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Molecular epidemiology of 16S rRNA methyltransferase in Brazil: RmtG in *Klebsiella aerogenes* ST93 (CC4)

HEMANOEL PASSARELLI-ARAUJO[1*], JUSSARA K. PALMEIRO[2,3*], KANHU C. MOHARANA[1], FRANCISNEI PEDROSA-SILVA[1], LIBERA M. DALLA-COSTA[3] AND THIAGO M. VENANCIO[1]

[1]Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego, 2000, P5, Sala 217, 28013-602 Campos dos Goytacazes, RJ, Brazil
[2]Laboratório de Bacteriologia e Biologia Molecular, Unidade do Laboratório de Análises Clínicas, Complexo Hospital de Clínicas, Universidade Federal do Paraná, Rua Padre Camargo, 280, 80060-240 Curitiba, PR, Brazil
[3]Faculdades Pequeno Príncipe, Instituto de Pesquisa Pelé Pequeno Príncipe, Av. Silva Jardim, 1632, 80050-540 Curitiba, PR, Brazil

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Abstract: Aminoglycosides are a class of antibiotics that play a key role in antimicrobial treatment of Multidrug resistant (MDR) Gram-negative bacilli, typically in combination with β-lactams. Ribosomal 16S RNA modification by methyltransferases (e.g. RmtG) is an aminoglycoside resistance mechanism that, along with the occurrence carbapenem-resistant Enterobacteriaceae (CRE), has become a clinical concern. In Brazil, rmtG genes were initially reported in Klebsiella pneumoniae, and monitoring isolates from other species carrying this gene is critical for epidemiological studies and to prevent dissemination. Here we report the presence of rmtG in Klebisella aerogenes D3 and characterize its genetic context in comparison to isolates from other species. Further, we performed a phylogenetic reconstruction of 900 16S rRNA methyltransferases (16S-RMTases) and methyltransferase-related proteins. We show that, in K. aerogenes D3, rmtG co-occurs with sul2, near a transposon with an IS91-like insertion sequence. Resistome analysis revealed the co-production of RmtG and CTX-M-59. Ongoing surveillance of 16S-RMTases is crucial to delay the dissemination of such multiresistant isolates. Our results also highlight the reduction in treatment options for CRE infections, as well as the need of expanding prevention measures of these pathogens worldwide.

Key words: *Klebsiella aerogenes*, aminoglycoside, 16S rRNA methyltransferases, multidrug resistance.

Correspondence to: Thiago Motta Venancio
E-mail: thiago.venancio@gmail.com
*These authors contributed equally to this work
ORCid: http://orcid.org/0000-0002-2215-8082
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INTRODUCTION

Aminoglycosides are broad-spectrum and highly potent antibiotics that bind to the 30S ribosomal subunit and compromise translation. The first identified aminoglycoside antibiotic was streptomycin, isolated from *Streptomyces griseus* in 1944 (Jones et al. 1944). Other notorious aminoglycosides are amikacin, kanamycin, gentamycin and neomycin, which are effective against aerobic and facultative anaerobic bacteria. Although nephrotoxicity and ototoxicity have been reported (Jiang et al. 2017, O'Sullivan et al. 2017), aminoglycosides have played an important role in antimicrobial therapy, especially against Carbapenem-Resistant Enterobacteriaceae (CRE) isolates (Iovleva and Doi 2017).

The expression of Extended-Spectrum Beta-Lactamases (ESBL) is commonly associated with the resistance against third-generation cephalosporins (e.g. ceftazidime, cefotaxime) and other types of beta-lactams (e.g. monobactam), being these resistant bacteria typically treated with carbapenems. Therefore, infections with CRE isolates have limited treatment options, requiring other antimicrobials such as aminoglycosides (Jonathan 2005, Legese et al. 2017). However, the evolution of resistant strains has increasingly reduced the potential of aminoglycosides over the years (Doi et al. 2016, Iovleva and Doi 2017).

The resistance to aminoglycosides can be mediated by different mechanisms, such as: (i) the expression of enzymes that modify and inactivate the antibiotic and; (ii) modification of the ribosomal binding sites by 16S ribosomal RNA methyltransferases (16S-RMTases) (Ramirez and Tolmasky 2010, Wachino and Arakawa 2012). Further, a decreased uptake, accumulation of the drug in bacteria due to membrane impermeabilization and/or overexpression of efflux pumps can also contribute to aminoglycoside resistance (Fernandez and Hancock 2012).

