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RmtF, a New Member of the Aminoglycoside Resistance 16S rRNA N7 G1405 Methyltransferase Family

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Multidrug-resistant clinical isolate *Klebsiella pneumoniae* BM4686 was highly resistant to 4,6-disubstituted 2-deoxystreptamines and to fortimicin. Resistance was due to the presence, on the 40-kb non-self-transferable plasmid pIP849, of the *rmtF* gene which was cotranscribed with the upstream *aac*(6′)-*Ib* gene. The deduced RmtF protein had 25 to 46% identity with members of the N7 G1405 family of aminoglycoside resistance 16S rRNA methyltransferases.

Aminoglycosides are used for the treatment of severe infections caused by Gram-negative organisms. They interfere with bacterial 16S rRNA function by binding at the A site where codon-anticodon accuracy is assessed (10). In Gram-negative pathogens, resistance to aminoglycosides is mediated primarily by enzymes that modify the drugs and less commonly by other mechanisms, including efflux (7). Substitution or methylation of bases involved in the binding of aminoglycosides to 16S rRNA can lead to loss of affinity and to resistance of the host (7, 9, 11). Methylation was initially observed in the actinomycete and streptomycete producers of aminoglycosides (1). Recently, 16S rRNA N7 G1405 methyltransferases which confer high-level resistance to the 4,6-disubstituted 2-deoxystreptamines and to fortimicin have emerged and disseminated among Gram-negative human pathogens. The first gene for this type of resistance was named *armA*, for aminoglycoside resistance methyltransferase (5). Reports followed of five other methyltransferases: RmtA (rRNA methyltransferase A), RmtB, RmtC, RmtD, and RmtE (4). In most instances, these genes are parts of large conjugative plasmids, together with quinolone resistance determinants and genes for extended-spectrum β-lactamases (6).

Klebsiella pneumoniae BM4686 was isolated from a patient in the Hôpital Sud, city of Saint-Pierre, La Réunion Island, in 2011. Antibiotic susceptibility was tested by disk diffusion on Mueller-Hinton agar according to the Comité de l'Antibiogramme de la Société Française de Microbiologie standards (3). MICs of antimicrobial agents were determined by dilution in Mueller-Hinton agar with 10^4 CFU per spot after 24 h of incubation. The strain was resistant to all β-lactams due to the presence of the genes bla_{OXA-1} and bla_{NDM-1}; no PCR products were obtained with primers internal to the genes for the PER, VEB, GES, KPC, VIM, IMP1, IMP2, and CTX-M β-lactamases. *K. pneumoniae* BM4686 was also resistant to all aminoglycosides, to fluoroquinolones, to rifampin, and to chloramphenicol but remained susceptible to the tetracyclines and colistin (Table 1; data not shown).

High-level resistance to aminoglycosides was indicative of the presence of a 16S rRNA methyltransferase, but attempts to amplify known genes were unsuccessful. Resistance could not be transferred to *Escherichia coli* J53 by conjugation, but plasmid DNA extracted from BM4686 and introduced by electrotransformation into *E. coli* TOP10 gave rise to cells growing on arbekacin (500 μg/ml). The transformants harbored ca. 40-kb plasmid pIP849, which conferred high-level resistance to the kanamycin and gentamicin classes, to fortimicin, and to rifampin (Table 1; data not shown). Plasmid pIP849 was nontypeable by PCR-based replicon typing (2). DNA from pIP849 and pUC19 was digested with EcoRI, mixed, ligated, and introduced by transformation into *E. coli* TOP10 with selection on arbekacin. The smallest plasmid in the transformants, pAT860, contained a ca. 4.5-kb insert which was sequenced.

TABLE 1 MICs of antimicrobial agents for the strains used in this study

Strain	MIC (μg/ml)[a]				
	AMI	GEN	TOB	APR	RIF
K. pneumoniae BM4686	>256	>256	>256	2	>256
E. coli					
TOP10	1	0.5	0.5	2	12
TOP10(pIP849)	>256	128	256	2	>256
TOP10(pAT860)	>256	256	256	2	12
TOP10(pAT861)	256	128	128	2	12

[a] Abbreviations: AMI, amikacin; GEN, gentamicin; TOB, tobramycin; APR, apramycin; RIF, rifampin.

The insert contained four open reading frames (ORFs) (Fig. 1). Comparison with sequences in the GenBank database revealed homology of the deduced products from ORF1 (positions 634 to 1188) with aminoglycoside 6′-*N*-acetyltransferase-Ib (99% identity over 193 amino acids; accession number BAE66666), from ORF3 (positions 2484 to 3767) with transposase InsE IS*CR5* of *E. coli* (95% identity over 429 amino acids; accession number YP_002891161), and from ORF4 (positions 4661 to 4028) with a putative xenobiotic acetyltransferase from *Paracoccus denitrificans* (74% identity over 155 amino acids; accession number YP_913929). ORF2 (positions 1512 to 2378) did not share homology with sequences in GenBank, but a BLASTx query of the deduced protein indicated similarity to G1405 16S rRNA methylases (25 to 46% identity over 254 amino acids) (Fig. 2) and it was designated RmtF (2). Within ORF2, a probable ribosome binding

