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IncFII$_k$ plasmid harbouring an amplification of 16S rRNA methyltransferase-encoding gene *rmtH* associated with mobile element IS*CR2*

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Objectives: To investigate the resistance mechanisms and genetic support underlying the high resistance level of the *Klebsiella pneumoniae* 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 *rmtH*- and bla_{SHV-12}-encoding genes were harboured by a novel ∼115 kb IncFII$_k$ plasmid designated pRmtH, and bla_{OXA-48} by a ∼62 kb IncL/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 *rmtH*-IS*CR2* unit arranged in tandem and inserted within a novel IS*26*-based composite transposon designated Tn*6329*.

Conclusions: This is the first known report of the 16S RMTase-encoding gene *rmtH* in a plasmid. The *rmtH*-IS*CR2* 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.[1] They are classified into two subgroups designated N7-G1405 16S-RMTases and N1-A1408 16S-RMTases.[1] Nine acquired N7-G1405 16S rRNA methyltransferases have been reported in Gram-negative bacteria and were designated ArmA and RmtA to RmtH.[2–10] NpmA is the sole acquired N1-A1408 16S-RMTase identified so far.[11] The ArmA-encoding gene, initially characterized from a *Klebsiella pneumoniae* strain isolated in France, is currently spread worldwide among Enterobacteriaceae.[12] *rmtB* has also been identified among Enterobacteriaceae and is mainly observed in East Asia, Europe and North America.[12] 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 *K. pneumoniae* strain isolated from a USA soldier wounded in Iraq in 2006.[10] In contrast to the other 16S RMTase-encoding genes, the *rmtH* gene was harboured by the chromosome of the strain.[10] In the present work, we report the complete sequence of an IncFII$_k$ plasmid harbouring an unusual gene amplification of *rmtH* in a *K. pneumoniae* strain isolated in Lebanon in 2012.

Materials and methods

K. pneumoniae 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 *aac(6′)-Ib* and *aac(3)-II* and the 16S RMTases genes *armA*, *npmA*

and *rmtA* to *rmtH*, as previously described.[2,3,5–11] Molecular identification of β-lactamases was performed by PCR amplification followed by Sanger sequencing, as previously described.[13] The genetic environment of the bla_{OXA-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 conferring 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′-CGCCTGTGTATTATCTCCCTGTTAGCC-3′ and 5′-CGTATGTACAAGGCGAACAGGCGTGAC-3′) and the QIAGEN 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 oxyimino-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 bla_{OXA-48} as the only carbapenemase-encoding gene. Genetic environment mapping showed that the bla_{OXA-48} gene was associated with the transposon composite Tn*1999.2*, 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 bla_{SHV-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, ∼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 oxyimino-cephalosporins, and was susceptible to penicillins combinations with β-lactamase inhibitors and carbapenems. PCR screening confirmed that this transconjugant harboured both *rmtH* and bla_{SHV-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 bla_{OXA-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 114 208 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 $IncFII_k$ incompatibility group[15] and IncFII pMLST group K:2 (http://pubmlst.org/plasmid/).

The pRmtH backbone, identified by comparison with related plasmids, is 71 055 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 (90 316–92 065 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 (*trbICEB*). 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*/*stB* 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*/*RelE*. The *hipB* gene is usually located in the *hipBA* operon and encodes a Cro-like repressor, which forms a complex with HipA and counteracts its toxicity.[16] In pRmtH, *hipA* is replaced by the gene encoding the RelE toxin, a global inhibitor of translation cleaving mRNA, whose corresponding antitoxin usually is RelB.[16]

As shown in Figure 1(b), the pRmtH plasmid contained an accessory module of 19 670 bp in length comprising antibacterial resistance genes (bases 94 978–113 044) and six copies of IS*26*, of which three were truncated. This antibiotic resistance island was bracketed by two intact IS*26* mobile elements. These two copies of IS*26* presented the same orientation and generated a novel IS*26*-mediated composite transposon of 16 419 bp in length. This novel transposon, designated Tn*6329*, was inserted within a truncated copy of IS*26*, and harboured bla_{SHV-12}, *rmtH* genes and IS*CR2* elements. Target sequence duplication, which is a hallmark of the transposition process, was not observed on either side of the IS*26*-flanked regions. The absence of such a transposition mark has been previously observed for IS*26*.[17] The six copies of IS*26* 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 Tn*6329* was the presence of *rmtH* and IS*CR2* as a 4-fold tandem repeat, which suggests a gene amplification process. The number of IS*CR2*-*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 IS*CR2*-*rmtH* units (Figure 2). The presence of repeats was confirmed by a long range PCR targeting