Among the resistance mechanisms against aminoglycosides, the most common in Enterobacteriaceae is the enzymatic inactivation of these antibiotics by aminoglycoside modifying enzymes (AMEs). In addition to AMEs, new acquired 16S-RMTases have been shown to confer high levels of resistance against clinically important aminoglycosides (e.g. amikacin, gentamicin and tobramycin), increasing their minimal inhibitory concentration (MIC). Most of the genes encoding these enzymes are carried by transposons and conjugative plasmids that can efficiently disseminate between different species, contributing for the dissemination of Multidrug Resistant (MDR) strains.

With regard to ribosomal modification, aminoglycoside-producing bacteria (e.g. *Micromonospora* and *Streptomyces* spp.) often have intrinsic 16S-RMTases for self-protection (Cundliffe 1989). In addition, 16S-RMTase genes are also found in pathogenic and non-aminoglycoside-producing bacteria, being known as acquired 16S-RMTases. Although the origins of these groups are not well understood, it has been shown that acquired and intrinsic 16S-RMTases have distinct GC contents (Wachino and Arakawa 2012).

Bacterial expression of acquired 16S-RMTases has recently become a concern to clinical treatment (Doi et al. 2016). The 16S rRNA modification occurs by methylation in two sites: (1) at the N7 position of G1405, by nine different enzymes (ArmA and RmtA to H), which confers high-level resistance to amikacin, kanamycin, gentamicin and tobramycin and; (2) at the N1 position of A1405, by NpmA, conferring high-level resistance to amikacin, kanamycin, gentamicin, tobramycin, neomycin, and apramycin (Wachino and Arakawa 2012).

Genes encoding 16S-RMTases are globally spread and have so far been found in Enterobacteriaceae, *Pseudomonas aeruginosa*, and

Acinetobacter baumannii (Doi et al. 2016). To date, RmtD, RmtG, ArmA, and RmtB were detected in Brazil (Doi et al. 2007, Yamane et al. 2008, Bueno et al. 2013, Quiles et al. 2015, Moura et al. 2017, Braun et al. 2018) and RmtD was first reported in *P. aeruginosa* isolates coproducing SPM-1 (Doi et al. 2007). In Brazil, RmtG was first reported in *Klebsiella pneumoniae* coproducing KPC-2 and CTX-M (Bueno et al. 2013). Recently, RmtG/D has been identified in *P. aeruginosa* and *Klebsiella aerogenes* (Francisco et al. 2015, Grazziotin et al. 2016). Regarding its genetic location, different Inc group plasmids have been shown to carry *rmtG* (N, A/C, B/O) (Bueno et al. 2013, Mancini et al. 2018), often within transposons containing additional resistance determinants.

The occurrence of RmtG is apparently more restricted to South America, although it has been recently reported in Europe (Mancini et al. 2018). Because of the risk involved in the spread of resistance genes such as those associated with 16S-RMTase production, continuous monitoring is required to prevent global transmission. To better evaluate the molecular epidemiology of RmtG, we performed an in-depth analysis of the genomic context of *rmtG* in a *K. aerogenes* clinical isolate. We provide strong evidence supporting the horizontal gene transfer of this region and also reconstructed the phylogeny of RmtG and other acquired 16S-RMTases to help assess the spreading potential of *rmtG*. Taken together, these results reinforce the need to keep an efficient system for the surveillance of resistance genes and their spread potential in the population.

MATERIALS AND METHODS

BACTERIAL ISOLATE

K. aerogenes D3 was isolated from bronchoalveolar lavage (BAL) from an elderly male patient under medical care at a tertiary teaching hospital located in Curitiba, Paraná, Brazil, in December 2006 (Grazziotin et al. 2016). The genome sequence of the *K. aerogenes* D3 was downloaded from GenBank (accession number LUTT00000000). *K. aerogenes* D3 showed high minimal inhibitory concentration (MIC) to amikacin (64 mg/L), gentamicin (> 64 mg/L), ceftazidime (16 mg/L), cefepime (128 mg/L), cefotaxime (128 mg/L), ertapenem (16 mg/L), imipenem (32 mg/L), ciprofloxacin (16 mg/L), levofloxacin (> 8 mg/L), doxycycline (64 mg/L), and fosfomycin (> 512 mg/L) (Grazziotin et al. 2016). Further, it was shown to be susceptible to meropenem, polymyxin, tigecycline, and minocycline, according to the Clinical and Laboratory Standard Institute and to the Brazilian Committee on Antimicrobial Susceptibility Testing standards (BrCAST, 2018, http://brcast.org.br/).

