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Indigenous and acquired modifications in the aminoglycoside binding sites of *Pseudomonas aeruginosa* rRNAs

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Abbreviations: MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; rRNA, ribosomal RNA; H44, helix 44; H69, helix 69; H45, helix 45; 2-DOS, 2- deoxystreptamines; MIC, minimal inhibitory concentration

Aminoglycoside antibiotics remain the drugs of choice for treatment of *Pseudomonas aeruginosa* infections, particularly for respiratory complications in cystic-fibrosis patients. Previous studies on other bacteria have shown that aminoglycosides have their primary target within the decoding region of 16S rRNA helix 44 with a secondary target in 23S rRNA helix 69. Here, we have mapped *P. aeruginosa* rRNAs using MALDI mass spectrometry and reverse transcriptase primer extension to identify nucleotide modifications that could influence aminoglycoside interactions. Helices 44 and 45 contain indigenous (housekeeping) modifications at $m^4Cm1402$, m^3U1498, m^2G1516, m^6_2A1518, and m^6_2A1519; helix 69 is modified at $m^3\Psi1915$, with m^5U1939 and m^5C1962 modification in adjacent sequences. All modifications were close to stoichiometric, with the exception of $m^3\Psi1915$, where about 80% of rRNA molecules were methylated. The modification status of a virulent clinical strain expressing the acquired methyltransferase RmtD was altered in two important respects: RmtD stoichiometrically modified m^7G1405 conferring high resistance to the aminoglycoside tobramycin and, in doing so, impeded one of the methylation reactions at C1402. Mapping the nucleotide methylations in *P. aeruginosa* rRNAs is an essential step toward understanding the architecture of the aminoglycoside binding sites and the rational design of improved drugs against this bacterial pathogen.

Introduction

Aminoglycosides are a group of broad-spectrum antibiotics that are widely used against Gram-positive and Gram-negative bacteria. The aminoglycoside tobramycin has found extensive application in the treatment of *Pseudomonas aeruginosa* infections, particularly for cystic fibrosis patients where this Gram-negative pathogen is a frequent cause of pulmonary complications.[1] Over 60% of cystic fibrosis patients are chronically infected with *P. aeruginosa* by the age of 20.[2] Treatment with tobramycin prolongs life expectancy, although the drug has a mainly palliative role reducing the intensity of infections without measurable success at eradicating them.[3] For want of better therapeutics, however, aminoglycosides will undoubtedly retain an important role in the immediate future.[4] Treatment is generally via tobramycin inhalation,[5] and recent improvements in therapy have been confined to the drug delivery systems. Clearly, more effective aminoglycosides are now required and any rational development of these compounds will require a thorough understanding of the rRNA targets. The rRNAs of *P. aeruginosa* have previously remained uncharted territory.

Aminoglycoside antibiotics, including tobramycin, exert their antimicrobial effects by disturbing protein synthesis on the bacterial ribosome.[6,7] There are two sites of aminoglycoside interaction on the ribosome, and the primary drug target is at the mRNA decoding region on the 30S ribosomal subunit (**Fig. 1A**). Here, aminoglycosides interact with highly conserved nucleotides at the ribosomal A site of 16S rRNA helix 44 (H44) where codon–anticodon interactions are monitored.[8-12] Drug interaction here causes loss of translational fidelity, the accumulation of erroneous proteins and, ultimately, bacterial death.[6,13] Recently, a second aminoglycoside site was discovered within another functionally important region on the 50S ribosomal subunit in helix 69 (H69) of 23S rRNA.[14,15] H69 interacts with H44 to form interbridge B2a at the interface of the two subunits.[16] Subsequent to termination of mRNA translation,[17] ribosome recycling factor binds to this interbridge and, in conjunction with elongation factor G, dissolves the H69-H44 interaction enabling the two ribosomal subunits to separate. Aminoglycoside binding within H69 (**Fig. 1B**) stabilizes the interbridge contacts and interferes with the subunit recycling process.[14,18]

