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Novel 16S rRNA Methyltransferase RmtH Produced by *Klebsiella pneumoniae* Associated with War-Related Trauma

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***Klebsiella pneumoniae* strain MRSN2404 was isolated from the chronic wound of a soldier who had been wounded in Iraq in 2006. The strain displayed very high MICs of all aminoglycosides, including arbekacin. A gene encoding a novel 16S rRNA methyltransferase, now designated RmtH, was identified. RmtH had 64% identity with RmtB1 and RmtB2. *rmtH* was bracketed by two copies of IS*CR2*, which may have played a role in its mobilization.**

Aminoglycosides, along with β-lactams and fluoroquinolones, remain one of the key classes of antimicrobial agents in the treatment of infections caused by Gram-negative bacteria. Mechanisms of resistance to aminoglycosides include enzymatic modification of the drugs, modification of the aminoglycoside-binding site, decreased permeability across the bacterial outer membranes, and augmented efflux. Among them, production of acquired 16S rRNA methyltransferase (16S RMTase) is the most worrisome since it compromises the activity of all aminoglycosides (1). Since the initial reports in 2003, nine such enzymes have been identified (1, 2, 18). With the exception of NpmA, which methylates residue A1408 of the 16S rRNA, they methylate residue G1405 and confer high-level resistance to all aminoglycosides formulated for intravenous use, including gentamicin, tobramycin, amikacin, and arbekacin. Among the G1405 16S RMTases, ArmA and RmtB appear to be the most widely distributed worldwide, having been found in multiple species of the *Enterobacteriaceae*, as well as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Production of 16S RMTases is frequently accompanied by coproduction of a carbapenemase or extended-spectrum β-lactamase (ESBL), which further facilitates multidrug resistance. In the United States, the most commonly encountered 16S RMTase is ArmA, produced by multidrug-resistant (MDR) and extensively drug-resistant (XDR) *A. baumannii*, whereas the presence of RmtB and RmtE has been reported for rare strains of *Escherichia coli*.

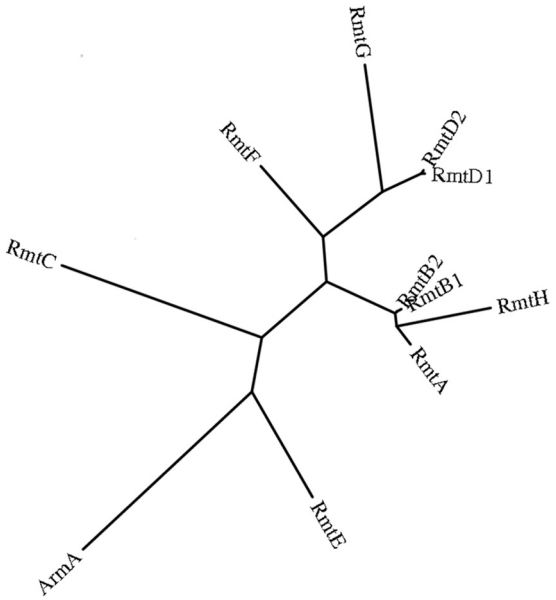

FIG 1 Phylogenetic tree of G1405 16S RMTases. The tree was generated using the tools available at http://www.phylogeny.fr (17). GenBank references are as follows: ArmA, AY220558; RmtA, AB120321; RmtB1, AB103506; RmtB2, JN968578; RmtC, AB194779; RmtD1, DQ914960; RmtD2, HQ401565; RmtE, GU201947; RmtF, JQ808129; RmtG, JX486113.

TABLE 1 MICs of aminoglycosides

Strain	MIC (μg/ml)		
	Gentamicin	Tobramycin	Amikacin
K. pneumoniae MRSN2404	>256	>256	>256
E. coli DH10B(pKp2404K1)	>256	>256	>256
E. coli DH10B(prmtHBX7)	>256	>256	>256
E. coli DH10B(pBC-SK(−))	0.25	0.5	1

In this article, we describe identification of RmtH, a novel 16S RMTase, in a clinical strain of *Klebsiella pneumoniae*. *K. pneumoniae* MRSN2404 was recovered from a 28-year-old male soldier who had suffered a tibial fracture from an explosion in Iraq in 2006. The strain was collected by the Multidrug-resistant Organism Repository and Surveillance Network (MRSN) to enhance infection prevention, inform empirical therapy, and influence policy (3). Following evacuation to the United States, a culture from the intramedullary wound of the right tibia grew XDR *A. baumannii*, ESBL-producing *K. pneumoniae*, and vancomycin-resistant enterococci. Subsequently, the patient had chronic draining wounds associated with the blast injury that occasionally expressed shrapnel. *K. pneumoniae* MRSN2404 was then isolated from the wound in 2009. This strain was found to have very high

