether the four newly confirmed 16S rRNA (m¹A1408) methyltransferases here possess similar properties, the binding of SAM and SAH to *CacKam*, *TcuKam*, *CarKam* and *UncKam* was measured using ITC (Fig. 4). The fits for all titrations produced reasonable stoichiometry (*n*), ranging from 0.7 to 1.1, indicating one SAM or SAH ligand bound per protein. Dissociation constants (*K_d*) obtained for SAM binding were in the low micromolar range (13–75 μM), consistent with the known 16S rRNA (m¹A1408) methyltransferases (Table 5). For three enzymes (*CacKam*, *TcuKam*, and *CarKam*), the characteristic higher SAH affinity was also observed, though the ~6-fold difference for both *TcuKam* and *CarKam* was less than the approximately 16-, 30- and 60-fold differences observed for *CacKam*, NpmA [19], and KamB (Savic *et al.*, manuscript in preparation), respectively. The most striking difference, however, was observed in the case of *UncKam* for which no detectable binding could be measured with SAH. Even with increased SAH concentration (2 mM) insufficient heat was released to detect an interaction. These cosubstrate binding analyses for the four new 16S rRNA (m¹A1408) methyltransferases indicate that a relatively low SAM affinity compared to SAH is a common but not universally conserved feature of this enzyme family.

4. Discussion

Acquired aminoglycoside-resistance methyltransferases have recently emerged as a significant new threat to the continued clinical use of these important drugs. To date, for the 16S rRNA (m¹A1408) methyltransferase family, the Gram-negative pathogen-derived enzyme NpmA and KamB from a Gram-positive aminoglycoside-producing bacterium have been structurally and functionally characterized. These recent studies have revealed likely conserved structural and functional features but also highlighted a number of potential critical differences in the details of their molecular mechanisms of action [19–21]. In particular, the observation that critical functional residues in one enzyme may not play a significant role in the other despite being highly conserved among all family members, underscores the fact that assignments of activity and mechanism are unreliable based upon sequence alone or a limited number of structures. Our goal in the present study was therefore to extend the known repertoire of aminoglycoside-resistance 16S rRNA (m¹A1408) methyltransferases. This work will serve as a platform for broadening our understanding of these important antibiotic resistance determinants that may identify novel variations of this resistance mechanism that could emerge clinically. Such understanding will be essential to circumvent the threat these enzymes pose when present in serious human pathogens.

4.1. Expansion of the 16S rRNA (m¹A1408) methyltransferase family

We expressed and functionally characterized four recently annotated hypothetical 16S rRNA (m¹A1408) methyltransferases and demonstrated each to be a *bona fide* resistance methyltransferase active in *E. coli*, and specifically modifying 16S rRNA residue A1408. MIC assays showed that each methyltransferase conferred a resistance spectrum characteristic of m¹A1408 modification, namely, high resistance to kanamycin, neomycin and apramycin, but low resistance to gentamicin. *CarKam* and *UncKam* conferred modestly lower MICs against each antibiotic which may reflect poorer methyltransferase expression or solubility under the conditions used resulting in decreased level of total 16S rRNA modification for these two enzymes. Reduction of *KamB* expression level can similarly modestly reduce the MIC values obtained under the experimental parameters used here (MAW and GLC, unpublished observations).

As more genome sequences are completed and with increased surveillance of resistant pathogens for the presence of aminoglycoside-resistance methyltransferases, many new enzymes are likely to be identified and classified by sequence homology to the currently annotated 16S rRNA (m¹A1408) methyltransferases. The present functional validation of a more diverse group of sequences encoding these enzymes than previously available will allow greater confidence that new assignments correctly reflect the likely function of the encoded protein. During the preparation of this manuscript, for example, one further hypothetical methyltransferase sequence was deposited from the candidate division WWE3 bacterium RAAC2_WWE3_1 (UniProt Accession: V5RTM5). With ~41% identity to *UncKam*, this sequence most likely also encodes an active 16S rRNA (m¹A1408) methyltransferase.