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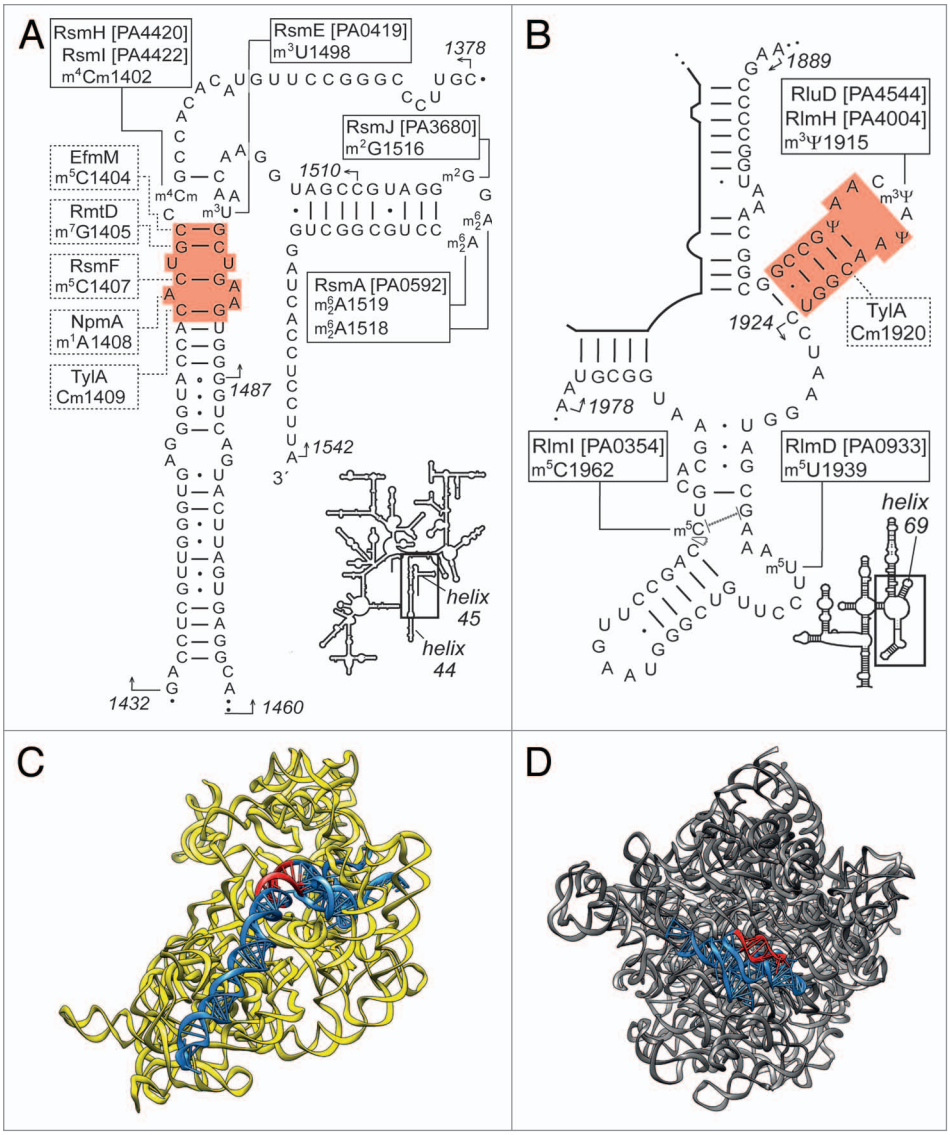

Figure 1. Aminoglycoside binding-sites (red) within the rRNAs of *P. aeruginosa*. (**A**) Helices 44 and 45 of 16S rRNA showing the indigenous housekeeping methylations mapped in this study at $m^4Cm1402$, m^3U1498, m^2G1516, m^6_2A1518, and A^6_2A1519, together with the respective enzymes and *P. aeruginosa* gene loci (unbroken lines and boxes). The acquired methylation catalyzed by RmtD at m^7G1405 (dashed lines and boxes) confers tobramycin resistance. Modifications seen in other bacteria (but not in *P. aeruginosa*) together with the associated methyltransferases include: m^5C1404 catalyzed by EfmM[33]; m^5C1407 by RsmF[22]; m^1A1408 by NpmA38; and Cm1409 by TlyA,[43] and are also indicated by dashed lines. (**B**) Modification at $m^3\Psi1915$ within the loop of 23S rRNA helix 69, and in neighboring structures at m^5U1939 and m^5C1962; *P. aeruginosa* lacks TlyA and, thus, the second target of this enzyme at C1920 is unmodified. Uridine isomerisation is mass neutral and the Ψ1911 and Ψ1917 modifications remain putative. Start and end points of the oligos used for rRNA isolation and MS analyses are shown by the arrows. (**C**) Positions of helices 44 and 45 (blue) with the aminoglycoside site highlighted (red) on the 16S rRNA tertiary structure (redrawn from PDB file 3I1M of the *E. coli* 30S subunit with r-proteins removed). (**D**) Helix 69 (red) with the surrounding 23S rRNA sequences in blue (redrawn from 3I1P, *E. coli* 50S subunit with r-proteins removed).

Helices 44 and 69 and adjacent rRNA sequences are targeted by several nucleotide modification enzymes, consistent with the notion that modifications tend to occur in rRNA regions that are of primary functional importance.[19,20] The sites of the indigenous (housekeeping) modifications have been comprehensively mapped within *Escherichia coli* rRNAs, and consist of 11 modified nucleotides in 16S rRNA and 25 in 23S rRNA. Housekeeping modifications are added within H44 by the methyltransferases RsmH and RsmI at nucleotide $m^4Cm1402$,[21] by RsmF at m^5C1407,[22] and by RsmE at m^3U1498.[23] Within the loop of the adjacent 16S rRNA helix H45, m^2G1516 is modified by RsmJ,[24] and m^6_2A1518 and m^6_2A1519 by RsmA.[25,26] At the second aminoglycoside side in H69 of 23S rRNA, three uridine isomerizations are introduced in the loop by the enzyme RluD to form Ψ1911, Ψ1915, and Ψ1917[19] and Ψ1915 is then methylated at the N3-position by RlmH.[27,28] Other methylations in the same region at nucleotides m^5U1939 and m^5C1962 are catalyzed by RlmD[29,30] and by RlmI,[31] respectively. Orthologs of some of the genes encoding these modification enzymes are evident in other Gram-negative bacteria.[32]

