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rmtD2, a New Allele of a 16S rRNA Methylase Gene, Has Been Present in *Enterobacteriaceae* Isolates from Argentina for More than a Decade▽

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The first allele of a 16S rRNA methyltransferase gene, *rmtD2*, conferring very high resistance to all clinically available aminoglycosides, was detected in 7/1,064 enterobacteria collected in 2007. *rmtD2* was located on a conjugative plasmid in a Tn*2670*-like element inside a structure similar to that of *rmtD1* but probably having an independent assembly. *rmtD2* has been found since 1996 to 1998 mainly in *Enterobacter* and *Citrobacter* isolates, suggesting a possible reservoir in these genera. This presumption deserves monitoring by continuous surveillance.

Posttranscriptional methylation of the 16S rRNA aminoglycoside binding site, common in aminoglycoside-producing microorganisms, has been described in nosocomial isolates highly resistant to all clinically available aminoglycosides. Currently, seven plasmid-mediated 16S rRNA methyltransferase (16S-methylase) genes have been identified: *armA*, *npmA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* (named *rmtD1* herein), and very recently, *rmtE*, from bovine origin (3, 4, 19). To date, only *Klebsiella pneumoniae* harboring *rmtD1* has been reported in Argentina (7). Herein, a nationwide survey of aminoglycoside resistance mediated by 16S-methylases among enterobacteria in Argentina was performed.

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Susceptibility and molecular detection of resistance mechanisms. To determine the prevalence of the 16S-methylases, a collection of 1,064 consecutive, nonduplicate enterobacterial isolates was analyzed. This sample set was collected during a 5-day period in 2007 from 66 hospitals belonging to the WHONET-Argentina Resistance Surveillance Network and submitted to the Servicio Antimicrobianos (the Argentinian National Reference Laboratory). Initial screening of 16S-methylase activity was performed using the disc diffusion susceptibility method for amikacin and gentamicin (inhibition zones of ≤10 mm) (1, 4). Although almost 80% of the collection was made up of *Escherichia coli* and *Klebsiella* spp., only 12 isolates belonging to other species (4 *Enterobacter cloacae*, 1 *Enterobacter aerogenes*, 3 *Serratia* species, 2 *Citrobacter freundii*, 1 *Morganella morganii*, and 1 *Proteus mirabilis* isolate) met the screening criteria used. Of these, 5 showed inhibition zones of ≥7 mm to amikacin, and 7 (all of the *Enterobacter* species and *C. freundii* isolates) showed an absence of inhibition zones for both aminoglycosides. Only these last 7 isolates gave positive results when tested for 16S-methylase genes by PCR (Table 1), and only the primers against *rmtD* genes rendered amplicons (Table 2). Complete gene amplification and DNA sequencing showed a unique 16S-methylase gene in these 7 isolates. This gene displayed 97.3% nucleotide identity (20 nucleotides of difference) and 96.4% amino acid identity (9 residues of difference) with *rmtD1*. Since this is the first description of a 16S-methylase gene allele, it was named *rmtD2*, as recommended by Doi and colleagues (6). The *rmtD2*-harboring isolates were from 6 hospitals in 4 geographic areas across Argentina, and pulsed-field gel electrophoresis (PFGE) of XbaI-digested DNA of the 4 *E. cloacae* and 2 *C. freundii* isolates showed that they were not clonally related (data not shown).

Antibiotic MICs, except that of apramycin (agar dilution method [1]), were determined by Etest (AB bioMérieux, Solna, Sweden). The 7 *rmtD2*-harboring isolates showed high MICs to all of the aminoglycosides tested except apramycin (Table 2). These results were comparable with those of Fritsche and colleagues for enterobacteria producing different enzymes that methylate the 16S rRNA at position G1405 (7). Conversely, Wachino and colleagues described NpmA, which methylates at position A1408, conferring intermediate resistance to amikacin (16 μg/ml) and low-level resistance to gentamicin (32 μg/ml) but high-level resistance to apramycin (>256 μg/ml) (19).