MULTILOCUS SEQUENCE TYPING (MLST), ANTIBIOTIC RESISTANCE-RELATED GENES AND GENETIC BACKGROUND ANNOTATION

To identify the sequence type (ST), the BIGSdb database version 3 (Jolley and Maiden 2010) was used to evaluate seven housekeeping *loci* (i.e. *dnaA, fusA, gyrB, leuS, pryG, rplB* and *rpoB*) in *K. aerogenes*. The Comprehensive Antimicrobial Resistance Database (CARD) version 1.1.8 (Jia et al. 2017) was used to compare all predicted proteins in *K. aerogenes* D3 with BLAST (Altschul et al. 1997), using minimum identity and query coverage thresholds of 70% and 50%, respectively. Short Read Sequence Typing (SRST2) version 0.2.0 (Inouye et al. 2014) was also used to map *K. aerogenes* D3 reads against the ResFinder database version 3.0 (Kleinheinz et al. 2014). Genes surrounding *rmtG* were manually curated with the aid of the UniProt database (Wu et al. 2006). The following RmtG-containing scaffolds from publicly available genomes were used for a comparative analysis of the genetic contexts: *K. pneumoniae* (NZ_CP004000.1), *E. cloaceae* (NZ_MUDF01000061.1) *E. hormaechei*

(NZ_JZXU01000048.1) and *P. aeruginosa* (NZ_LZDA01000022.1).

PHYLOGENETIC ANALYSIS OF THE 16S rRNA METHYLTRANSFERASES

The following reference protein sequences from the acquired N7-G1405 16S-RMTases were used to find 16S-RMTases to be included in phylogenetic reconstructions: ArmA (ADC55560.1); RmtA (BAC20579.1); RmtB1 (BAC81971.1); RmtB2 (AFC75738.1); RmtC (AIA09786.1); RmtD1 (ABJ53409.1); RmtD2 (ADW66527.1); RmtE (ALD03565.1); RmtF (AFJ11385.1); RmtG (AGE00988.1); RmtH (AGH19769.1).

These reference proteins were used to search the NCBI non-redundant (nr) database using BLASTP (Altschul et al. 1997) with a minimum coverage and maximum e-value thresholds of 50% and 1e-10, respectively. Proteins annotated as hypothetical were not considered. All detected homologs were aligned with T-Coffee v11 (Di Tommaso et al. 2011) and the alignment was processed with TrimAl version 1.2 (Capella-Gutierrez et al. 2009). Maximum likelihood phylogenetic trees were built using RaxML v8.2.11 (Stamatakis 2014), using gamma correction for among-site rate variation and 1,000 bootstrap replicates.

RESULTS AND DISCUSSION

K. aerogenes D3 showed a MDR profile, exhibiting resistance against multiple antibiotics belonging to at least three classes, including aminoglycosides, beta-lactams and fluoroquinolones. MLST analysis assigned this isolate to the Clonal Complex 4, ST93. Because the release of *K. aerogenes* MLST profile (https://pubmlst.org/kaerogenes/) is very recent (accessed on February 2018), there is no sufficient information about lineage global distribution and clonal characteristics.

The *K. aerogenes* D3 scaffold containing *rmtG* most likely belongs to a plasmid, as it shares 99% identity and is almost completely covered (99%) by the reference plasmid pKP13f (CP004000.1) of a *K. pneumoniae* isolate identified at the same state of *K. aerogenes* D3 (Ramos et al. 2014). We were unable to recover the *K. aerogenes* D3 plasmid replicon type by using PlasmidFinder version 1.3 (Carattoli et al. 2014). However, we found that pKP13f is a conjugative plasmid belonging to IncFIB incompatibility group, which also harbors $bla_{CTX\text{-}M\text{-}2}$. Hence, we hypothesize that the *K. aerogenes* D3 *rmtG*-carrying plasmid may have similar features, including a high dissemination power.