In addition to the enzymes that add the rRNA housekeeping modifications, several other methyltransferases modify 16S rRNA H44, and in doing so confer resistance to aminoglycosides. Such is the case with methylation at m^5C1404,[33] m^7G1405, and m^1A1408 (**Fig. 1A**). Methyltransferases directing the latter two modifications are more prevalent in clinical pathogens and show similarity to enzymes originally identified in aminoglycoside-producing actinomycetes.[34,35] The m^7G1405 methyltransferases include ArmA and RmtA-RmtH,[36-39] and confer high-level resistance to 4,6-disubstituted 2-deoxystreptamines (2-DOS) aminoglycosides, including tobramycin; the m^1A1408 methyltransferases include NpmA, and these enzymes confer resistance to both 4,5-disubstituted 2-DOS and 4,6-disubstituted 2-DOS aminoglycosides.[40]

Recently, the *rmtD* methyltransferase gene was shown to be prevalent in clinical isolates of *P. aeruginosa* that are highly resistance to tobramycin.[41] Here, we map the housekeeping modifications within the aminoglycoside binding sites of 16S and 23S rRNAs from the aminoglycoside-sensitive *P. aeruginosa* strain PAO1 and a resistant clinical strain containing *rmtD*. We address the questions as to whether any of the housekeeping modifications could affect drug binding at either of the

Table 1. Modified nucleotides within the aminoglycoside binding sites of 16S and 23S rRNAs

	rRNA modification	*E. coli* genes encodingmodifying enzymes		Reference for *E. coli*	Putative PAO1 genes encoding modifyin enzymes	% ID PAO1	% G+C content of gene orthologs	
		Name	Synonym(s)		Name		*E. coli*	PAO1
16S rRNA								
1402	m^4C	*rsmH*	*mraW*	21	PA4420	53.3	55.6	64.4
1402	Cm	*rsmI*	*yraL*	21	PA4422	58.3	54.6	67.4
1407	m^5C	*rsmF*	*yebU*	22	*no*	*no*	51.8	*no*
1498	m^3U	*rsmE*	*yggJ*	23	PA0419	46.1	53.0	71.2
1516	m^2G	*rsmJ*	*yhiQ*	24	PA3680	43.3	58.2	71.4
1518, 1519	m^6_2A	*rsmA*	*ksgA*	25, 26	PA0592	48.3	53.0	66.0
23S rRNA								
1911, 1915, 1917	Ψ	*rluD*	*yfiI*	19	PA4544	54.6	52.8	66.9
1915	$m^3\Psi$	*rlmH*	*ybeA*	27, 28	PA4004	53.8	58.5	68.6
1939	m^5U	*rlmD*	*ygcA, rumA*	29, 30	PA0933	36.5	51.5	67.1
1962	m^5C	*rlmI*	*yccW*	31	PA0354	33.5	51.8	67.4

Putative methyltransferases were identified in silico in the genome of *P. aeruginosa* strain PAO1 by their similarity to the *E. coli* enzymes (gene annotations shown here). The percentages of identical amino acids in the encoded enzymes are indicated (% ID PAO1). The G–C base-pair content is given for each gene (% G+C), and can be compared with the overall G–C content within the *E. coli* genome (50.8%) and the *P. aeruginosa* PAO1 genome (66.6%). *no*, no orthologous gene.

aminoglycoside sites, and whether the acquisition of RmtD activity hindered any of the indigenous methyltransferases in gaining access to their nucleotide targets.

Results and Discussion

Modifications at 16S rRNA nucleotides $m^4Cm1402$, m^3U1498, m^2G1516, m^6_2A1518, and m^6_2A1519. The *P. aeruginosa* PAO1 genome contains genes for several enzymes that share significant similarity with characterized *E. coli* rRNA methyltransferases (**Table 1**). To ascertain whether the PAO1 orthologs are expressed and do in fact modify the main aminoglycoside binding site at the decoding region of 16S rRNA, this region was analyzed using MALDI-MS and reverse transcriptase primer extension.

The sequences 1460–1510 and 1487–1542, which make up H45 and the 3'-portion of the decoding site in H44, were shown to contain several methylated nucleotides. The MS spectra clearly showed the presence of m^6_2A1518 and m^6_2A1519 in the RNase T1 fragment $[m^6_2A][m^6_2A]CCUG$ (A1518-G1523) at *m/z* 1976 (**Fig. 2A**), as well as in the RNase A fragment $AGG[m^2G]G[m^6_2A][m^6_2A]C$ (A1513-C1520) at *m/z* 2761 (**Fig. 2B**). There was no evidence of peaks with masses that were smaller by multiples of 14 Da (indicating a proton rather than a methyl group), and it was concluded from this that both A1518 and A1519 were completely (stoichiometrically) dimethylated. These modifications are added in *E. coli* by RsmA (KsgA)[25,26] at a late assembly stage of the 30S ribosomal subunit,[42] and are presumably mediated in the same manner by the homologous *P. aeruginosa* enzyme encoded by the PA0592 gene (**Table 1**). The *m/z* 2761 peak also contained stoichiometric amounts of a fifth methyl group that was mapped to nucleotide G1516. Modification at m^2G1516 is catalyzed on the *E. coli* 30S subunit by RsmJ,[24] and the homologous enzyme is presumably encoded by PA3680 in *P. aeruginosa*.