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TABLE 1. Sequences of primers used for specific gene detection

Target[a]	Primer name[b]	Primer sequence (5′ to 3′)	Size (bp)	T_a (°C)[c]	Source or reference
armA	armA-F	AGGTTGTTTCCATTTCTGAG	405	51	This work
	armA-R	ACCTATACTTTATCGTCGTC			
rmtA	rmtA-F	CGTGACATAACATCTGTATG	333	51	This work
	rmtA-R	TTCAAATTCATCAGGCAGTG			
rmtB	rmtB-F	ATTGGGATTTTACCTTTGCC	290	51	This work
	rmtB-R	TATAAGTTCTGTTCCGATGG			
rmtC	rmtC-F	AGATACCAAATCCAACTACG	369	51	This work
	rmtC-R	TAAGTAGAAGATCACTCTCG			
rmtD	rmtD-F	TCAAAAAGGAAAAGGACGTG	500	51	This work
	rmtD-R	CGATGCGACGATCCATTC			
	rmtD-F2[d]	ATGAGCGAACTGAAGGAAAAAC	744	57	This work
	rmtD-R2[d]	TCATTTTCGTTTCAGCACGTAAA			
npmA	npmA-F	CTCAAAGGAACAAAGACGG	641	51	7
	npmA-R	GAAACATGGCCAGAAACTC			
bla_{TEM}	tem1-F	ATGAGTATTCAACATTTTCGTG	861	55	This work
	tem1-R	TTACCAATGCTTAATCAGTGAG			
bla_{SHV}	shv1-F	ATGCGTTATATTCGCCTGTG	861	55	This work
	shv1-R	TTAGCGTTGCCAGTGCTCG			
bla_{CTX-M}	ctx-MU1	ATGTGCAGYACCAGTAARGT	803	55	13
	ctx-MU2	TGGGTRAARTARGTSACCAG			
bla_{KPC}	Uni-KPC-F	ATGTCACTGTATCGCCGTCT	882	55	17
	Uni-KPC-R	TTACTGCCCGTTGACGCCC			
qnrB	qnrB-F	CCGACCTGAGCGGCACTGA	523	55	18
	qnrB-R	CGCTCCATGAGCAACGATGCCT			

[a] *rmtE* was reported after the conclusion of this work, and its analysis was not included.
[b] F, forward; R, reverse.
[c] T_a, PCR annealing temperature.
[d] Primers to amplify the complete *rmtD* gene.

Taken together, these data suggest that the new variant RmtD2 methylates at position G1405. Moreover, our phenotypic results support the approach of Doi and Arakawa for detecting the 16S-methylases that act at that last position (4).

The *rmtD2*-harboring isolates displayed other resistance mechanisms (Table 2). Under the new carbapenem breakpoints (2), all 7 clinical strains showed resistance/intermediate resistance to ertapenem, and several of them showed intermediate resistance to imipenem and/or meropenem. This is probably due to impermeability combined with hyperproduced AmpC and/or CTX-M-type enzymes (11, 16). Production of metallo-β-lactamases was discarded by EDTA-sodium mercaptoacetic acid (SMA) phenotypic screening (8). All of the isolates were resistant to ciprofloxacin (Q4079 showed reduced susceptibility), tetracycline, and chloramphenicol as well. Biparental conjugations were performed between each *rmtD2*-harboring clinical isolate and sodium azide-resistant *E. coli* J53 or rifampin- and nalidixic acid-resistant *E. coli* ER1793 as recipients, using amikacin (50 μg/ml) and gentamicin (50 μg/ml) plus sodium azide (100 μg/ml) or rifampin (300 μg/ml), respectively, to select the transconjugants. All conjugations rendered *rmtD2*-harboring transconjugants highly resistant to aminoglycosides (Table 2). Resistance to ampicillin, piperacillin, extended-spectrum cephalosporins, and chloramphenicol was cotransferred to *E. coli* in some cases. Transmission of reduced susceptibility to ciprofloxacin was observed in the transconjugant J-4143. The characterization of relevant genetic determinants responsible for these resistant phenotypes, such as bla_{TEM-1}, $bla_{CTX-M-2}$, and *qnrB10*, was performed by PCR and DNA sequencing (Tables 1 and 2). Therefore, RmtD2 was coexpressed with, but not necessarily linked to, other resistance mechanisms.

