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Heterologous Expression and Functional Characterization of the Exogenously Acquired Aminoglycoside Resistance Methyltransferases RmtD, RmtD2, and RmtG

Laís L. Corrêa,[a,b] Marta A. Witek,[a] Natalia Zelinskaya,[a] Renata C. Picão,[b] Graeme L. Conn[a]

Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia, USA[a]; Laboratório de Investigação em Microbiologia Médica, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil[b]

The exogenously acquired 16S rRNA methyltransferases RmtD, RmtD2, and RmtG were cloned and heterologously expressed in *Escherichia coli*, and the recombinant proteins were purified to near homogeneity. Each methyltransferase conferred an aminoglycoside resistance profile consistent with m^7G1405 modification, and this activity was confirmed by *in vitro* 30S methylation assays. Analyses of protein structure and interaction with *S*-adenosyl-L-methionine suggest that the molecular mechanisms of substrate recognition and catalysis are conserved across the 16S rRNA (m^7G1405) methyltransferase family.

The retained potency of aminoglycoside antibiotics has renewed interest in their use in clinical practice (1). However, among several resistance mechanisms, production of acquired 16S rRNA methyltransferases has emerged as a significant threat to clinical efficacy (2, 3). These enzymes modify the aminoglycoside 16S rRNA binding pocket to confer high-level aminoglycoside resistance. The predominant 16S rRNA modification in pathogenic bacteria is methylation of the N7 position of G1405 (m^7G1405), with nine distinct enzymes (ArmA and RmtA to -H) reported to date from clinical and veterinary isolates (3, 4).

Acquired 16S rRNA (m^7G1405) methyltransferases are globally distributed, and the genes that encode them typically reside within mobile elements that may coharbor additional resistance determinants (3). For example, RmtD was first detected in Brazil in *Pseudomonas aeruginosa* coproducing SPM-1, while both RmtD and RmtG were identified in *Klebsiella pneumoniae* coproducing KPC-2 and CTX-M (5, 6). Both enzymes and a variant of RmtD (RmtD2) were subsequently identified in *Enterobacter* spp., *Citrobacter freundii*, and *Escherichia coli* isolates from South and North America (7–10).

To extend our understanding of these resistance determinants, we cloned, expressed, and purified the acquired 16S rRNA (m^7G1405) methyltransferases RmtD, RmtD2, and RmtG. The genes that encode RmtD, RmtG, and RmtD2 were PCR amplified from template DNA extracted from an endemic SPM-1-producing *P. aeruginosa* isolate and two separate isolates from hospital-based outbreaks of *K. pneumoniae* in Brazil, respectively (5, 11; L.L.C. and R.C.P., unpublished data). Amplicons were cloned via the TOPO TA vector (Invitrogen) into a modified pET44a vector to generate 6×His-tagged methyltransferases with a thrombin cleavage site as described previously (12, 13). An equivalent construct was also generated by using an *E. coli* codon-optimized gene obtained by commercial chemical synthesis for the intrinsic 16S rRNA methyltransferase Sgm from *Micromonospora zionensis* for which m^7G1405 activity has been directly experimentally verified (14–16).

Recombinant proteins were expressed at 37°C in *E. coli* BL21(DE3) using lysogeny broth (500 ml) containing ampicillin (100 μg/ml). Protein expression was induced at mid-log phase (optical density at 600 nm, 0.6 to 0.8) with 0.5 or 1.0 mM isopropyl-β-D-thiogalactopyranoside, and growth was continued for 6 h at 30°C or for 3 h at 37°C for RmtD and all other proteins, respectively. Cells were harvested by centrifugation; resuspended in lysis buffer (5 ml/g of wet cells) containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol, and 10 mM imidazole; and lysed by sonication. Insoluble cell debris was removed by centrifugation, and target proteins were purified on an ÄKTApurifier10 system. First, Ni^{2+} affinity chromatography (HisTrap FF) was performed in lysis buffer with target protein elution accomplished by using a gradient of imidazole (10 to 300 mM). Target protein-containing fractions were pooled, concentrated, and further purified by gel filtration chromatography (Superdex 75 16/60) preequilibrated with 20 mM Tris buffer (pH 8.0) containing either 300 mM NaCl and 20% glycerol (RmtD and RmtD2) or 200 mM NaCl and 10% glycerol (RmtG). Sgm was purified by the same procedure but under previously established solution conditions (17). All of the proteins eluted from the gel filtration column and exhibited SDS-PAGE mobilities in good agreement with their calculated molecular weights (data not shown and Fig. 1A, respectively).