Regarding the co-occurrence of other resistance genes along with *rmtG* in *K. aerogenes* D3, we performed a resistome analysis (see methods for details) that uncovered major genes conferring resistance against beta-lactams ($bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}59}$, $bla_{OXA\text{-}9}$ and bla_{CMY}), aminoglycosides (*aac(6')-Ib10* and *aadA*), fluoroquinolones (*oqxAB* and *qnrS*), sulfonamides (two copies of *sul1* and one of *sul2*) and fosfomycin (*fosA*) (Supplementary Material - Table SI). These genes were associated with the high MICs exhibited by this isolate. In addition, it is very important to emphasize that CTX-M-59 is associated with cephalosporin resistance and the co-production of RmtG, CTX-M-59 and other aminoglycoside resistance genes could further limit the treatment options for *K. aerogenes* infections.

To further investigate the potential interspecies exchange of antibiotic resistance genes, we examined the sequences flanking *rmtG* for evidence supporting horizontal gene transfer (HGT) events. A schematic representation of the region where *rmtG* is located in *K. aerogenes* D3 is shown in Figure 1. The *rmtG* gene is part of an operon that includes genes related with rRNA and tRNA modification such as *rsmH, tgt* and *rsmL*. The gene *sul2* was also found upstream to *rmtG*, associated with *glmM*. This genomic region was delimited by transposases, supporting its acquisition by HGT. Moreover, one transposase located downstream

was associated with an IS91 insertion sequence suggesting that IS91 may have played a role in the mobilization of *rmtG*. It is important to note that the group of IS91-like elements are associated with the evolution by accumulation and dissemination of a variety of resistance genes (Garcillan-Barcia and de la Cruz 2002, Toleman et al. 2006). Table I indicates some characteristics of other methyltransferases and shows that IS91-like elements have been previously found in association with *rmtF* and *rmtE*.

The genomic context of *rmtG K. aerogenes* D3 is highly similar to that of other Enterobacteriaceae, as well as to that of *Pseudomonas aeruginosa* (Figure 1). However, in Enterobacteriaceae species (*E. cloaceae*, *E. hormaechei* and *K. pneumoniae*), the similarity was beyond the limits of the operon containing *rmtG*. This suggests that *rmtG* was acquired as part of a larger DNA segment present

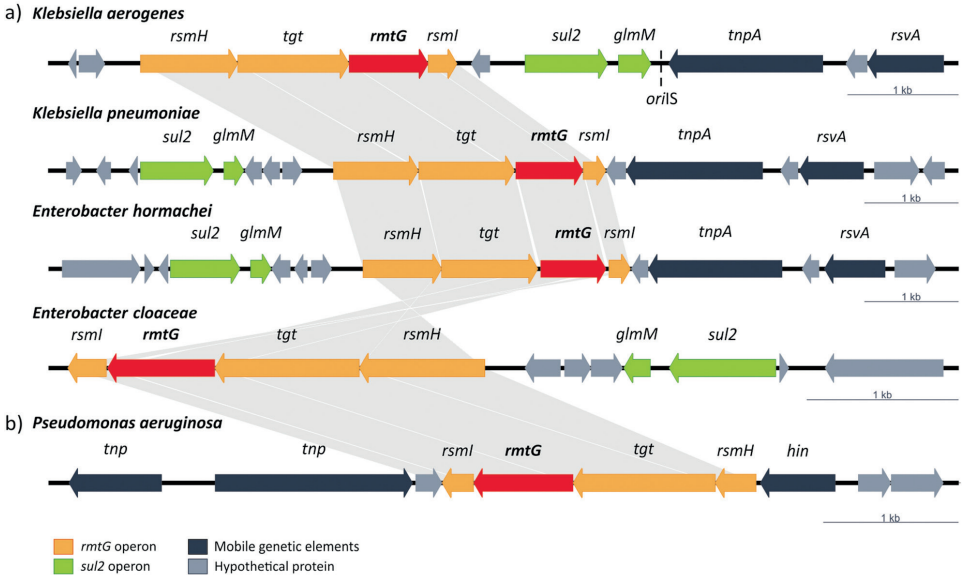

Figure 1 - Genetic context of: (**a**) Enterobacteriaceae species (*K. aerogenes, K. pneumoniae, E. hormachei and E. cloaceae*) and (**b**) *Pseudomonas aeruginosa*. Conserved regions are connected by grey shadows. The *ori*IS represents the start position of the insertion sequence; *tnpA* encodes a transposase from *IS91* family, which has been reported as associated with the accumulation of various resistance genes. It is important to notice that the *sul2* operon is absent in the *rmtG* neighborhood in *P. aeruginosa*.