4.2. Common and divergent features of 16S rRNA (m¹A1408) methyltransferase activity

Detailed structural studies have been reported for the aminoglycoside-resistance methyltransferases NpmA and KamB in isolation, and for the former in complex with the 30S subunit [19–21]. These recent studies revealed a number of likely conserved mechanistic features such as the exploitation of a large, highly conserved tertiary surface of 16S rRNA to control substrate specificity, and ‘flipping’ of A1408 into the active site of the enzyme. However, the same studies also highlighted potentially significant differences among these enzymes including apparent differences in the role of conserved residues in interactions with 16S rRNA or in control of base flipping. Deeper understanding of the structural and mechanistic conservation (or otherwise) among these important resistance determinants will require further high-resolution structural and detailed mechanistic studies of other members of this enzyme family such as those reported here. The present studies have shown that each of the four newly confirmed 16S rRNA (m¹A1408) methyltransferases possess similar secondary structures in solution to each other and to other well characterized family members.

Each enzyme also binds SAM with an affinity comparable to that previously measured for NpmA and KamB. More substantial differences were observed in the relatively higher binding affinity for the reaction by-product (SAH) compared to SAM, which does not appear to be a universally conserved hallmark of these enzymes. Most remarkably, *UncKam*

showed a complete reversal of this trend; although we could measure a modest binding affinity for SAM with the same protein preparation, no binding was detected with SAH. The origin of this striking difference in SAH affinity for *UncKam* is not immediately obvious as the residues critical for cosubstrate binding in other family members are conserved [19,20]. However, the future elucidation of the *UncKam* structure promises to shed light on this question as well as potentially providing insight into the structural origins and functional implications of the unusual relative SAM/ SAH affinities of other the 16S rRNA (m¹A1408) methyltransferases.

4.3. Conclusions

Four hypothetical RNA methyltransferases from different environmental bacterial species were cloned, expressed in *E. coli* and experimentally placed in the 16S rRNA (m¹A1408) methyltransferase family. Our results further underscore the potential for novel functional and mechanistic variation among this group of enzymes and set the scene for further detailed structure-function studies that promise to uncover novel insights into the mechanisms of action of this important class of resistance determinants. Detailed knowledge of their common mechanisms of action will provide a platform for the future development of strategies to circumvent the high-level resistance they confer and facilitate the continued use of current drug treatments against otherwise resistant pathogens.

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Abbreviations used

CD	circular dichroism
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITC	isothermal titration calorimetry
MIC	minimum inhibitory concentration
NRSMD	normalized root mean square deviation
RT	reverse transcriptase
SAM	S-adenosyl-L-methionine
SAH	S-adenosylhomocysteine

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Highlights

- Four hypothetical aminoglycoside-resistance methyltransferases expressed.
- All are functional, conferring high resistance to kanamycin and apramycin.
- A1408 in the 30S ribosome subunit is the methylation target.
- Protein secondary structures consistent with known A1408 methyltransferases.
- Measured SAM/SAH affinities suggest differences in cosubstrate binding properties.

FIGURE 1. Cloning, expression and purification of putative aminoglycoside-resistance 16S rRNA methyltransferases acting at A1408

(A) Maximum likelihood (ML) phylogenetic tree of A1408 aminoglycoside-resistance methyltransferases inferred with MAFFT/UPGMA phylogeny [33] using the WAG amino acid substitution model. The robustness of each node was estimated by bootstrap analysis of 1000 replicates of the original data set. The values represent the confidence score for each branch and the bar represents amino acid replacements per position per unit evolutionary time. (B) Schematics of the pET and pET-HT expression constructs. Proteins expressed from pET-HT contain an N-terminal 6 × His (H) tag followed by a thrombin (T) recognition sequence for proteolytic cleavage at the site indicated (arrowhead). (C) SDS-PAGE analysis of the purification procedure (shown for CacKam, left) and other final purified proteins (right). The lanes indicated are uninduced cells (-), induced cells (+), soluble protein (S) following lysis by sonication, and after purification by Ni²⁺-affinity (1) and gel filtration chromatography (2). M is protein molecular weight marker (kDa).

FIGURE 2. Analysis of protein secondary structure using circular dichroism (CD) spectroscopy
 CD spectroscopic analysis of *CacKam* (open square/ orange), *TcuKam* (solid circle/ black), *CarKam* (open circle/ green), and *UncKam* (solid square/ blue). Each spectrum was deconvoluted to produce the estimated protein secondary structure contents shown in Table 3.