***rmtD2* flanking regions.** XbaI DNA libraries from *E. cloacae* Q4010, *E. aerogenes* Q4079, and *C. freundii* Q1174 were generated in *E. coli* TOP10 using the cloning vector pACYC184. The *rmtD2*-harboring clones were selected with chloramphenicol (34 μg/ml), gentamicin, and kanamycin (4 μg/ml each), and DNA inserts were sequenced using pACYC184-specific and sequence-based primers. The inserts from isolates Q4010 and Q1174 were completely sequenced, while a 19.7-kb partial sequence was obtained from a longer fragment of Q4079. The genetic environments of *rmtD2* in these three isolates differed only in the cassette arrays of the class 1 integrons and showed the same IS*CR14*-bracketed genetic architecture as those surrounding *rmtD1* in *Pseudomonas aeruginosa* PA0905 and *K. pneumoniae* R2 from Brazil (5) (Fig. 1A). However, several key differences between the environments of *rmtD1* and *rmtD2* were found (Fig. 1B to D). We concluded that the IS*CR14*-bracketed structures surrounding *rmtD1* and *rmtD2* are composed of very similar blocks of genes but having different edges. Moreover, it is noteworthy that the second IS*CR14* in *E. aerogenes* Q4079, named herein IS*CR14B*, is actually a chimera, since the last 337 nucleotides of *orf494* and its 256-bp downstream sequence to *ori*IS showed a maximal identity of 96% with IS*CR5B* (GenBank accession no. AM849110) but only 79% with IS*CR14* (9). Therefore, IS*CR14B* might have resulted from a recombination between two IS*CR*s (e.g., IS*CR14* and IS*CR5B*), as has already been reported for IS*CR5B*, which is itself a chimeric element (9). All of the data strongly suggest that the IS*CR14*-bracketed region found in the enterobacterial isolates from Argentina and that in *P. aeruginosa* PA0905 from Brazil were assembled in independent processes through a series of IS*CR* transposition/homologous recombination events rather than variants derived

TABLE 2. Antimicrobial susceptibility and molecular typing of resistance mechanisms of clinical isolates and transconjugant strains

Antimicrobial agent or genetic determinant[a]	MIC (μg/ml) or molecular typing result[b]															
	Donor clinical isolates[c]							*E. coli* transconjugant strains[d]							*E. coli* transf.[e]	*E. coli* acceptors	
	Ecl Q4010	Ecl Q3039	Ecl Q2054	Ecl Q5161	Cfr Q1174	Cfr Q4143	Eae Q4079	ER-4010	ER-3039	J-2054	J-5161	J-1174	J-4143	J-4079		ER1793	J53
Antimicrobial susceptibility testing																	
AMK	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	0.5	1.5
GEN	≥1,024	≥1,024	256	256	≥1,024	≥1,024	256	≥1,024	≥256	≥256	≥256	768	768	256	≥256	0.125	1.5
KAN	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	0.75	2
NET	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	0.125	0.125
TOB	≥1,024	≥1,024	≥1,024	≥1,024	≥1,024	768	256	≥1,024	≥256	256	≥256	≥1,024	256	256	≥256	0.094	1
APR	8	4	8	8	8	4	8	ND	ND	ND	ND	ND	ND	ND	ND	4	4
AMP	≥256	≥256	≥256	≥256	≥256	≥256	≥256	64	≥256	48	≥256	96	≥256	≥256	ND	6	6
PIP	≥256	≥256	≥256	≥256	≥256	≥256	≥256	8	16	6	≥256	4	≥256	≥256	ND	1	2
FOX	≥256	≥256	≥256	≥256	≥256	≥256	≥256	2	4	12	8	12	12	16	ND	8	8
CAZ	192	≥256	128	≥256	≥256	24	12	0.125	0.25	0.125	0.5	0.19	8	16	ND	0.125	0.19
CTX	≥256	≥256	≥256	≥256	≥256	≥256	≥256	0.047	0.064	0.094	0.38	0.094	192	≥256	ND	0.094	0.094
FEP	6	192	4	24	12	256	128	0.094	0.094	0.125	0.38	0.094	24	48	ND	0.047	0.064
IPM	0.75	1.5	2	2	0.75	0.38	0.75	0.25	0.25	0.25	0.25	0.75	0.25	0.25	ND	0.25	0.38
ERT	8	24	16	≥32	6	0.75	0.5	0.012	0.016	0.012	0.012	0.012	0.032	0.023	ND	0.016	0.008
MEM	1	0.5	1.5	3	3	0.25	0.094	0.023	0.023	0.023	0.023	0.032	0.047	0.023	ND	0.023	0.023
CIP	≥32	≥32	≥32	≥32	≥32	≥32	0.19	0.125	0.19	0.016	0.008	0.016	0.75	0.016	ND	0.125	0.012
TET	48	48	96	256	256	48	24	1.5	4	2	6	4	4	4	ND	0.5	1
CHL	256	96	128	256	≥256	24	≥256	12	24	16	16	48	48	≥256	ND	3	6
PCR																	
armA	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
rmtA	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
rmtB	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
rmtC	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
rmtD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND	ND
npmA	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
bla_{TEM}	+	+	−	−	−	+	+	+	−	ND	ND	ND	−	+	ND	ND	ND
bla_{SHV}	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
bla_{KPC}	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
bla_{CTX-M}	−	−	−	+	−	+	+	ND	ND	ND	+	ND	+	+	ND	ND	ND
qnrB	−	−	−	−	−	+	−	ND	ND	ND	ND	ND	+	ND	ND	ND	ND