Screening the sequence between H44 and H45 by primer extension produced an intense stop band indicating the presence of a modification on nucleotide U1498 (**Fig. 3A**). Methylation at the 5-position of the uracil or 2'-*O* of the ribose could be excluded, as these would have respectively resulted in either no stop or merely a partial pausing of reverse transcription,[43] so it was concluded that this modification was m^3U1498. The level of m^3U modification was quantified as being > 97% (**Fig. 3A**). The primer extension data was fully consistent with the MS analyses

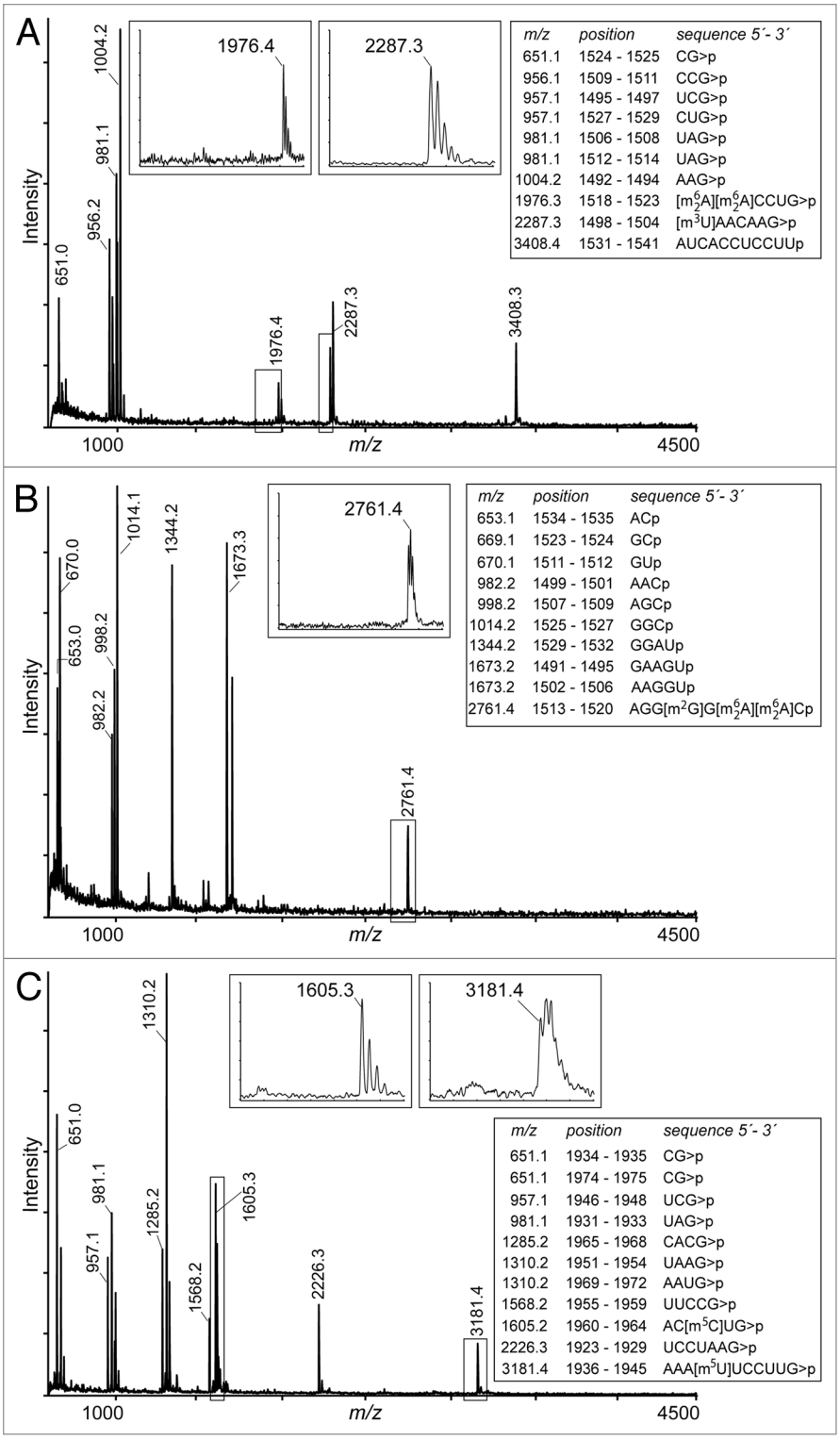

Figure 2. MALDI-MS analyses of the aminoglycoside sites within *P. aeruginosa* rRNAs. (**A**) RNase T1 digestion products of the 16S rRNA sequence G1487 to A1542 reveals the dimethylated adenosines m^6_2A1518 and m^6_2A1519 in the *m/z* 1976 peak. The lack of significant peaks smaller by multiples of 14 Da (box) indicates that addition of these four methyl groups by RsmA is essentially stoichiometric. The m^3U1498 modification is seen in the *m/z* 2287 peak, and is also stoichiometric. Masses are shown for the 2'-3'-cyclic phosphate fragments (> *P*) with the exception of the 3'-fragment (*m/z* 3408) where the ultimate adenosine A1542 was missing. (**B**) RNase A analysis of the same sequence. The two adenosines m^6_2A1518 and m^6_2A1519 are in the *m/z* 2761 peak with m^2G1516; all five methylations are stoichiometric (box). Masses are with a linear (hydrated) 3'-phosphate (p). (**C**) RNase T1 digestion products of the 23S rRNA sequence C1924 to G1978. Nucleotide m^5C1962 migrates in the fragment at *m/z* 1605, and m^5U1939 at *m/z* 3181. Enlargements of these regions (boxes) show that methylation reactions by RlmI and RlmD, respectively, are close to complete. Spectra from these rRNA regions of *P.aeruginosa* strains BB1285 and PAO1 (with and without RmtD) were indistinguishable. The theoretical (boxed) and empirical masses (on peaks) matched to within 0.2 Da.

where the U1498-G1504 sequence flew exclusively at *m/z* 2287, corresponding to [m^{3}U]AACAAG, with no evidence of an unmethylated fragment at *m/z* 2273 (**Fig. 2A**). The m^{3}U1498 modification is added by RsmE in *E. coli*[23] and requires a fully assembled 30S subunit as a substrate for methylation.[44] The equivalent methyltransferase would be encoded by PA0419 in *P. aeruginosa*.

In the 1378–1432 region, two modifications were observed at nucleotide C1402 and these were shown to be equivalent to those added by the *E. coli* N^4-methyltransferase RsmH and the 2'-*O*-methyltransferase RsmI.[21] The peak from the *P. aeruginosa* rRNA at *m/z* 1307 corresponds to the fragment [m^{4}Cm]CCG (C1402-G1405), and both methylations appear to be stoichiometric, with no peaks at *m/z* 1279 or *m/z* 1293 (**Fig. 4A**). The genes encoding the *P. aeruginosa* RsmH/RsmI methyltransferases in are listed in **Table 1**. No other modifications were evident in the H44 and H45 regions of the *P. aeruginosa* 16S rRNA.