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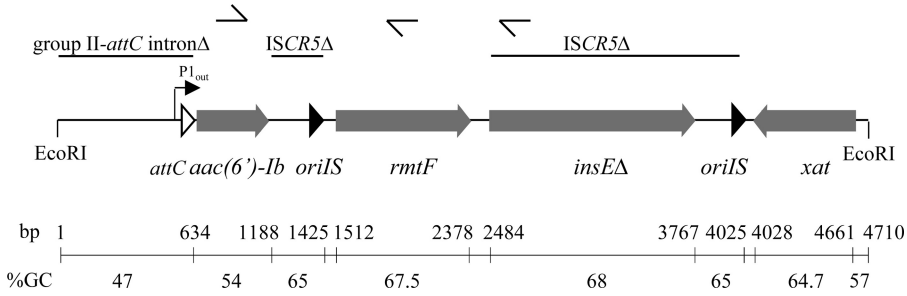

FIG 1 Schematic representation of the genetic environment of *rmtF*. Arrows indicate ORFs and directions of transcription. Closed arrowheads, *oriIS* of IS*CR5*. Open arrowhead, *attC*. Broken arrow, group IIC-*attC* $P1_{out}$ promoter. The genetic elements are indicated by thin lines. The primers used for reverse transcription and amplification are indicated by thin arrows at the top, and the G+C contents and scale are shown at the bottom.

site, GGAGA (positions 1585 to 1589), was present 9 nucleotides upstream from a putative ATG initiation codon leading to a 777-bp coding sequence. *In silico* analysis of the sequence upstream from *rmtF* did not reveal any putative promoter. Synthesis of cDNA from total RNA of *E. coli* TOP10 harboring pIP849 was carried out using either a random hexamer (Thermoscientific RevertAid premium first-strand cDNA synthesis kit) or *rmtF*- and *insE*Δ-specific primers (Fig. 1). PCR products were obtained with the three cDNAs and primers specific for *aac*(6′)-*Ib*, *rmtF*, and *insE*Δ, indicating that the *aac*(6′)-*Ib* and *rmtF* genes are cotranscribed. A 5′ rapid amplification of cDNA ends experiment (Ambion/Life Technologies FirstChoice RLM-RACE kit) indicated that adenine 490 is the transcription initiation site for the *aac*(6′)-*Ib*-*rmtF* operon. This suggests the $P1_{out}$ promoter (−35 motif [TTGCCA] and −10 motif [TTTAAT], positions 455 to 483), which is part of the group IIC-*attC* intron (Fig. 1) (8), for expression of the genes. The 1.5-kb PCR fragment obtained with the 5′-ATTCGAGCGAACACGCAGTGA and 5′-AGAACCCGC GCTTCTTGCAGG primers encompassing *rmtF* was cloned into pCR-Blunt (Invitrogen) and resequenced, and the pAT861 recombinant plasmid conferred high-level resistance to 4,6-disubstituted 2-deoxystreptamines and to fortimicin on *E. coli* TOP10 (Table 1; data not shown).

The overall G+C contents of the *rmtF* gene (67.5 mol%), flanking *oriIS* IS*CR5* (65 mol%), *insE* (68 mol%), and *xat* (64.7 mol%) were similar and significantly higher than that of the *aac*(6′)-*Ib* (54 mol%) and the adjacent upstream region (47 mol%) (Fig. 1). The G+C content of *rmtF* was significantly different from that of the genome of members of the family *Enterobacteriaceae*, which is ca. 50 mol%, but was close to those of the 16S rRNA methyltransferase structural genes from producers such as "*Streptomyces tenebrius*" (*kgmB*; 71 mol%) and *Micromonospora purpurea* (*grmA*; 65 mol%), favoring a direct and recent origin in the family *Actinomycetaceae*.

The *rmtF* gene was bracketed by a 3′ portion of IS*CR5* including *oriIS* and by a 5′-truncated *insE* gene for the IS*CR5* transposase together with *oriIS* (Fig. 1). This genetic organization is consistent with the hypothesis that *rmtF* was recruited through IS*CR* transposition or homologous recombination (13), as has been proposed from *rmtD1*-*rmtD2* and IS*CR14* (12). A transposition-associated deletion would then be responsible for the transcriptional fusion with the *aac*(6′)-*Ib* gene.

Nucleotide sequence accession number. The nucleotide sequence of the insert in pAT860 has been deposited in the GenBank database and assigned accession number JQ808129.

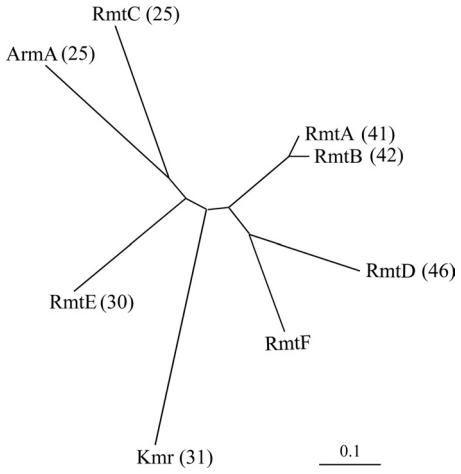

FIG 2 Phylogenetic relationships among 16S rRNA methyltransferases determined using CLUSTALW. The GenBank accession numbers of plasmid-borne 16S rRNA methyltransferase genes are as follows: ArmA, AY220558; RmtA, AB083212; RmtB, AB103506; RmtC, AB194779; RmtE, GU201947; RmtF, JQ808129. Kmr (accession no. Y15838) is a chromosomal 16S rRNA methyltransferase from aminoglycoside-producing *Streptomyces kanamyceticus*. The scale bar represents a 10% amino acid sequence difference. The values in parentheses are percentages of identity relative to RmtF.

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