[a] AMK, amikacin; GEN, gentamicin; KAN, kanamycin; NET, netilmicin; TOB, tobramycin; APR, apramycin; AMP, ampicillin; PIP, piperacillin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; ERT, ertapenem; MEM, meropenem; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol.
[b] + and −, positive and negative amplification, respectively; ND, not determined.
[c] Ecl, *E. cloacae*; Cfr, *C. freundii*; Eae, *E. aerogenes*.
[d] Transconjugant strains are designated with the letter from the acceptor *E. coli* strain (ER- and J- indicate derivation from *E. coli* ER1793 and J53, respectively) and the number from the donor clinical strain.
[e] *E. coli* transformed with the *rmtD2* gene.

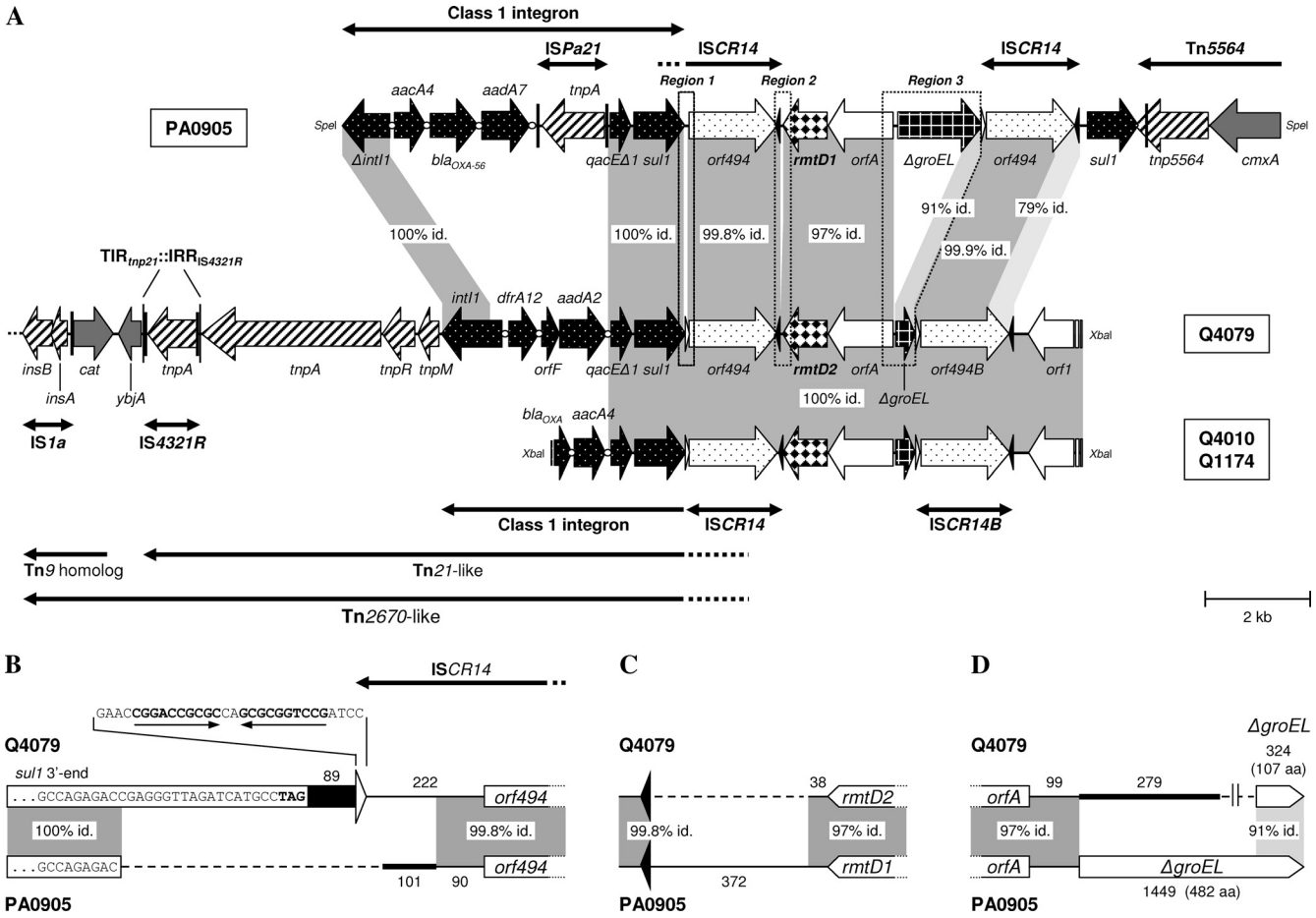

FIG. 1. Schematic representation of the genetic platform of *rmtD2*. (A) Comparison of the fragments harboring *rmtD2* in *E. aerogenes* Q4079 and *C. freundii* Q1174 or *E. cloacae* Q4010 with the genetic environment of *rmtD1* in *P. aeruginosa* PA0905 (5). The gray-shaded areas indicate nucleotide identity. Regions 1, 2, and 3 (framed by dotted lines), which included key differences between both genetic platforms, are depicted in more detail in panels B, C, and D, respectively. The broad horizontal arrows indicate genes and their transcriptional orientations. The *attI* and *attC* sites of class 1 integrons are symbolized by open circles. The black and white triangles represent *ori*IS and *ter*IS, respectively. The thick lines with single or double arrowheads indicate the insertion elements (ISs) and transposons found (black vertical bars show inverted repeats [IRs]). (B) Region 1. A gap (dashed line) was introduced to maximize the alignment, and the numbers (nucleotides) indicate fragment lengths. The last nucleotides of *sul1* are shown for each class 1 integron (sequences are not to scale). The black bar and the thick line indicate the sequences downstream to *sul1*, i.e., the end of the 3′ conserved sequence (3′CS) and a sequence without significant similarity in GenBank, respectively. The white triangle represents the *ter*IS found only in *E. aerogenes* Q4079, and its sequence is shown (arrows indicate IRs). (C) Region 2. The dashed line and numbers are described for panel B. The black triangles represent *ori*ISs. (D) Region 3. The dashed line and numbers are described for panel B. The thick line indicates a sequence without significant similarity in GenBank. Δ*groEL* genes are not to scale, and the number of amino acids (aa) of each putative encoding protein is shown.

from a common original structure. This is similar to the proposed model of the IS*CR19*-bracketed bla_{OXA-18} structure (12).

To the best of our knowledge, this is the first report of a 16S-methylase gene linked to a Tn*21*-like element (Fig. 1A). Another Tn*21*-like transposon containing the same class 1 integron (*dfrA12-orfF-aadA2*) as that found in *E. aerogenes* Q4079 was previously described in a 65-kb conjugative plasmid from a fecal *E. coli* isolate (20). Unlike in this plasmid, the Tn*21*-like element of Q4079 has its terminal inverted repeat at the *tnp* end (TIR_{tnp21}) disrupted by the insertion of IS*4321R* (Fig. 1A). However, it was shown that the precise excision of IS*4321* can re-create the original TIR_{tnp21}, allowing Tn*21* transposition (14). In *E. aerogenes* Q4079, the Tn*21*-like element is inserted in the same genetic context as Tn*21* in the 94-kb multiresistant plasmid NR1 (R100) of *Shigella flexneri*, i.e., upstream of the *ybjA* gene and disrupting a Tn*9* homolog (10, 20) (Fig. 1A). Since the Tn*21*-harboring Tn*9* element of NR1 is itself transposable as Tn*2670* (10), the location of *rmtD2* in a putative Tn*2670*-like element constitutes another potential means of dissemination. In summary, the finding of *rmtD2* in this genetic context suggests that its dissemination could be highly facilitated.