***P. aeruginosa* lacks a homolog of RsmF that modifies m^{5}C1407 in *E. coli* 16S rRNA.** The *P. aeruginosa* fragment UCACACCAUG (U1406-G1415) contains no modification, and flew at *m/z* 3183 (**Fig. 4**). Thus, the m^{5}C1407 modification added in *E. coli* rRNA by the housekeeping methyltransferase RsmF[22] is absent in *P. aeruginosa*. Consistent with this, no *rsmF* homolog could be identified in the *P. aeruginosa* PAO1 genome (**Table 1**). This gene is conserved in the *Enterobacteriaceae* including *Salmonella*, *Klebsiella*, and *Shigella* homologs with > 80% amino acid identity; other Orders, including Aeromonadales, Alteromonadales, and Vibrionales, contain ORFs that are around 58% identical to RsmF. A dendrogram

for genetic similarity in Gammaproteobacteria shows that those bacteria lacking RsmF are genetically related, while the bacteria possessing RsmF also cluster together. Thus, RsmF seems to have an early origin during speciation of Gram-negative bacteria in prokaryotes, and has been lost in *P. aeruginosa* most likely through a single deletion event in a common ancestor (**Fig. S1**). Also absent in *P. aeruginosa* is the 2'-*O*-methyltransferase TlyA, which modifies nucleotide C1409 in 16S rRNA and C1920 in 23S rRNA (**Fig. 1**) and is linked with capreomycin resistance in mycobacteria.[45]

Modifications at 23S rRNA nucleotides $m^3\Psi$1915, m^5U1939, and m^5C1962. Screening the *P. aeruginosa* genome in silico revealed several candidates for genes encoding enzymes that might modify the secondary aminoglycoside binding site within H69 of 23S rRNA. These included the gene encoding the pseudouridine synthase RluD, which isomerizes the 23S rRNA nucleotides U1911, U1915 and U1917 in the *E. coli* helix 69 loop.[17,46] Nucleotide Ψ1915 is subsequently methylated by RlmH to form $m^3\Psi$1915,[27,28] and RlmH activity has been shown to be dependent on prior RluD-catalyzed isomerization of this nucleotide.[28] Species analyzed thus far have been shown to possess an RlmH ortholog only when accompanied by an RluD ortholog. *P. aeruginosa* is no exception, and possesses both of these enzymes, respectively, encoded by PA4004 and PA4544. Methylation of the *P. aeruginosa* rRNA was indicated by the strong primer extension stop before Ψ1915 (**Fig. 3B**), consistent with the RlmH-directed $m^3\Psi$ modification. The level of methylation at this nucleotide (no more than 80% of rRNA molecules) appears to reflect a mode of substrate recognition similar to that seen in *E. coli*, where RluD methylates Ψ1915 only after association of the newly assembled 50S subunit into the 70S ribosome complex.[28]

