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To cite this version:
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Received 18 May 2016; returned 7 July 2016; revised 2 September 2016; accepted 17 September 2016

**Objectives:** To investigate the resistance mechanisms and genetic support underlying the high resistance level of the <i>Klebsiella pneumoniae</i> strain CMUL78 to aminoglycoside and β-lactam antibiotics.

**Methods:** Antibiotic susceptibility was assessed by the disc diffusion method and MICs were determined by the microdilution method. Antibiotic resistance genes and their genetic environment were characterized by PCR and Sanger sequencing. Plasmid contents were analysed in the clinical strain and transconjugants obtained by mating-out assays. Complete plasmid sequencing was performed with PacBio and Illumina technology.

**Results:** Strain CMUL78 co-produced the 16S rRNA methyltransferase (RMTase) RmtH, carbapenemase OXA-48 and ESBL SHV-12. The <i>rmtH</i>- and <i>bla</i><sub>SHV-12</sub>-encoding genes were harboured by a novel ∼115 kb IncFII<sub>k</sub> plasmid designated pRmtH, and <i>bla</i><sub>OXA-48</sub> by a ∼62 kb IncI/L/M plasmid related to pOXA-48a. pRmtH plasmid possessed seven different stability modules, one of which is a novel hybrid toxin–antitoxin system. Interestingly, pRmtH plasmid harboured a 4-fold amplification of an <i>rmtH-ISC</i>R2 unit arranged in tandem and inserted within a novel IS<sub/>26</sub>-based composite transposon designated Tn6329.

**Conclusions:** This is the first known report of the 16S RMTase-encoding gene <i>rmtH</i> in a plasmid. The <i>rmtH-ISC</i>R2 unit was inserted in a composite transposon as a 4-fold tandem repeat, a scarcely reported organization.

**Introduction**

The production of acquired 16S rRNA methyltransferases (16S RMTases) is an emerging mechanism of aminoglycoside resistance among Gram-negative bacteria.<sup>1</sup> They are classified into two subgroups designated N7-G1405 16S-RMTases and N1-A1408 16S-RMTases.<sup>2</sup> Nine acquired N7-G1405 16S rRNA methyltransferases have been reported in Gram-negative bacteria and were designated ArmA and RmtA to RmtH.<sup>2–10</sup> NpmA is the sole acquired N1-A1408 16S-RMTase identified so far.<sup>11</sup> The ArmA-encoding gene, initially characterized from a <i>Klebsiella pneumoniae</i> strain isolated in France, is currently spread worldwide among Enterobacteriaceae.<sup>12</sup> <i>rmtB</i> has also been identified among Enterobacteriaceae and is mainly observed in East Asia, Europe and North America.<sup>12</sup> This broad diffusion of 16S RMTases is a major concern because they confer a high level of resistance to all clinically relevant aminoglycosides.

A RmtH-encoding gene was recently characterized in a <i>K. pneumoniae</i> strain isolated from a USA soldier wounded in Iraq in 2006.<sup>10</sup> In contrast to the other 16S RMTase-encoding genes, the <i>rmtH</i> gene was harboured by the chromosome of the strain.<sup>10</sup> In the present work, we report the complete sequence of an IncFII<sub>k</sub> plasmid harbouring an unusual gene amplification of <i>rmtH</i> in a <i>K. pneumoniae</i> strain isolated in Lebanon in 2012.

**Materials and methods**

<i>K. pneumoniae</i> strain CMUL78 was isolated from a blood sample recovered from a 5 day old newborn admitted to the Tripoli Government Hospital, Lebanon in 2012. It was identified with the MALDI-TOF MS system VITEK MS (bioMérieux). The ST was assigned with the MLST scheme available at www.pasteur.fr/mlst. Antibiotic susceptibility and MICs and ESBL production were assessed according to the guidelines of EUCAST (http://www.eucast.org/). Carbapenemase production was detected by a modified Hodge test.