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ArmA  -----MDKNDVVKKILESKKYENLDSDIVEKVVSISEKKYK-LKEVENYSKKKLHQIWGS 54
RmtA  -----MSFDDALASILSSKKYRSLCPDTVRRILDQEWGRHKSPKLAVEATRTRLHGICGA 55
RmtB1 -----MNINDALTSILASKKYRALCPDTVRRILTEEWGRHKSPKQTVEAARTRLHGICGA 55
RmtB2 -----MNINDALTSILASKKYRALCPDTVRRILTEEWGRHKSPKQAVEAARTRLHGICGA 55
RmtC  MKTNDNYIEEVTAKVLTSGKYSTLYPPTVRRVTERLFDRYP-PKQLEKEVRKKLHQAYGA 59
RmtD1 -------MSELKEKLLASKKYRDVCPDTIERIWRECSAKFKKEKDVDKAAREALHGVTGA 53
RmtD2 -------MSELKEKLLASKKYRDVCPDTIERIWRECSAKFKKEKDADKAAREALHGVTGA 53
RmtE  -----MNIDEMVAEVLSSKKYTSVDPAVVRRVCMETAPKYPKKKEAIKAVKNELHIIHEV 55
RmtF  ---MDERAQAALDALLSAKNLRDVCPETVRRVFMELLPRYRKPKDAEKAARTHLHQITGA 57
RmtG  ------MRDPLFEKLAASKKYRDVCPDTIARILTECRAKYRREKEIDKAAREKLHGITAA 54
RmtH  -----MTIEQAAADILSSKKYQLLCPDTVVRILTQEWGRHKKPKQAVERTRERLHGICGA 55

ArmA  YYSAYPNWDKLLKKYNQGQ-----------------LSIEDLLKIHSSTNERVA--TLN 94
RmtA  YVTPES-LKAAAAALSVG-------------------DVQKALSLHASTKERLA--ELD 92
RmtB1 YVTPES-LKAAAAALSAG-------------------DVKKALSLHASTKERLA--ELD 92
RmtB2 YVTPES-LKAAAAALSAG-------------------DVKKALSLHASTKERLA--ELD 92
RmtC  YIGGID-GKRLEKKIEKIIHEIPNPTTDEATRTEWEKEICLKILNLHTSTNERTV--AYD 116
RmtD1 FMTERE-YKRAMEMAAAR-------------------DWEALLGMHASTRERLPVESMD 92
RmtD2 FMTERE-YKRAMELAATR-------------------DWEALLGMHASTRERLPVESMD 92
RmtE  FLQNEC-YKNALSFLSQLSLDFNN---------AQLIDITMQIMQSHTSTKERLG--DIE 103
RmtF  FMTADA-QKKARALLARWNEGDE--------------SALAAALSLHASTRERLP--GAD 100
RmtG  FMTDAE-YRRAMEIAVRGG-------------------ELAELMECHASTRERLPLEETD 94
RmtH  YLAPQV-EKQASTALAAG-------------------DVQKALALHASTRERLD--TYP 92

ArmA  DFYTYVFGNIKHVSSILDFGCGFNPLALYQWN-ENEKIIYHAYDIDRAEIAFLSSIIGKL 153
RmtA  CLYDFIFSG-GVPHRVLDIACGLNPLALFI----RDITSVWACDIHQGLGDVITPFAHHQ 147
RmtB1 TLYDFIFSA-ETPRRVLDIACGLNPLALYE----RGIASVWGCDIHQGLGDVITPFAREK 147
RmtB2 TLYDFIFSA-ETPRRVLDIACGLNPLALYE----RGVASVWGCDVHQGLGDVITPFAREK 147
RmtC  ELYQKIFEVTGVPTSITDAGCALNPFSFPFFTEAGMLGQYIGFDLDKGMIEAIEHSLRTL 176
RmtD1 RVFDQLFEASGTPARILDLACGLNPVYLAHR---LPNAAITGVDISGQCVNVI----RAF 145
RmtD2 RVFDQLFEAIGTPARILDLACGLNPVYLAHR---LPNAAIAGVDISGQCVNVI----RAF 145
RmtE  AVCSFLSTHISKEGSVMDIGCGFNPFALPLL--HEFPATYYAYDICSEGINILNKYFSIL 161
RmtF  EWMRRVSPFLGADARVLDLACGLNPILLGS----MGVTNALGMDIHLGCVRLVNETARAR 156
RmtG  AVYARLLGAPDESA--LDLACGLNPAYLQNR---YPEMRVTGIDISGQCVRVL----RAL 145
RmtH  QLYQFVFEN-NLPARVLDIACGLNPLMLHR----QGVASVWGCDIHQGLGNVLTPYAQKH 147

