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The Pathogen-Derived Aminoglycoside Resistance 16S rRNA Methyltransferase NpmA Possesses Dual m^1A1408/m^1G1408 Specificity

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Chemical modification of 16S rRNA can confer exceptionally high-level resistance to a diverse set of aminoglycoside antibiotics. Here, we show that the pathogen-derived enzyme NpmA possesses dual m^1A1408/m^1G1408 activity, an unexpected property apparently unique among the known aminoglycoside resistance 16S rRNA (m^1A1408) methyltransferases. Although the biological significance of this activity remains to be determined, such mechanistic variation in enzymes acquired by pathogens has significant implications for development of inhibitors of these emerging resistance determinants.

Methylation of 16S rRNA has emerged as a significant new threat to the efficacy of aminoglycoside antibiotics, particularly against Gram-negative pathogens (1), with two families of resistance methyltransferase defined by the modifications they produce, m^7G1405 and m^1A1408 (Fig. 1A). Structural and functional studies of enzymes from each family, and of both aminoglycoside producer and pathogenic origin, have firmly established them as class I methyltransferases (2) and have begun to reveal the molecular details of their interactions with the cosubstrate *S*-adenosyl-L-methionine (SAM) and the 30S subunit substrate (3–7).

Despite these advances, important questions remain about the molecular mechanisms of action of these enzymes. For example, studies of the pathogen-derived 16S rRNA (m^1A1408) methyltransferase NpmA (8) and the orthologous enzyme Kmr from *Sorangium cellulosum* So ce56 (9) revealed a critical but undefined role for the 30S in promoting SAM binding and/or catalysis. We therefore sought to establish a system to dissect the mechanism by which the 30S subunit regulates m^1A1408 methyltransferase activity. To this end, we obtained the wild-type (Δ7prrn) *Escherichia coli* strain SQZ10 and its 16S rRNA A1408G variant (Δ7prrn-A1408G), in which all chromosomal rRNA operons are replaced by a single plasmid-borne copy (10, 11). Our expectation was that subunits isolated from the A1408G strain would allow analysis of 30S enzyme-SAM interactions in the absence of the modification reaction.

We first used an established *in vitro* methylation assay with [^{3}H]SAM (12), to verify that neither NpmA nor KamB, a 16S rRNA (m^1A1408) methyltransferase from the tobramycin producer *Streptoalloteichus tenebrarius*, had activity against the 30S-A1408G subunits. To our surprise, however, we found that NpmA, but not KamB, did in fact appear to methylate the variant 30S subunits (Fig. 1B), and we decided to examine this unexpected enzymatic activity.

To identify the site of modification in 30S-A1408G, we performed two additional assays. First, reverse transcription (RT) of 16S rRNA extracted from untreated and NpmA- or KamB-treated wild-type and 30S-A1408G subunits was used to visualize the modified nucleotide (Fig. 1C and D). Correlating with the [^{3}H]SAM assays, a strong stop corresponding to m^1A1408 modification was observed for both enzymes with wild-type 30S, whereas only NpmA treatment resulted in a band at the equivalent position for 30S-A1408G subunits. Additionally, we observed no difference in NpmA modification of wild-type 30S isolated from the Δ7prrn strain and *E. coli* MRE600, typically used in these assays (12, 13). Second, we isolated wild-type 30S subunits from *E. coli* BL21(DE3) cells grown without and with KamB overexpression, and tested the activity of NpmA and KamB against these unmodified and m^1A1408 premodified substrates using the [^{3}H]SAM assay (Fig. 1E). Neither NpmA nor KamB could incorporate ^{3}H into subunits previously modified with m^1A1408, demonstrating that NpmA does not possess activity at an alternative 16S rRNA nucleotide outside the region examined in our RT assay. From these experiments, we conclude that NpmA methylates 30S-A1408G subunits at nucleotide 1408 and thus possesses dual A1408/G1408 modification activity.

To address the question of whether this novel activity is unique to NpmA, we tested the ability of Kmr and five other 16S rRNA (m^1A1408) family members (13, 14) to modify the 30S-A1408G subunits. Although all enzymes robustly modified wild-type subunits *in vitro*, none possessed catalytic activity with the 30S-A1408G substrate (Fig. 1F). Thus, among the collection of enzymes tested, dual A1408/G1408 activity is unique to the sole pathogen-derived 16S rRNA (m^1A1408) methyltransferase, NpmA.