The molecular characterization of aminoglycoside resistance genes was performed by PCRs targeting the aminoglycoside acetyltransferase genes <i>aac(6′)-Ib</i> and <i>aac(3)-II</i> and the 16S RMTases genes <i>armA</i>, <i>npmA</i>
and rmtA to rmtH, as previously described.10–11 Molecular identification of β-lactamases was performed by PCR amplification followed by Sanger sequencing, as previously described.13 The genetic environment of the blaOXA-48 gene was further investigated by PCR and sequencing, as previously described.13

The transferability of carbapenem and aminoglycoside resistance was assessed by broth mating-out assay. Transconjugant selection was performed on agar plates supplemented with ertapenem (0.5 mg/L) or gentamicin (50 mg/L). The plasmid content of the bacteria and the size of plasmids were determined with plasmid DNA extracted by alkaline lysis, as previously described.13 Plasmid confering resistance to aminoglycosides was extracted from the Escherichia coli transconjugant and sequenced with the Pacific Biosciences RS II SMRT technology (http://www.pacb.com/). The raw reads were de novo assembled by the Celera-based hierarchical genome assembly process with SMART portal software (http://www.pacb.com/). The genomic DNA of K. pneumoniae CMUL78 was also sequenced with Illumina sequencing technology with 300 bp paired-end libraries (Illumina, San Diego, CA, USA), which were assembled de novo to obtain genomic fragments and were mapped on plasmid pRmtH resulting from the hierarchical genome assembly process to assess the quality of sequences. ORFs were predicted and annotated with RAST server.14 The resulting annotation was manually checked. The long-range PCR was performed using specific primers (5′-CGCTGTTATCTCCCTGTTAGGC-3′ and 5′-CGATGAATCGGAGCGAACCCTGAC-3′) and the QIAEN Long Range PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The resulting complete sequence of rmtH-encoding plasmid pRmtH was submitted to EMBL/GenBank under the accession numbers LT576116.

Results and discussion

K. pneumoniae strain CMUL78 had high-level resistance to amikacin, gentamicin, tobramycin, netilmicin and arbekacin (MICs >256 mg/L) as usually observed for resistance conferred by the 16S rRNA methyltransferases. PCR targeting aminoglycoside resistance genes only detected the 16S methyltransferase rmtH gene recently described.10 CMUL78 was also resistant to all tested penicillins and their combinations with β-lactamase inhibitors. The oxymino-cephalosporins had MICs in the resistance range (cefotaxime, 2 mg/L; ceftazidime 2 mg/L) except cefepime (<0.5 mg/L). The strain exhibited susceptibility to carbapenems (imipenem, 1 mg/L; meropenem and doripenem, <0.125 mg/L), except ertapenem (MIC, 1 mg/L). PCR followed by sequencing showed that CMUL78 harboured blaOXA-48 as the only carbapenemase-encoding gene. Genetic environment mapping showed that the blaOXA-48 gene was associated with the transposon composite Tn1999.2, as previously reported.13 The ESBL synergy test was positive and the molecular characterization of the corresponding gene showed the presence of the ESBL-encoding gene blaSHV-12. Overall, the CMUL78 strain co-produced the 16S RMTase RmtH, ESBL SHV-12 and carbapenemase OXA-48, which has been observed in 1.5% of Enterobacteriaceae recovered from clinical settings in north Lebanon.13

The analysis of plasmid content revealed three plasmids, whose sizes were estimated to be ~62 kb, ~90 and ~115 kb by agar gel migration (data not shown). Two transconjugants were obtained by mating-out assays. The first transconjugant contained only the ~115 kb plasmid. It exhibited resistance to aminoglycosides and oxymino-cephalosporins, and was susceptible to penicillins combinations with β-lactamase inhibitors and carbapenems. PCR screening confirmed that this transconjugant harboured both rmtH and blaSHV-12, suggesting they are encoded by the same plasmid, designated pRmtH. The second transconjugant was characterized by the presence of the ~62 kb plasmid conferring resistance only to ertapenem, penicillins and their combinations with β-lactamase inhibitors. PCR assays showed the presence of blaOXA-48 in addition to the canonical genes repA, traU and parA genes of the 62 kb pOXA-48a plasmids.

Plasmid pRmtH was sequenced at >300× coverage depth with SMRT technology, which generates long DNA sequences. The errors in long DNA sequences were corrected with Illumina short- and high-fidelity reads. The resulting nucleotide sequence formed a circular 114208 bp plasmid with an average G+C content of 55. It contained a total of 130 ORFs, including 10 ISs. Replicon analysis showed that it belongs to the IncFII, incompatibility group15 and IncFII pMLST group K:2 (http://pubmlst.org/plasmid/).