Putative gene homologs encoding the methyltransferases RlmD and RlmI were also evident in *P. aeruginosa*, which in *E. coli* are responsible for methylation at m^5U1939[29] and m^5C1962,[31] respectively. MALDI-MS spectra of RNase T1 digestion products showed that a methyl group was present on C1962 in the fragment AC[m^5C]UG (A1960-G1964) at *m/z* 1605 and on U1939 in the fragment AAA[m^5U]UCCUUG (A1936-G1845) at *m/z* 3181 (**Fig. 2C**). Enlargements of these spectral regions show only trace amounts of products with 14 Da lower mass (*m/z* 1591 and 3167), indicating that methylation at these targets is almost complete. No other modifications were evident in this region of the *P. aeruginosa* 23S rRNA. The predictions of modification enzymes made in silico matched the empirical data—all the rRNA modifications predicted by the search were shown to occur and, conversely, there were no modifications at sites where enzymes were predicted to be absent (**Table 1**).

Acquisition of the RmtD methyltransferase confers high-level resistance to aminoglycosides. The minimal inhibitory concentrations (MICs) of aminoglycoside drugs required to stop growth of *P. aeruginosa* BB1285 are considerably higher than for the susceptible strain PAO1 (**Table 2**). Expression of *rmtD* in BB1285 facilitates growth of this pathogenic strain at a tobramycin concentration that is over 200-fold higher than required to inhibit cells lacking this methyltransferase gene.

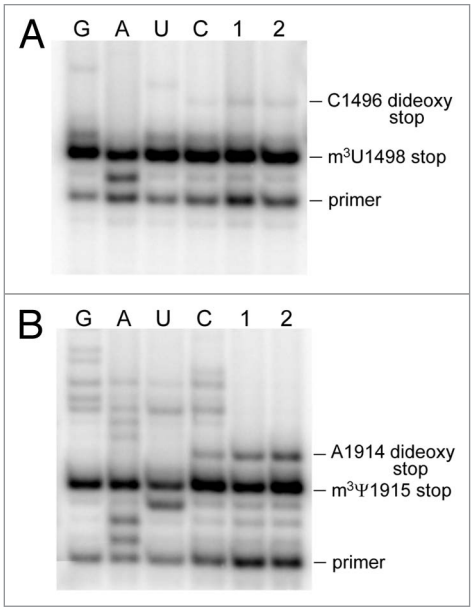

Figure 3. Gel autoradiograms of primer extension on *P. aeruginosa* rRNAs. (**A**) 16S rRNA showing the strong stop directly before the m^3U1498 modification, and (**B**) on 23S rRNA with the stop before $m^3\Psi$1915. Lanes 1 and 2 are from *P. aeruginosa* strains BB1285 and PAO1, respectively. Read-through at the modification sites was terminated by ddGTP or ddTTP (dideoxy stop) and the intensities of these bands reflect the amounts of unmethylated U1498 and Ψ1915. The dideoxy sequencing reactions C, U, A, and G were performed on PAO1 rRNA.

The *P. aeruginosa* BB1285 rRNAs were analyzed to define the methylation site of RmtD, and to see whether acquisition of this resistance determinant influenced the functioning of any of the housekeeping methyltransferases at adjacent sites. MALDI-MS analysis of 16S rRNA from the BB1285 strain revealed the loss of the two fragments [m^4Cm]CCG and UCACACCAUG, seen for the susceptible strain at *m/z* 1307 and *m/z* 3183, respectively, with the concomitant appearance of a new fragment [m^4Cm]CC[m^7G]UCACACCAUG (C1402-G1415) at *m/z* 4485 (**Fig. 4B**). This fragment arises because hydrolysis by RNase T1 at G1405 is prevented by the N^7-methylation. The sequence and base position of this methylation were confirmed by primer extension (**Fig. S2**). The catalysis of m^7G1405 modification by RmtD and the subsequent high-level resistance to the subset of 4,6-disubstituted 2-deoxystreptamines aminoglycosides (**Table 2**) fit well with the function of other Arm/Rmt methyltransferase orthologs characterized to date.[47]

Interference between acquired and indigenous methyltransferases. Closer scrutiny of the spectral region around *m/z* 4485 in the BB1285 strain reveals a second peak at *m/z* 4471, corresponding to a proportion of 16S rRNA molecules with two (rather than three) methyl groups in the C1402-G1415 sequence. Obviously the m^7G1405 methylation is still present, otherwise the sequence would have been cleaved to lower masses and, therefore, the methyl group at the 2'-*O*- or the N^4-position of C1402 must be missing. In the PAO1 strain where there is no m^7G1405 methylation, nucleotide C1402 is present in the fragment at *m/z* 1307, and the lack of any smaller peaks in this spectral region (**Fig. 4A**)

