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 co-transcribed 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 (11). In Gram-negative pathogens resistance to aminoglycosides is mediated primarily by enzymes that modify the drugs and less commonly by other mechanisms including efflux (8). 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 (8, 10, 12). Methylation was initially observed in the actinomycetes and streptomycetes producers of aminoglycosides (2). 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 (6). Reports followed of five other methyltransferases: RmtA (Ribosomal RNA methyltransferase A), RmtB, RmtC, RmtD, and RmtE (5). In most instances these genes are part of large conjugative plasmids, together with quinolone resistance determinants and genes for extended-spectrum β-lactamases (7). 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 (4). 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 genes blaOXA-1 and blaNDM-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, rifampin, and to chloramphenicol but remained susceptible to the tetracyclines and colistin (Table and 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 electrottransformation into *E. coli* TOP10 gave rise to cells growing on arbekacin (500 µg/ml). The transformants harbored plasmid pIP849 of ca. 40 kb which conferred high-level resistance to the kanamycin and gentamicin classes, to fortimicin, and to rifampin (Table and data not shown). Plasmid pIP849 was non-typable 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.

The insert contained four open reading frames (ORFs) (Fig. 1). Comparison with sequences in the GenBank data library 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), of ORF3 (positions 2484 to 3767) with transposase InsE ISCR5 of *E. coli* (95% identity over 429 amino acids; accession number YP_002891161), and of 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 with G1405 16S rRNA
methylases (25 to 46% identity over 254 amino acids) (Fig. 2), and was designated RmtF (3). Within ORF2, a probable ribosome binding site, GGAGA (positions 1585 to 1589), was present nine nucleotides upstream from a putative ATG initiation codon leading to a 777-bp coding sequence. \textit{In silico} analysis of the sequence upstream from \textit{rmtF} did not reveal any putative promoter. Synthesis of cDNA was carried out from total RNA of \textit{E. coli} TOP10 harboring pIP849 using either a random hexamer (14) or \textit{rmtF} and \textit{insE}Δ specific primers (Fig. 1). PCR products were obtained with the three cDNAs and primers specific for \textit{aac(6')-Ib} and \textit{rmtF} and \textit{insE}Δ indicating that the \textit{aac(6')-Ib} and \textit{rmtF} genes are co-transcribed. A 5'-RACE experiment (1) indicated that adenine 490 was the transcription initiation site for the \textit{aac(6')-Ib-rmtF} operon. This suggests the P1\textsubscript{out} promoter [-35 motif (TTGCCA) and -10 motif (TTTAAT), positions 455-483], which is part of the group IIC-\textit{attC} intron (Fig. 1) (9), for expression of the genes. The 1.5-kb PCR fragment obtained with the 5'-ATTCGAGCGAACACGCAGTGA and 5'-AGAACCCGCGCTTCTTGCAGG primers encompassing \textit{rmtF} was cloned in pCR-Blunt (Invitrogen), resequenced, and the pAT861 recombinant plasmid conferred to \textit{E. coli} TOP10 high-level resistance to 4,6-disubstituted 2-deoxystreptamines and to fortimicin (Table 1 and data not shown).

The overall mol % G+C of the \textit{rmtF} gene (67.5%), of flanking \textit{oriIS ISCR5} (65%), \textit{insE} (68%), and \textit{xat} (64.7%) was similar and significantly higher than that of the \textit{aac(6')-Ib} (54%) and adjacent upstream region (47%) (Fig. 1). The mol % G+C of \textit{rmtF} was significantly different from that of the genome of members of the family \textit{Enterobacteriaceae} which is ca. 50% but was close to those of the 16S rRNA methyltransferases structural genes from the producers such as \textit{Streptomyces...
tenebrius (kgmB, 71%) or Micromonospora purpurea (grmA, 65%) favoring a direct and recent origin in the Actinomycetaceae.

The rmtF gene was bracketed by a 3’ portion of ISCR5 including oriIS and by a 5’-truncated insE gene for the ISCR5 transposase together with oriIS (Fig. 1). This genetic organization is consistent with the hypothesis that rmtF was recruited through ISCR transposition or homologous recombination (15), as has been proposed from rmtD1-rmtD2 and ISCR14 (13). A transposition associated deletion would then be responsible for the transcriptional fusion with the aac(6’)-Ib gene.

The nucleotide sequence of the insert in pAT860 has been assigned GenBank accession number JQ808129.
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REFERENCES


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Abbreviations: AMI, amikacin; GEN, gentamicin; TOB, tobramycin; APR, apramycin; RIF, rifampin.
FIG. 1. Schematic representation of the genetic environment of rmtF. Arrows indicate open reading frames and direction of transcription. Closed arrowheads, oriIS of ISCR5. Open arrowhead, attC. Broken arrow, group IIC-attC P1out promoter. The genetic elements are indicated by thin lines. Primers used for reverse transcription and amplification are indicated by thin arrows at the top and the mol % G+C and the scale in bp at the bottom of the figure.

FIG. 2. Phylogenetic relationship among 16S rRNA methyltransferases determined using CLUSTALW. 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, Y15838, is a chromosomal 16S rRNA methyltransferase from aminoglycoside producing Streptomyces kanamyceticus. The scale bar represents a 10% difference in amino acid sequence.