The m^1A1408 methyltransferases are structurally most similar to the tRNA methyltransferase TrmB (3, 5), which catalyzes an m^7G modification (at tRNA position 46). NpmA might therefore feasibly catalyze either m^1G or m^7G modification, which would require either altered catalysis (but with similar

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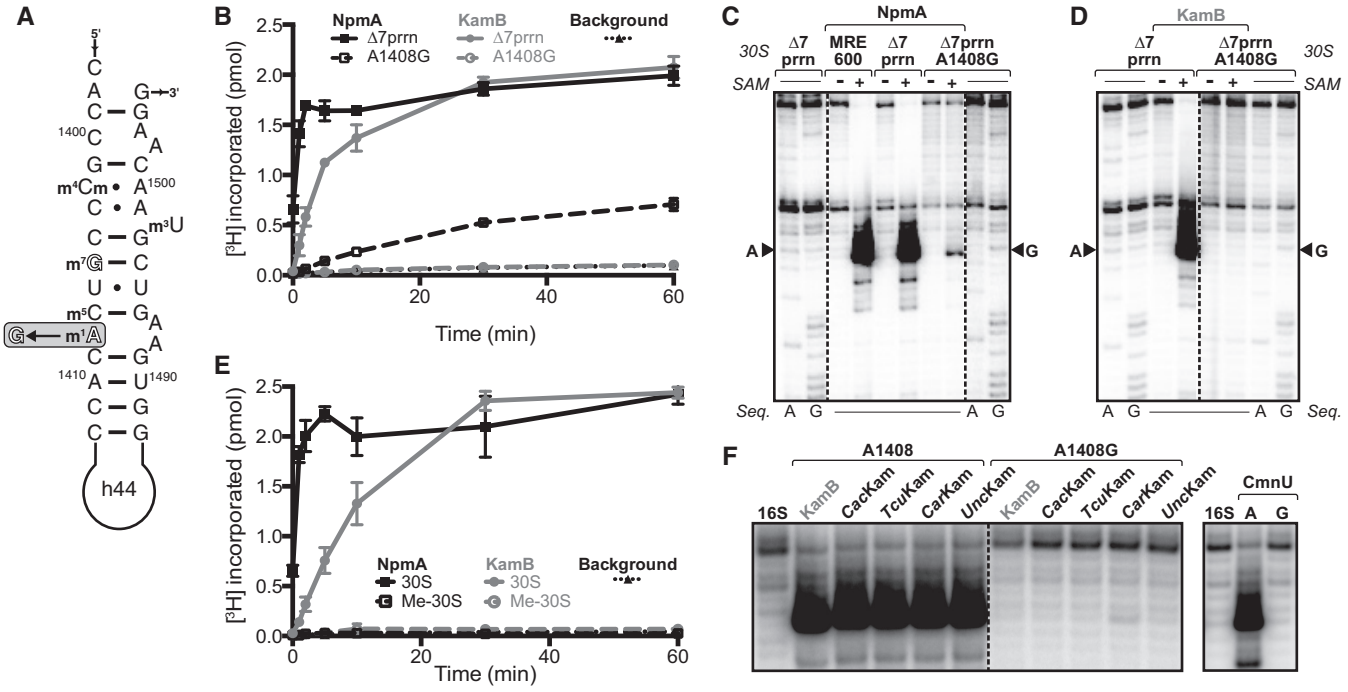

FIG 1 NpmA methylates 30S subunits with G1408. (A) Sequence and secondary structure of the h44 region containing the target nucleotides (outline font) of the m^{7}G1405 and m^{1}A1408 aminoglycoside-resistance 16S rRNA methyltransferases. The mutation in the 30S-A1408G subunit (shaded box) used in this study and the locations of intrinsic 16S rRNA modifications in this region are also indicated. (B) *In vitro* methylation in the presence of [^{3}H]SAM of Δ7prrn wild-type and 30S-A1408G subunits by NpmA (black) and KamB (gray). NpmA, but not KamB, can methylate 30S-A1408G subunits (dashed black line). (C and D) RT analysis of 16S rRNA methylation by NpmA (C) and KamB (D) using 30S subunits from *E. coli* MRE600 (A1408), Δ7prrn (A1408), and Δ7prrn-A1408G. Sequencing lanes (Seq.) contain the complementary dideoxynucleotide in the RT reaction. The position of modified 1408 is marked by arrowheads. (E) *In vitro* methylation in the presence of [^{3}H]SAM of wild-type unmethylated (30S) and m^{1}A1408 premethylated (Me-30S) subunits by NpmA (black) and KamB (gray). (F) RT analysis of modification of wild-type or 30S-A1408G subunits by five other 16S rRNA (m^{1}A1408) methyltransferases (13, 14); each enzyme is capable of incorporating only the m^{1}A1408 modification. The origins of the enzymes not described in the text are *Catenulispora acidiphila* (*Cac*Kam; UniProt accession C7Q5P8), *Thermomonospora curvata* (*Tcu*Kam; D1A6K4), *Candidatus* Arthromitus sp. SFB-mouse-NYU (*Car*Kam; F9VLU6), an uncultured bacterium (UncKam; K2DC64), and *Saccharothrix mutabilis* subsp. *capreolus* (CmnU; A6YEH1).