The pRmtH backbone, identified by comparison with related plasmids, is 71055 kb in length and composed of three distinct modules: 1.7 kb plasmid replication module, 33.5 kb plasmid transfer module and 7.9 kb plasmid stability module (Figure 1a). The replication module (90316–92065 bp) comprised genes repA2 and repA. The conjugative transfer module comprised 21 tra genes (traA to traN, traQ and traS to traX) and four trb genes (trbIV/CEB). The stability module of pRmtH plasmid comprised a multimeric resolution system, the partition systems and toxin–antitoxin systems. The resolution system consisted of the site-specific resolvase encoded by resA gene. The partition system encoding genes identified within the pRmtH plasmid consisted of the segregation module-encoding genes stbA/stbB and psiA/psiB. The pRmtH plasmid also contained four toxin–antitoxin system-encoding genes: hok/sok, ccdA/ccdB, vapB/vapC systems and the novel gene combination hiPB/ReFC. The hiPB gene is usually located in the hiPB a operon and encodes a C-lox-like repressor, which forms a complex with HipA and counteracts its toxicity.16 In pRmtH, HipA is replaced by the gene encoding the ReFC toxin, a global inhibitor of translation cleaving mRNA, whose corresponding antitoxin usually is ReFB.16

As shown in Figure 1b, the pRmtH plasmid contained an accessory module of 19670 bp in length comprising antibiotic resistance genes (bases 94978–113044) and six copies of IS26, of which three were truncated. This antibiotic resistance island was bracketed by two intact IS26 mobile elements. These two copies of IS26 presented the same orientation and generated a novel IS26-mediated composite transposon of 16419 bp in length. This novel transposon, designated Tn6329, was inserted within a truncated copy of IS26, and harboured blaSHV-12, rmtH genes and ISCR2 elements. Target sequence duplication, which is a hallmark of the transposition process, was not observed on either side of the IS26-flanked regions. The absence of such a transposon mark has been previously observed for IS26.17 The six copies of IS26 within pRmtH suggest a high activity of this IS that can contribute to the formation of antibiotic resistance clusters.

However, the most intriguing finding in Tn6329 was the presence of rmtH and ISCR2 as a 4-fold tandem repeat, which suggests a gene amplification process. The number of ISCR2-rmtH units embedded within pRmtH was assessed from the long-read sequences generated by the SMRT method and analysis of the depth of sequencing of six pRmtH variants constructed in silico and differing by the number of ISCR2-rmtH units (Figure 2). The presence of repeats was confirmed by a long range PCR targeting
the region containing the repeats (data not shown). Few examples depicted gene amplification, such as the case of the amplification of IS26-\textit{bla}_{SHV-5}-\textit{IS26} units.\textsuperscript{18} In our case, gene amplification was associated with the ISCR2 mobile element. The ISCR element family comprised 19 members related to element \textit{IS91}.\textsuperscript{19} \textit{IS91} is responsible for gene amplifications, when the transposase
Figure 2. Depth of sequencing of pRmtH according to the rmtH-ISR2 unit number. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

misidentified the ter1S element during the rolling-circle transposition mechanism. A similar process may be involved in the ISCR2-rmtH amplification in pRmtH.

At last, genomic assembly also revealed that the strain belonged to the new ST ST1157, exhibited the wzi-273 capsular genotype and harboured no major virulence factor (K1, K2, rmpA, rmpA2 and accumulation of siderophores). We also detected fosA5 as an additional resistance gene and the well-known 62 kb OXA-48-encoding IncI plasmid.

In conclusion, to the best of our knowledge, this is the first observation of gene amplification for 16S RMTases. Such gene amplification is usually associated with an increased expression of the amplified gene. Such strain benefit is not obvious for rmtH, because a single copy of this gene confers very high aminoglycoside MICs. The repetitions may also facilitate the horizontal spread of rmtH. The beneficiary may be the antibiotic-susceptible bacterial population more than the rmtH-encoding bacteria, which must assume the burden of repeats. This hypothesis suggests an altruist behaviour of resistant bacteria, a paradigm opposed to the usual view of bacterial relationships. However, it is also a state easily reversible by homologous recombination, which allows the bacteria to adapt to their environment. Although gene amplification has hitherto been a scarcely reported mechanism, long-read sequencing technology should make the identification of such genetic structures much easier.

Acknowledgements

We are grateful to Professor Patrick McGann and Professor Yohei Doi for kindly providing the clinical K. pneumoniae strain MRSN2404. We also thank Alexis Pontvianne and Laurent Guillouard for their technical assistance.

Funding

This work was supported by the National Institute of Agronomic Research (USC-2018) and the Centre Hospitalier Regional Universitaire de Clermont-Ferrand, France and Ecole Doctorale en Sciences et Technologies, Université Libanaise, Lebanon.

Transparency declarations

None to declare.

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