Measurements of aminoglycoside MICs were made as previously described (12) in liquid cultures of *E. coli* BL21(DE3) transformed with the empty pET vector, pET-HT*rmtD*, pET-HT*rmtD2*, or pET-HT*rmtG*. All three enzymes conferred high-level resistance to gentamicin and kanamycin but not to apramycin or neomycin (Table 1), a profile consistent with the m^7G1405 modification which confers resistance to 4,6-disubstituted deoxystreptamines but not other structural classes of aminoglycoside (3). All three enzymes also efficiently methylated 30S subunits in *in vitro* assays

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Address correspondence to Graeme L. Conn, gconn@emory.edu.

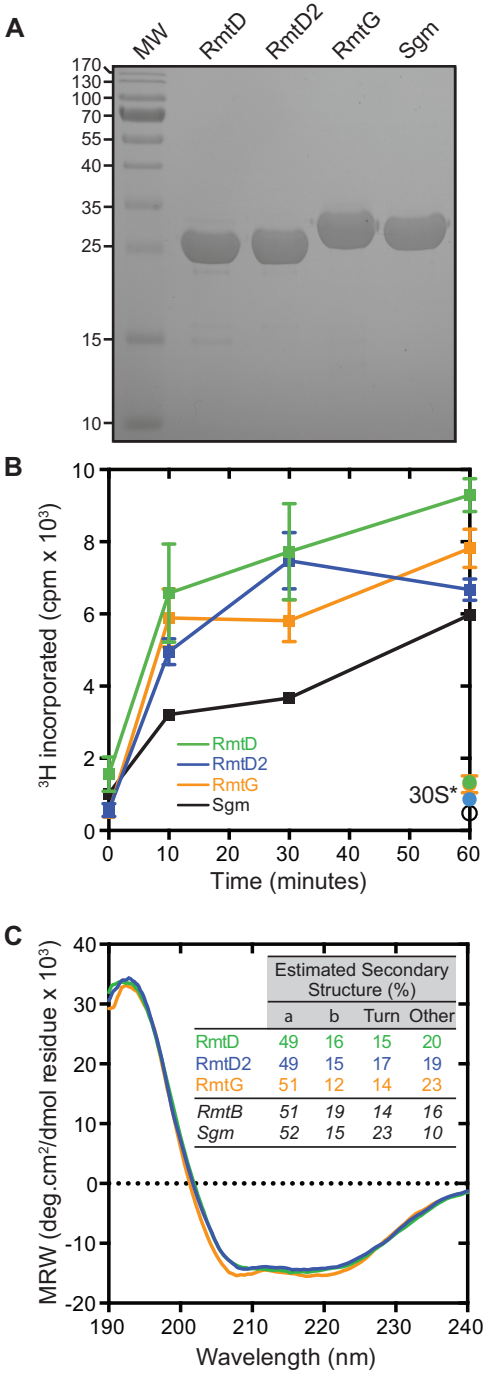

FIG 1 Expression and purification of active recombinant RmtD, RmtD2, and RmtG. (A) SDS-PAGE analysis of purified recombinant RmtD (29.5 kDa), RmtD2 (29.5 kDa), and RmtG (31.5 kDa). The intrinsic 16S rRNA (m^{7}G1405) methyltransferase Sgm (32.4 kDa) is shown for comparison. MW, protein molecular weight standards (protein masses in kDa are shown to the left). (B) *In vitro* time course methyltransferase assays of RmtD (green), RmtD2 (blue), and RmtG (orange) using 30S subunits isolated from *E. coli* MRE600 in the presence of [^{3}H]SAM. Single 60-min time points are also shown for assays using m^{7}G1405 modified 30S (30S*) isolated from *E. coli* BL21(DE3) expressing Sgm. (C) Circular dichroism spectroscopy analyses of RmtD, RmtD2, and RmtG. Spectra were deconvoluted to estimate the secondary-structure content shown in the inset. Values for RmtB (Protein Data Bank code 3FRH) and Sgm (Protein Data Bank code 3LCV) were calculated from X-ray crystal structures via the STRIDE webserver (24). MRW, mean residue molar ellipticity.