target base orientation) or substantially altered target positioning (with similar chemistry), respectively. Modification at the guanine 7 position produces a weak RT stop but can be enhanced by sodium borohydride ($NaBH_4$) reduction of the modification and subsequent cleavage of the RNA chain by β-elimination at the abasic site generated (15, 16). We measured the effect of $NaBH_4$ treatment on the intensity of RT stop generated by NpmA modification of G1408 and the adjacent m^{7}G1405 modification catalyzed by Sgm (17, 18) as a positive control. Whereas the band corresponding to m^{7}G1405 was enhanced 3.8-fold, no significant change was observed for G1408 (~1.1-fold increase) (Fig. 2A). Next, to directly examine the modified nucleotide, we isolated the rRNA fragment corresponding to nucleotides C1378-G1432 using a complementary

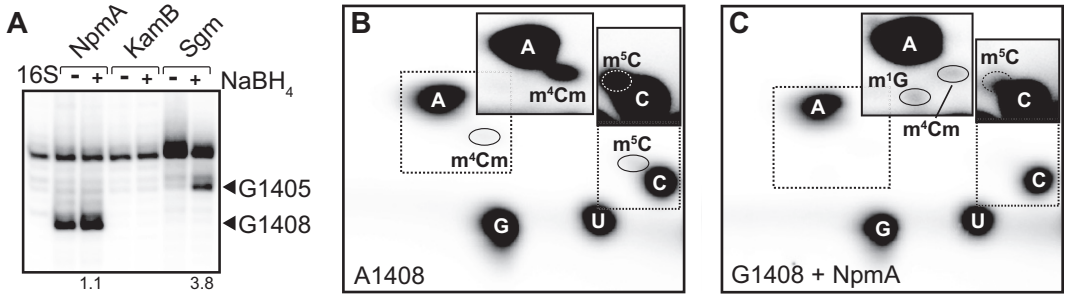

FIG 2 NpmA methylates G and A at nucleotide 1408 at the same position (N1). (A) RT analysis of (left to right) unmodified 16S rRNA (16S), NpmA or KamB *in vitro*-modified 16S rRNA extracted from Δ7prrn-A1408G 30S subunits with and without subsequent sodium borohydride treatment, and equivalent analysis of *in vivo*-modified 16S rRNA from wild-type *E. coli* BL21(DE3) cells expressing the m^{7}G1405 methyltransferase Sgm. The values below the gel are fold enhancement with $NaBH_4$ treatment (+). (B and C) TLC analysis of the nucleoside composition of the 16S rRNA fragment corresponding to nucleotides 1378 to 1432 from unmodified wild-type (B) and NpmA-modified Δ7prrn-A1408G (C) 30S subunits. Regions boxed with a dotted line are duplicated in the insets (solid lines) with 20×-increased contrast to allow visualization of the low-abundance modified nucleotides.

deoxyribonucleotide (18) and examined the rRNA nucleotide composition using an established thin-layer chromatography (TLC) system (solvents A and B in reference 19). Comparison of unmodified wild-type and NpmA-modified 30S-A1408G rRNA revealed a new spot at the known position of m^1G, confirming this as the modification incorporated by NpmA (Fig. 2B and C).

The TLC analyses also identified the previously unmapped position of the m^4Cm modification in this solvent system (Fig. 2B and C). The reduction in intensity of the spot derived from $m^4Cm1402$ and that from m^5C1407 (Fig. 1) further suggests that A1408G mutation and/or N1 methylation directly impacts the activity of the enzymes (RsmH/RsmI and RsmF, respectively) which otherwise stoichiometrically modify these rRNA residues (20).

Several instances have been documented of RNA methyltransferases that catalyze the same modification on more than one site, either in the same or on two different substrates. However, dual nucleotide specificity may be much rarer, with only a single example of a class IV (SPOUT family) methyltransferase from the euryarchaeon *Thermococcus kodakaraensis* being reported to catalyze tRNA m^1A9/m^1G9 modification (21). Our finding that the class I NpmA possesses such dual activity is particularly remarkable given that NpmA makes only a single direct base interaction with the 16S rRNA (to the A1408 N6 amine), which should uniquely select for adenine (7). How NpmA adjusts its active site to accommodate the guanine base and accomplish methylation of the protonated N1 atom remains to be determined. We speculate that NpmA may have retained ancestral m^1G activity, but given that A1408G itself confers exceptionally high-level aminoglycoside resistance, it is unclear why NpmA would have retained this activity. G1408 is found in a small fraction of bacteria, and we speculated that N1 methylation might overcome a growth deficit caused by guanine at this position in the ribosomal decoding center. However, we could not detect a significant difference in growth between the wild-type and A1408G Δ7prrn strains.

In conclusion, we have found that the pathogen-derived 16S rRNA (m^1A1408) methyltransferase NpmA possesses dual nucleotide specificity and is capable of catalyzing both m^1A1408 and m^1G1408 modifications. While the biological significance of NpmA's dual specificity is presently unclear, the finding that a pathogen-derived enzyme might possess a catalytic capacity significantly different from that of orthologous enzymes from other bacteria has the potential to confound development of specific inhibitors of such resistance determinants.

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