Prevalence of *rmtD2*. We observed an *rmtD2* prevalence of 0.7% in *Enterobacteriaceae*. Considering the distribution of species in the collection studied, the prevalence rates of this gene were 9.3% in *Enterobacter* spp. and 13.3% in *Citrobacter* spp. To better estimate these rates across a broader time period, an analysis of the WHONET-Argentina database (year 2007, 19,077 enterobacteria) was performed. The total corre-

TABLE 3. Analysis of *Enterobacteriaceae* from WHONET-Argentina database, 2007

Group of isolates analyzed (total no.)	% (no.) of isolates with no inhibition zone for AMK or GEN[a]
All *Enterobacteriaceae* (19,077)	2.1 (403)
Enterobacteriaceae minus *Enterobacter* and *Citrobacter* spp. (16,906)	1.2 (211)*†
Enterobacter spp. (1,730)	9.1 (157)*
Citrobacter spp. (441)	7.9 (35)†

[a] Absence of inhibition zones for both amikacin and gentamicin. Only one isolate per patient was tested. Symbols indicate significant differences ($P < 0.0001$) by the chi-square test.

lation between the presence of *rmtD2* and the absence of inhibition zones for both amikacin and gentamicin was used as an indicator of the presence of this gene. As observed in the surveillance collection, the overall prevalence of *rmtD2* was low (2.1%) but significantly higher in *Enterobacter* spp. and *Citrobacter* spp. than in other enterobacteria (Table 3), suggesting a possible reservoir in these species.

In order to establish a continuous surveillance, the National Reference Laboratory had recommended to the WHONET-Argentina hospitals to search for enterobacterial isolates showing an absence of inhibition zones for both amikacin and gentamicin. Twelve strains (4 *E. cloacae*, 1 *Enterobacter agglomerans*, 1 *C. freundii*, 4 *K. pneumoniae*, and 2 *P. mirabilis* strains), isolated from January 2008 to April 2009, were selected for molecular characterization. By PCR screening, 10/12 isolates were positive only for *rmtD* genes. The 2 *P. mirabilis* isolates were negative for all of the 16S-methylase genes tested and remain under study. By sequencing, *rmtD1* was found in 2 *K. pneumoniae* isolates, while *rmtD2* was observed in the remaining 8 isolates.

***rmtD2* has been present in Argentina for more than a decade.** The first 16S-methylase producer described in Argentina was a *K. pneumoniae* strain isolated in 2005 (7). To determine whether this resistance mechanism was present earlier, 153 AmpC-producing enterobacterial strains isolated in 1996 to 1998 from a previous national survey were analyzed (15). Thirteen isolates (1 *Enterobacter* sp., 8 *E. cloacae*, 3 *C. freundii*, and 1 *Serratia marcescens* isolate) were highly resistant to amikacin and gentamicin (MICs of >256 μg/ml). Four of them (2 *E. cloacae*, 1 *C. freundii*, and 1 *S. marcescens* isolate), selected for 16S-methylase gene characterization, showed *rmtD2* only. These results point out the presence of *rmtD2* in *Enterobacter* spp. and *C. freundii* isolated in the 1990s and strengthen the hypothesis of a reservoir in these species, with a further dissemination to other enterobacteria, such as *K. pneumoniae*. This presumption indicates that continued surveillance is necessary to monitor the spread of *rmtD* genes.

Nucleotide sequence accession numbers. The nucleotide sequences determined here have been assigned GenBank accession numbers HQ401565 (*E. aerogenes* Q4079), HQ401566 (*E. cloacae* Q4010), and HQ401567 (*C. freundii* Q1174).

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