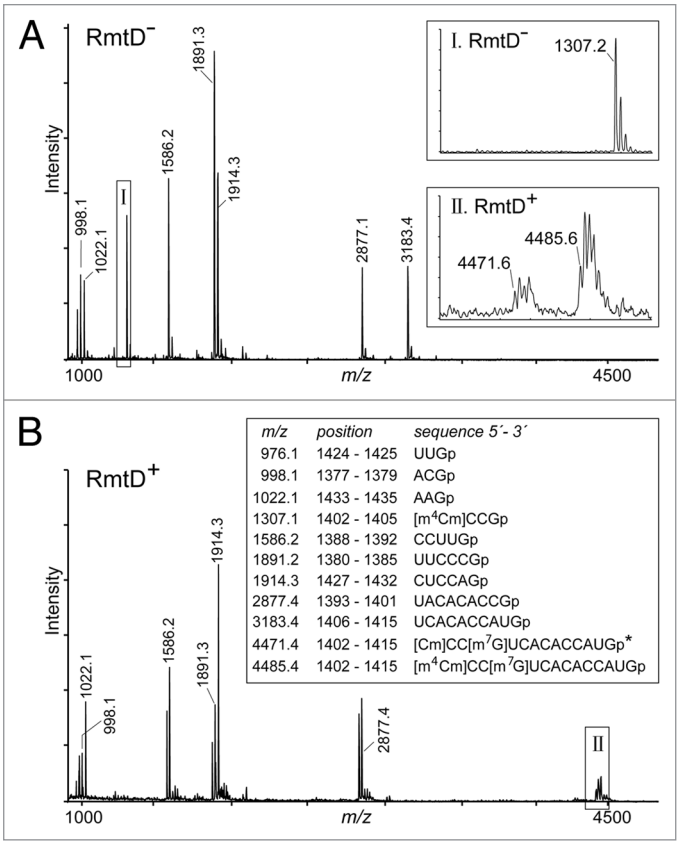

Figure 4. MALDI-MS spectra of RNase T1 fragments from the 16S rRNA sequence C1378-G1432 derived from aminoglycoside-susceptible and -resistant *P. aeruginosa* strains. (**A**) In the RmtD⁻ strain PAO1, G1405 is unmodified and migrates in the *m/z* 1307 fragment; nucleotide C1407 is in the RNase T1 fragment at *m/z* 3183 and contains no modification. (**B**) In the resistant RmtD⁺ strain BB1285, these two fragments are completely absent, and a new peak appears at *m/z* 4485, indicating stoichiometric methylation at m⁷G1405. The minor peak at *m/z* 4471 (box II) arises from partial loss of one of the C1402 methylations, both of which were stoichiometric prior to RmtD expression (box I). There was insufficient fragment material to determine whether RmtD interferes with the 2'-*O*- or N^4-methylation at C1402 (*). The multiple tops in the enlargement (boxes) reflect the natural distribution of ^{12}C and ^{13}C isotopes; the ^{12}C monoisotopic masses of products with linear 3'-phosphates are given here; in spectrum B, smaller amounts of 2'-3'-cyclic products are evident to the left of each main peak.

shows that both the 2'-*O*- and N^4-methylations are added stoichiometrically. Incomplete methylation at C1402 in the BB1285 strain indicates that RmtD is impeding one of the C1402 methylation reactions.

Other recent studies have shown a reduction in modification by the *E. coli* enzyme RsmF at 16S rRNA m⁵C1407 in the presence of Sgm[48] or Arm/Rmt methyltransferases,[49] all of which methylate the 7-position of G1405. Despite this interference with the function of a housekeeping methyltransferase, expression of the chromosome-coded *arm/rmt* genes did not entail any detectable fitness cost, measured in terms of growth rate and competition under laboratory conditions.[49] The RsmI and RsmH enzymes responsible for the 2'-*O*- and N^4-methylations of C1402 are present in all bacterial species and are thus more highly conserved than RsmF. Interference with modification at C1402 might therefore be expected to have a more distinct (but as yet undefined) biological cost.

Common for all these housekeeping enzymes that methylate the decoding region (**Table 1**), as well as for those associated with resistance such as Armt/Rmt[47] and TlyA,[50] is that they all require a 16S rRNA substrate that has been assembled with its r-proteins. The reactions performed by these methyltransferases coincide within a time-frame subsequent to 30S subunit assembly, but prior to its association with the 50S. Considering the sizes of the methyltransferases and the spatial proximity of their targets, it is perhaps not surprising that steric hindrance occurs when an extra enzyme such as RmtD is introduced. The fact that modifications by the indigenous rRNA methyltransferase are for the most part stoichiometric reflects how these enzymes have evolved in conjunction with the ribosomal components such that they function effectively in a highly orchestrated manner.

Materials and Methods

Bacterial strains and isolation of rRNA. The strains used in this study were the wild-type *P. aeruginosa* PAO1 and *P. aeruginosa* strain BB1285, an aminoglycoside-resistant clinical isolate from Brazil harbouring *rmtD* and *bla* (SPM-1). The strains were cultured at 37 °C in 200 ml Luria-Bertani broth (Difco) containing kanamycin at 50 μg/ml where appropriate. Cells were grown to mid-log phase, harvested by centrifugation, washed twice with 50 mM TRIS-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl at 4 °C, and then lyzed by sonication in the same buffer. Cell debris was removed by centrifugation, the supernatant was extracted with phenol and chloroform to remove proteins, and the total cellular RNA was recovered by ethanol precipitation.