TABLE I
Comparison between acquired 16S methyltransferases location based on previous studies.

16S rRNA Methyltrasferase	Plasmid Replication Origin	IS family	Reference
armA	IncN	IS26	(Gonzalez-Zorn et al. 2005)
rmtA	-	IS6100	(Yamane et al. 2004)
rmtB	IncFII	IS26	(Liu et al. 2018)
rmtC	IncA/C	ISKPn14	(Gruber et al. 2015)
rmtD	RepA	IS26	(Bueno et al. 2016)
rmtE	IncI1	IS91	(Li et al. 2017)
rmtF	IncN	IS91	(Hidalgo et al. 2013)
rmtG	-	IS91	This study
rmtH	IncFII	IS26	(Beyrouthy et al. 2017)

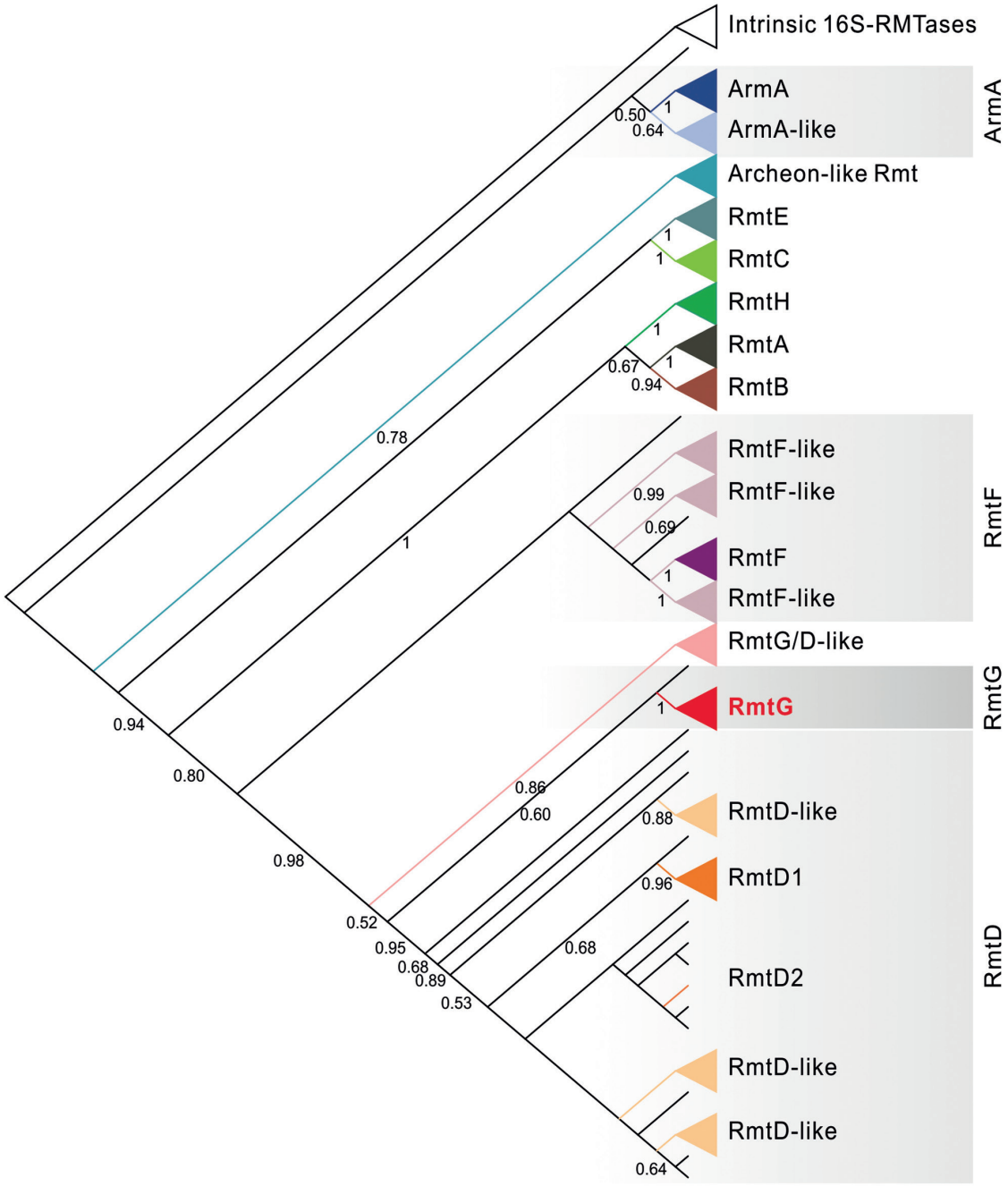

Figure 2 - Phylogenetic reconstruction of 16S rRNA methyltransferases. Intrinsic 16S-RMTases were arbitrarily used as outgroups. Labels at internal nodes represent bootstrap support values. Nodes with support values lower than 0.5 were not labeled. Clades containing canonical 16S-RMTases were collapsed (in solid colors) at a minimum bootstrap threshold of 0.8, except for Intrinsic 16S-RMTases, ArmA-like and RmtD/F-like clades (which were collapsed for better visualization, even with low support). RmtB1 and RmtB2 formed a monophyletic group and were collapsed into the RmtB clade. We have not found strong support for the separation of the RmtD1 and RmtD2 clades, although the monophyly of the RmtD group is supported. The suffix "-like" were used for clades containing potentially new subfamilies of 16S-RMTases derived from the metagenome study reported by Pehrsson et al. (2016) (except ArmA-like). These clades are represented in light versions of colors used for the clades containing their canonical counterparts. Notably, these metagenome sequences are restricted to the Rmt(F, G/D, D)-like branch.

in several Enterobacteriaceae. When the genomic context from Enterobacteriaceae is compared with *P. aeruginosa*, only the *rmtG* operon was identified and the *sul2* gene was absent.

We also explored the phylogenetic relationships between 900 16S-RMTases available in the nr database (Figure 2). The RmtG clade is closely related to RmtD. However, when the genetic context of these genes are compared, the presence of *sul1* instead of *sul2* was observed in *rmtD* genetic context (Doi et al. 2008). While *sul1* is usually found in class 1 integrons linked with