TABLE 1 Aminoglycoside MICs for *E. coli* harboring plasmids encoding acquired resistance methyltransferases

Plasmid	MIC (μg/ml)			
	Apramycin	Gentamicin	Kanamycin	Neomycin
pET vector	32	4	16	16
pET-HT_*rmtD*	32	>1,024	>1,024	16
pET-HT_*rmtD2*	32	>1,024	>1,024	16
pET-HT_*rmtG*	32	>1,024	>1,024	16

using *S*-adenosyl-L-[^{3}H]methionine ([^{3}H]SAM) (13), with activity comparable to that of Sgm (Fig. 1B) and other Rmt enzymes (18, 19). In contrast, in assays using 30S from Sgm-expressing cells, no additional methylation was observed (Fig. 1B, 30S*). These results demonstrate that each purified recombinant protein is active and that RmtD, RmtD2, and RmtG modify the N7 position of 16S rRNA nucleotide G1405.

We next used circular dichroism spectroscopy and deconvolution using the CDSSTR algorithm via DICHROWEB (20) to assess the solution structure of each methyltransferase (Fig. 1C) as previously described (12). All three methyltransferases were well folded with predicted secondary-structure contents in excellent agreement with those calculated from the high-resolution structures of Sgm and RmtB (21, 22).

The aminoglycoside resistance methyltransferases require SAM as their obligatory cosubstrate (methyl group donor) and produce *S*-adenosylhomocysteine (SAH) as the methylation reaction by-product. Analyses of enzymes that catalyze the m^{1}A1408 modification have revealed a characteristic, though not universal, higher relative affinity for SAH than for SAM (12, 23). In contrast, for Sgm, SAH binding was reported to be several hundred times weaker despite its comparable affinity for SAM (21). We therefore used isothermal titration calorimetry (ITC) to measure the affinities of RmtD, RmtD2, and RmtG for SAM and SAH (Fig. 2 and Table 2). Purified proteins were dialyzed against a mixture of 20 mM Tris (pH 8.0), 300 mM NaCl, 20% glycerol, and 10 mM β-mercaptoethanol, except Sgm, for which previously established solutions were used (17). SAM (1.0 to 1.5 mM) and SAH (0.4 to 0.8 mM) were prepared by using the final dialysis solutions, and titrations were performed on an Auto-iTC$_{200}$ microcalorimeter (Malvern/Microcal) as described previously (12). All SAM binding affinities were in the low micromolar range (Table 2), consistent with previous measurements for Sgm and other RNA methyltransferases (17, 21). Surprisingly, all methyltransferases had significantly greater affinity for SAH, including Sgm, contradictory to the prior report (21). We considered whether differences in solution conditions might have affected the measured affinities. We first attempted to dialyze Sgm against the conditions used previously, where weaker SAH binding was observed (21), but found that the protein consistently precipitated from solution. As an alternative comparison, titrations were performed with Sgm under the conditions used for the acquired methyltransferases and for RmtD2 under our prior conditions for Sgm (17). Regardless of the solution conditions used, essentially identical SAM and SAH affinities for Sgm and RmtD2 were measured in each case.