ArmA  KTT--IKYRFLNKESD--VYKGTYDVVFLLKMLPVLKQQDVN-ILDFLQLFHTQNFVISF 208
RmtA  GLD--FTFALQDVMCTP--PTETGDLALVFKLLPLLEREQAGAAMALLQALATPRIAVSF 203
RmtB1 DWD--FTFALQDVLCAP--PAEAGDLALIFKLLPLLEREQAGSAMALLQSLNTPRMAVSF 203
RmtB2 DWD--FTFALQDVLCAP--PAETGDLALIFKLLPLLEREQAGSAMALLQSINTPRMAVSF 203
RmtC  NAPEGIVVKQGDILSD---PSGESDLLLMFKLYTLLDRQEEASGLKILQEWKYKNAVISF 233
RmtD1 GG---AEARLGDLLCE--IPEDEANAALLFKVLPLLERQRAGAAMDALMRVNAEWIVASF 200
RmtD2 GG---AEARLGDLLCE--IPEDEADAALMFKVLPLLERQRTGAAMEALMRVNAEWIVASF 200
RmtE  KKGE-YRAELLDAVSVT--PKEKVDVALLFKLLPLLQQQKKGRGFSILEELDFDKAIVSF 218
RmtF  GWH--TRARACDLLSE--IPAEEADAALLMKLLPVLEAQKTGRAAELLASLRAPRLVVTF 212
RmtG  G----VDARLGDLLAENAIPRARYSVALLFKILPLLDRQSAGAARRILEAVNADALICSF 201
RmtH  GWD--FTFALHDVLCAP--VAASGDMALVFKLLPLLEREQPGAALALLRTLDAPVICVSF 203

ArmA  PIKSLSGKEKGMEENYQLWFESFTK-GWIKILDSKVIGNELVYITSGFQK---------- 257
RmtA  PTRSLGGRGKGMEANYSAWFEGALP-DEFEIEDTKTIGIELVYMIKRNK---------- 251
RmtB1 PTRSLGGRGKGMEANYAAWFEGGLP-AEFEIEDKKTIGTELIYLIKKNG---------- 251
RmtB2 PTRSLGGRGKGMEANYAAWFEGGLP-TEFEIEDKKTIGTELIYLIKKNG---------- 251
RmtC  PIKTISGRDVGMEENYTVKFENDLVGSDLRIMQKLKLGNEMYFIVSRL---------- 281
RmtD1 PTRSLGGRNVGMEKHYSEWMEAHVP-ENRAIAARLTGENELFYVLKRK---------- 247
RmtD2 PTRSLGGRNVGMEKHYSEWMEAHVP-ENRAIAARLTGENELFYVLKRK---------- 247
RmtE  PIKSLGGKQKGMETFYSNLFEENLP-SSLEIIEKQTFSNEMFYVIQNKTKNGGNQS---- 273
RmtF  PTRTLGGRGVGMEKHYADWFERILP-DTLSVRDRFTVSDELVYLVERT---------- 259
RmtG  PTRSLSGRNVGMAVHYAAWMRDQLP-EKWRIERTVETDNELYYVLKEKQDGEAVRGGDSH 260
RmtH  PTRSLGGRGKGMHQHYATWFEGLVA-PHFTVQHHTLIGDELLYRIQPNPA---------- 252

ArmA  ----
RmtA  ----
RmtB1 ----
RmtB2 ----
RmtC  ----
RmtD1 ----
RmtD2 ----
RmtE  ----
RmtF  ----
RmtG  RESE 264
RmtH  ----
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FIG 2 Amino acid sequence alignment of G1405 16S RMTases. The alignment was generated using the ClustalW software program (www.ebi.ac.uk/Tools/msa/clustalw2/).

MICs (>256 μg/ml) of all aminoglycosides tested, including gentamicin, tobramycin, amikacin, and arbekacin, when tested by the standard broth microdilution method recommended by the Clinical and Laboratory Standards Institute (4). It was also resistant to ceftriaxone, ceftazidime, cefepime, aztreonam, and ciprofloxacin but remained susceptible to ertapenem and imipenem. The strain was phenotypically confirmed as an ESBL producer, and screening of β-lactamase genes with PCR and sequencing showed that it carried $bla_{\text{CTX-M-15}}$ as well as $bla_{\text{SHV-1}}$ and $bla_{\text{OXA-1}}$. Multilocus sequence typing (www.pasteur.fr/mlst) (5) assigned the strain to sequence type (ST) 48, an ST which has been reported in association with ESBL production worldwide (6–10).