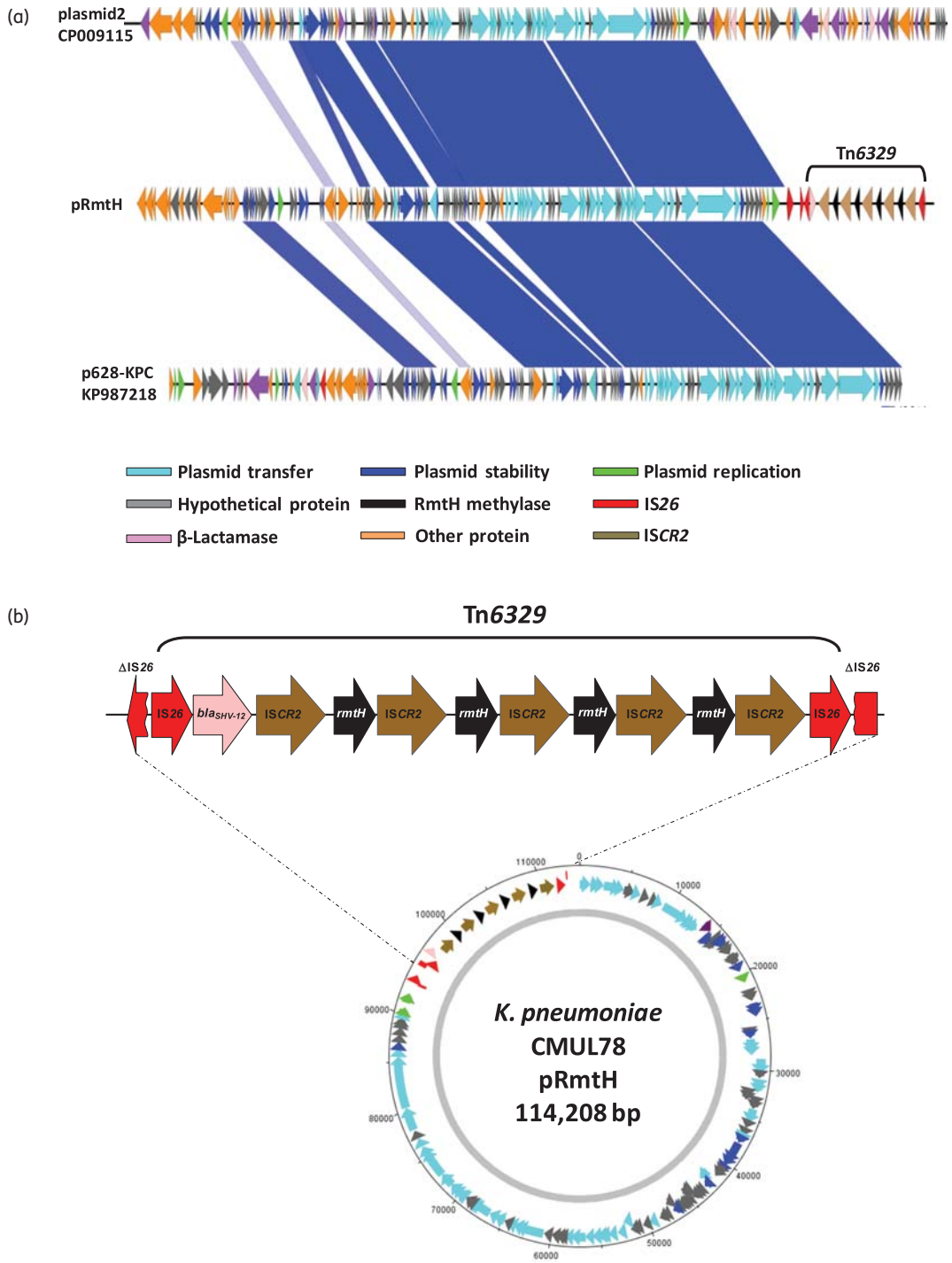

Figure 1. Complete sequence of IncFII$_k$ plasmid pRmtH. (a) Comparative analysis of pRmtH plasmid backbone and related IncFII$_k$ plasmids. Plasmids p628-KPC (*K. pneumoniae*, KP987218) and plasmid2 (*K. pneumoniae*, CP009115) shared similar regions involved in plasmid transfer, stability and replication. ORFs are indicated with arrows directed according to their transcription. Plasmid features are coloured as per the key. (b) Schematic representation of plasmid pRmtH with a focus on the IS*26*-based Tn*6329* composite transposon harbouring the 4-fold tandem repeat of the IS*CR2-rmtH* unit. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

the region containing the repeats (data not shown). Few examples depicted gene amplification, such as the case of the amplification of IS*26*-*bla*$_{SHV-5}$-IS*26* units.[18] In our case, gene amplification was associated with the IS*CR2* mobile element. The IS*CR* element family comprised 19 members related to element IS*91*.[19] IS*91* is responsible for gene amplifications, when the transposase

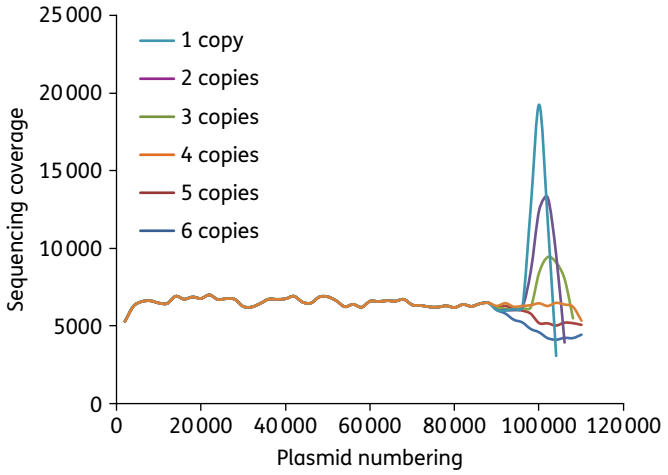

Figure 2. Depth of sequencing of pRmtH according to the *rmtH*-IS*CR2* unit number. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

misidentified the *ter*IS element during the rolling-circle transposition mechanism.[19,20] A similar process may be involved in the IS*CR2*-*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 IncL 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.

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Transparency declarations

None to declare.

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