Defining the molecular mechanisms of antimicrobial resistance is critical to support the development of new effective strategies to combat multidrug-resistant pathogens. Here, we have es-

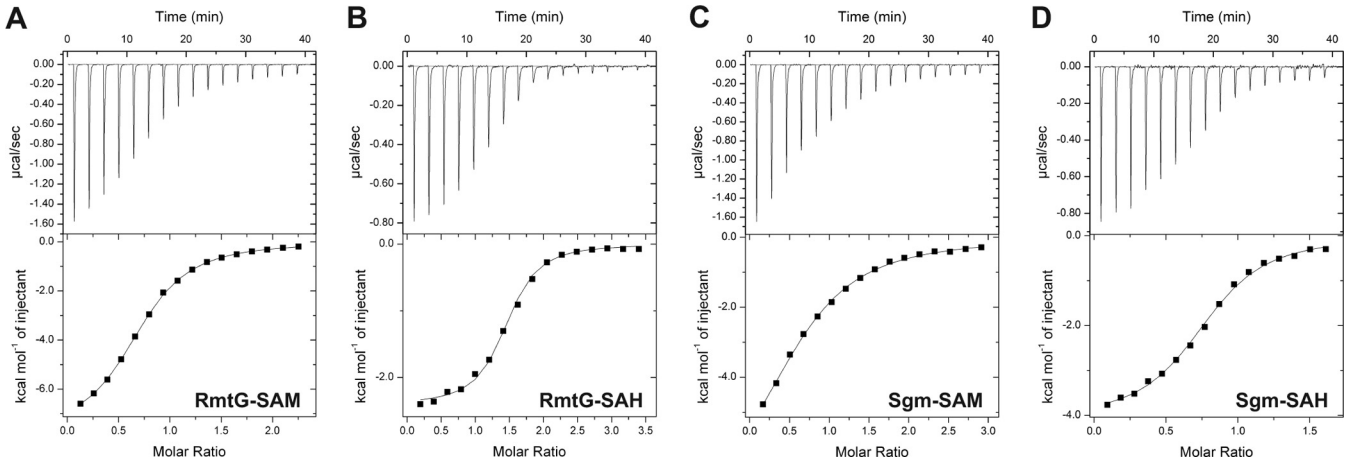

FIG 2 ITC analysis of RmtG and Sgm ligand binding. Example titrations of RmtG with SAM (A) and SAH (B) and of Sgm with SAM (C) and SAH (D). Titrations were fitted with a one binding site model to obtain binding affinities (K_d) for all protein ligand pairs (see Table 2).

tablished recombinant expression of active RmtD, RmtD2, and RmtG methyltransferases, providing protein samples suitable for detailed structure-function studies. Our results show that each protein likely adopts a structure very similar to that of characterized m^{7}G1405 enzymes and indicate that higher affinity for SAH than for SAM may be a feature common to all aminoglycoside resistance methyltransferases. For both groups of enzymes, the potential for product inhibition may contribute to the regulation of methyltransferase activity and/or control of substrate specificity. In contrast, contrary to the emerging evidence for the m^{1}A1408 methyltransferase family, our findings suggest that among the 16S rRNA (m^{7}G1405) methyltransferases, the molecular mechanisms of substrate recognition and catalysis are likely to be highly conserved between intrinsic and acquired enzymes. Therefore, the development of inhibitors that are broadly effective against this resistance enzyme family may be feasible.

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TABLE 2 Binding affinities of aminoglycoside resistance 16S rRNA (m^{7}G1405) methyltransferases for SAM and SAH measured by ITC

Class and enzyme	K_d (μM)[a]		Reference
	SAM	SAH	
Acquired m^{7}G1405			
RmtD	42 ± 19	2.0 ± 0.2	This study
RmtD2	29 ± 5[b]	1.2 ± 0.3[b]	
RmtG	11.4/10.0	3.7/1.0	
Producer m^{7}G1405			
Sgm	25 ± 16[b]	4.8 ± 2.6[b]	This study
Sgm	17.6	ND[c]	17
Sgm	18	300	21

[a] Measurements were made in triplicate with independent samples and are shown as means ± standard deviations, except for RmtG, for which only two independent measurements were made (values from both experiments are shown). Errors associated with fits to one binding site model were negligible compared to those between measurements.
[b] Binding was assessed under two different solution conditions with essentially identical results.
[c] ND, not determined.

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