other resistance genes (Martinez et al. 2007), *sul2* is typically found in small plasmids. We observed that *sul2* in the D3 strain was not located in an integron, as *sul1* does in *P. aeruginosa* (Doi et al. 2008). On the other hand, *tgt* was shared by both operon architectures, indicating that it might be important in *rmtD/G*-containing strains. We also observed that the RmtG clade was closely related to a clade of predicted proteins from a metagenomics study using the PARfuMS (Parallel Annotation and Reassembly of Functional Metagenomic Selection) method (Forsberg et al. 2012) to sequence DNA fragments containing resistance genes from antibiotic-selected colonies from soil, feces and latrine (Pehrsson et al. 2016). We found several distinct 16S-RMTase clades comprising sequences from metagenomic studies (Figure 2), suggesting that unculturable bacteria constitute a reservoir of novel or divergent methyltransferases that might confer resistance against aminoglycosides. This study also allowed the discovery of a number of genes associated with antibiotic resistance in low-income habitats (Pehrsson et al. 2016). Because several sequences from the above mentioned study were derived from samples obtained in South America, we also tried to analyze their genetic context to trace potential HGT events. However, this analysis was not possible because the genes of interest were located in short scaffolds that did not allow a proper collinearity analysis.

In this work, we reported the presence of *rmtG* in *K. aerogenes*, analyzed its genetic context and potential associations with the general resistance profile of the isolate. The co-production of RmtG and CTX-M-59 constitutes a clinical concern due the limited treatment options to combat this bacterium. Moreover, the global proliferation capacity of this *K. aerogenes* should be taken into account in order to avoid hospital outbreaks, food and domestic animal infections. This work also alerts to the importance of maintaining surveillance practices to control the global spread of these pathogens.

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SUPPLEMENTARY MATERIAL

Table SI - Resistome Analysis of Klebsiella aerogenes D3.