FIGURE 3. The four putative 16S rRNA methyltransferases require 30S as substrate and modify nucleotide A1408

Reverse transcription primer extension analysis of *in vitro* methylation reactions using (A) 30S subunits and (B) naked 16S rRNA as substrate. With 30S subunit as the substrate, reverse transcription was strongly terminated at nucleotide C1409, corresponding to modification of A1408 for KamB (positive control), CacKam, TcuKam, CarKam and UncKam. Nucleotide positions were identified by sequencing of unmodified 16S rRNA with ddNTPs (shown on left for ddTTP). No termination is observed for the negative control (no enzyme) which lacks A1408 modification, or for any enzyme with naked 16S rRNA as the substrate.

FIGURE 4. Isothermal titration calorimetry (ITC) analysis of SAM and SAH binding to the four methyltransferase enzymes

Example titrations of SAM with (A) *CacKam*, (B) *TcuKam*, (C) *CarKam*, and (D) *UncKam*.

(E)-(H) as (A-D) but with SAH.

Table 1

Origins and identifiers for the four putative A1408 16S rRNA methyltransferases

Bacterial Strain	NCBI Taxonomy	Phylum	Gene Name	UniProt Accession	Predicted Protein
<i>Catenulispora acidiphila</i> DSM 44928	479433	Actinobacteria	Caci_9046	C7Q5P8	CacKam 250 aa
<i>Thermomonospora curvata</i> DSM 43183	471852	Actinobacteria	Teur_0890	D1A6K4	TcuKam 221 aa
<i>Candidatus Arthromitus</i> sp. SFB-mouse-NYU	1073972	Fermicute	SFBNYU_002210	G4CBF6	CarKam 214 aa
Uncultured Bacterium	77133	Unknown (environmental sample)	ACD_24C00409G 0003	K2DC64	UncKam 214 aa

Table 2

Sequence comparisons among putative and confirmed A1408 methyltransferases

Sequence identity (similarity), %					
	<i>TcuKam</i>	<i>CarKam</i>	<i>UncKam</i>	<i>KamB</i>	<i>NpmA</i>
<i>CacKam</i>	55.8 (68.5)	17.4 (36.4)	23.5 (40.8)	32.3 (50.6)	25.6 (42.6)
<i>TcuKam</i>		22.5 (38.6)	24.9 (47.5)	35.4 (51.6)	29.7 (45.8)
<i>CarKam</i>			28.4 (52.3)	20.2 (41.7)	29.6 (51.3)
<i>UncKam</i>				21.0 (44.1)	29.8 (51.8)

^aSequence comparisons were performed with EMBOSS Needle [32].

Table 3

Predicted protein secondary structures from CD spectra deconvolution

Estimated secondary structure (%)					
	α	β	Turn	Other	NRSMD
<i>CacKam</i>	15	28	24	33	0.021
<i>TcaKam</i>	16	28	24	32	0.021
<i>CarKam</i>	14	32	23	31	0.041
<i>UncKam</i>	17	27	24	32	0.034
KamB, CD spectrum ^a	17	32	19	32	-
PDB: 3MQ2	30	29	24	17	-
NpmA, CD spectrum ^a	24	29	17	30	-
PDB: 3MTE	34	28	16	22	-

^aData from reference [23].

Table 4

Aminoglycoside resistance spectra

Enzyme (plasmid)	Minimum Inhibitory Concentration (µg/ml)			
	Kanamycin	Gentamicin	Neomycin	Apramycin
None (pET44a)	8	8	16	32
KamB (pET-HT)	>1024	16	256	>1024
CacKam (pET-HT)	>1024	64	512	>1024
(pET)	>1024	64	512	>1024
TcuKam (pET-HT)	>1024	32	256	>1024
(pET)	>1024	16	256	>1024
CarKam (pET-HT)	512	16	128	512
(pET)	512	16	128	1024
UncKam (pET-HT)	1024	16	64	1024
(pET)	1024	16	64	512

Table 5

SAM and SAH binding affinities

Enzyme	Kd (μM)	
	SAM	SAH
<i>CacKam</i>	44	2.7
<i>TcuKam</i>	13	1.9
<i>CarKam</i>	24	4.1
<i>UncKam</i>	75	NB ^a
<i>KamB</i> ^b	65	ND ^a
<i>NpmA</i> ^c	20	0.6

^aNB, no binding detected; ND, not determined.^bData from reference [23].^cData from reference [19].