Given the resistance phenotype consistent with 16S RMTase production, the strain was screened for 16S RMTase genes using PCR (2, 11, 12). However, PCR was negative for all known 16S RMTase genes. We therefore proceeded with further experiments to identify the determinant of high-level aminoglycoside resistance. The genomic DNA of *K. pneumoniae* MRSN2404 was extracted, digested with KpnI (New England BioLabs, Ipswich, MA), and ligated with the vector pBC-SK(−) (Agilent Technologies, Santa Clara, CA), which had been digested with the same enzyme. Electrocompetent *Escherichia coli* DH10B was transformed with this genomic library, and transformants were selected on tryptic soy agar (TSA) plates containing chloramphenicol (30 μg/ml) and gentamicin (50 μg/ml). This procedure yielded a single colony, which was cross-resistant to other aminoglycosides as well. The recombinant plasmid harbored by this transformant (pKp2404K1) was then fully sequenced. The sequencing revealed the presence of a 3.1-kb insert, which contained an open reading frame (ORF) corresponding to a 252-amino-acid sequence. This ORF showed 64% amino acid identity with the 16S RMTases RmtB1 and RmtB2 and 63% identity with RmtA. Identity with other 16S RMTases was much lower, ranging from 25% with ArmA to 39% with RmtD1, RmtD2, and RmtF (Fig. 1). The ORF was designated *rmtH* according to the proposed nomenclature of acquired 16S RMTases (13). We then performed PCR cloning of *rmtH* using the primers rmtH-XbaI-fwd (5′-CGCTCTAGAATG ACCATTGAACAGGCAGC-3′) and rmtG-BamHI-rev (5′-CGC GGATCCTCAAGCTGGGTTTGGCTGGA-3′) (the restriction sites are underlined). The PCR product was digested with XbaI and BamHI and ligated with pBC-SK(−) digested with the same enzymes. Transformants were obtained using the method above. A transformant harboring a recombinant plasmid with the intact *rmtH* structural gene (prmtHBX7), as confirmed by sequencing, was used for susceptibility testing. Susceptibility testing was performed using Etest (bioMérieux, Hazelwood, MO) according to the manufacturer's instructions. As shown in Table 1, the original genomic clone, as well as the PCR clone, showed high-level resistance to gentamicin, tobramycin, and amikacin, as expected. Based on the pattern of aminoglycoside resistance and the amino acid alignment with known 16S RMTases, RmtH likely functioned as a G1405 16S RMTase (Fig. 2).

The full sequence of pKp2404K1 revealed that *rmtH* was bracketed by two copies of IS*CR2* in tandem. IS*CR2* is an IS*91*-like transposable element which is found in association with various resistance genes, including those for sulfonamide, trimethoprim, and florfenicol (14), and also tetracycline and cephalosporin (15, 16). It is believed to facilitate mobilization of the genetic elements downstream. To our knowledge, this is the first instance where a 16S RMTase gene was found in association with IS*CR2*. IS*CR2* is usually found intact upstream of a resistance gene, while the second copy downstream is typically truncated (14). Since IS*CR2* possesses a KpnI restriction site, we were not able to assess whether the two copies of IS*CR2* were intact or not. Nonetheless, this unique arrangement suggested that they likely played a role in the initial mobilization of *rmtH* to *K. pneumoniae* MRSN2404.

Attempts to mobilize *rmtH* to *E. coli* by either transformation or conjugation were not successful. DNA hybridization of S1 nuclease-treated genomic DNA separated by pulsed-field gel electrophoresis (PFGE) with an *rmtH*-specific probe did not yield any band despite the presence of multiple plasmids on the PFGE gel. Finally, the *rmtH* probe hybridized to an approximately 500-kb band which was generated by PFGE following XbaI digestion (data not shown). These findings suggested that *rmtH* was likely located on the chromosome.

In summary, we report a novel 16S RMTase, RmtH, identified in an ESBL-producing *K. pneumoniae* strain which was recovered from a soldier who had been wounded during an operation in Iraq. The finding underscores the diversity of 16S RMTases and highlights the importance of continued surveillance in identifying emerging antimicrobial resistance mechanisms.

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been submitted to the GenBank under accession number KC544262.

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This material has been reviewed by the Walter Reed Army Institute of Research. There was no objection to its presentation. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

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