Bioinformatic analyses. BLAST searches[51] were restricted to the *P. aeruginosa* PAO1 genome[52] using the sequences of modification enzymes from *E. coli* strain K-12 sub-strain W3110 (GenBank accession number NC_007779) as queries. These *E. coli* enzymes were previously characterized as being responsible for methylation and uridine isomerization adjacent to the aminoglycoside binding sites, and include RsmA, RsmE, RsmF, RsmH, RsmI, and RsmJ modifying *E. coli* 16S rRNA, and RlmD, RlmH, RlmI, and RluD that are specific for 23S rRNA (**Table 1**).

MALDI mass spectrometry analysis. Specific sequences of approximately 50 nucleotides were isolated from the 16S and 23S rRNAs of *P. aeruginosa* strains PAO1 and BB1285. Five complementary deoxyoligonucleotides were hybridized to the *P. aeruginosa* 16S rRNA sequences C1378 to G1432, C1460 to C1510, and G1487 to A1542 and the 23S rRNA sequence C1924 to G1978 and A1889 to G1948 (strain PAO1 GenBank accession number AE004091); the *E. coli* numbering system is used throughout (**Fig. 1**). 100 pmol of rRNA was heated with 500 pmol of deoxyoligonucleotide at 80 °C for 5 min and cooled to 35 °C over 2 h. Regions of the rRNAs that were not protected by hybridization were removed by digestion with 20 U of mung bean nuclease (NE Biolabs) and 0.25 μg of RNase A (Sigma-Aldrich), and the protected rRNA sequences were separated by

gel electrophoresis.[43,53] Each isolated rRNA sequence was digested overnight with 3 U of RNase T1 (Roche Diagnostics) or 0.25 μg of RNase A (Sigma-Aldrich) at 37 °C in 3 μl aqueous solution of 60 mM 3-hydroxypicolinic acid. Samples were analyzed by Matrix Assisted Laser Desorption/Ionization mass spectrometry (MALDI-MS, Voyager Elite, Perseptive Biosystems) recording in reflector and positive ion mode.[54] Spectra were interpreted with the program *m/z* (Proteometrics Inc.).

Primer extension analysis. Several rRNA modifications, including m^3U and $m^3\Psi$, can be detected by their ability to impede reverse transcription. Primers complementary to 16S rRNA nucleotides 1501–1517 and 23S rRNA nucleotides 1920–1939 were used to evaluate the modifications at m^3U1498 and $m^3\Psi1915$, respectively. Extensions were performed with 1 mM dTTP, dATP, dCTP, and 5 mM ddGTP; the reactions stop immediately before m^3U or $m^3\Psi$, or run through on unmethylated rRNA templates to be terminated by ddG at the next cytosine in the sequence. Primer 1459–1479 was used for detection of methylation at the N^7-position of G1405 (m^7G1405) after reduction of the rRNAs with sodium borohydride ($NaBH_4$), followed by cleavage with aniline.[55] The cleavage reaction is generally incomplete[56] and was improved by including hypermethylated tRNA carrier in the reactions.[57] Each deoxynucleotide primer was 5'-end labeled with ^{32}P, and 3 pmol was extended on 2 μg of rRNA with 1.5 U of AMV reverse transcriptase (Finnzymes).[58] Extensions products were run on denaturing polyacrylamide/urea gels alongside dideoxy sequencing reactions performed on *P. aeruginosa* PAO1 rRNAs. Gel bands were visualized and quantified by phosphorimaging (Typhoon, Amersham Biosciences), and the stoichiometry of m^3U and $m^3\Psi$ modifications was calculated from the ratio of the methylated:didedoxy bands.

Antimicrobial susceptibility testing. *P. aeruginosa* strains were studied using in-house microtiter plates according to the CLSI guidelines[59] to evaluate their susceptibility to the 4,6-disubstituted 2-DOS aminoglycosides, gentamicin, kanamycin, tobramycin, amikacin, and arbekacin, and also to the 4,5-disubstituted 2-DOS, neomycin, and the 4-substituted 2-DOS, apramycin (**Table 2**).

Table 2. Susceptibilities to aminoglycosides of the *P. aeruginosa* strains PAO1 and BB1285 *rmtD*$^+$

	P. aeruginosa PAO1	*P. aeruginosa* BB1285 (*rmtD*$^+$)
Aminoglycoside	**MIC (μg/mL)**	
Gentamicin	2	> 512
Kanamycin	128	> 512
Tobramycin	0.5	128
Amikacin	2	> 512
Arbekacin	4	> 512
Neomycin	8	32
Apramycin	8	8

The relatively high tolerance of the PAO1 strain to kanamycin is due to a mechanism unrelated to rRNA modification. Here, a chromosome-coded N^3-acetyltransferase[50] confers resistance to a subset of aminoglycosides.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/25984

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