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Coexistence of three bla_{KPC-2} genes on an IncF/IncR plasmid in ST11 *Klebsiella pneumoniae*

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ABSTRACT

Objectives: Here we report the finding of three copies of the bla_{KPC-2} gene on a plasmid in ST11 *Klebsiella pneumoniae*.
Methods: Carbapenem-resistant *K. pneumoniae* clinical strain WCHKP2 was subjected to whole-genome sequencing (WGS) using both a short-read Illumina HiSeq X10 platform and long-read MinION sequencer. Hybrid assembly was performed using Unicycler, and contigs were corrected using Pilon. Based on WGS, the sequence type (ST), capsular type, plasmid replicon type and plasmid multilocus sequence type were determined and virulence and antimicrobial resistance genes were identified. Mating was performed to identify a self-transmissible plasmid mediating carbapenem resistance.
Results: Strain WCHKP2 was resistant to imipenem [minimum inhibitory concentration (MIC) = 64 μg/mL] and meropenem (MIC = 128 μg/mL). Strain WCHKP2 had a 5 477 148-bp circular chromosome, two small ColRNAI-like plasmids (5596 bp and 10 060 bp), and one large plasmid (177 516 bp, designated pKPC2_020002) containing an IncR and an IncFII replicon. Surprisingly, there were three copies of the bla_{KPC-2} carbapenemase gene on pKPC2_020002, which was not self-transmissible. Each of the bla_{KPC-2} genes was located in the same genetic context with insertion sequence IS*Kpn27* upstream and IS*Kpn6* downstream and bracketed by IS*26*. The three copies of the IS*26*–IS*Kpn27*–bla_{KPC-2}–IS*Kpn6*–IS*26* unit were present in tandem.
Conclusion: Here we report the surprising co-existence of three copies of bla_{KPC-2} on an IncR/IncF plasmid due to the action of IS*26*. Multiple copies of IS*26* are a key factor generating genetic plasticity and could mediate the multiplication of resistance genes.

1. Introduction

Carbapenem-resistant *Klebsiella pneumoniae* has emerged as a major challenge for clinical management and infection control in human health [1] and is labelled as 'critical' in the World Health Organization (WHO) list of antimicrobial-resistant 'priority pathogens' that pose the greatest threat to human health [2]. Carbapenem resistance in *K. pneumoniae* is largely due to the production of carbapenem-hydrolysing enzymes (carbapenemases) [3]. *K. pneumoniae* carbapenemase (KPC) is the most common type of carbapenemase in *K. pneumoniae*. The KPC-encoding gene bla_{KPC} is commonly present in a single copy on plasmids and sometimes on the chromosome. Here we report the presence of three copies of bla_{KPC-2} on a single plasmid of a carbapenem-resistant *K. pneumoniae* clinical strain.

2. Materials and methods

2.1. Strain identification, antimicrobial susceptibility testing and string test

K. pneumoniae clinical strain WCHKP2 was recovered in China in August 2016 from the sputum of a male patient with pneumonia that was sent for routine clinical microbiology examination. The minimum inhibitory concentrations (MICs) of amikacin, aztreonam, aztreonam/avibactam, ceftazidime, ceftazidime/avibactam,

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ciprofloxacin, colistin, imipenem, meropenem, piperacillin/tazobactam, trimethoprim/sulfamethoxazole and tigecycline were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [4]. As there are no breakpoints for colistin and tigecycline from the CLSI, those defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/) were applied. The length of viscous strings was measured when colonies were stretched by an inoculation loop in the string test [5], with hypermucous ST23:K1 *K. pneumoniae* WCHKP030925 used as a control strain.

2.2. Genomic sequencing and analysis

Genomic DNA of strain WCHKP2 was prepared using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) and was subjected to whole-genome sequencing using both an Illumina HiSeq X10 platform (Illumina Inc., San Diego, CA) and the long-read MinION sequencer (Oxford Nanopore Technologies, Oxford, UK). De novo hybrid assembly both of short Illumina reads and long MinION reads was performed using Unicycler v.0.4.3 [6] under conservative mode for increased accuracy. Complete circular contigs were then corrected using Pilon v.1.22 [7] with Illumina reads for several rounds until no further improvements were reported.

The sequence type (ST) was determined using the genome sequence to query the multilocus sequence typing (MLST) database available at the Institut Pasteur (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Capsular typing was performed using Kaptive (https://github.com/katholt/Kaptive). Virulence genes were identified by querying the Institut Pasteur database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Antimicrobial resistance genes were identified from genome sequences using the ABRicate program (https://github.com/tseemann/abricate) to query the ResFinder database (http://genomicepidemiology.org/). Plasmid replicon type and plasmid multilocus sequence type were determined using the PlasmidFinder and pMLST tools available from the Center for Genomic Epidemiology (http://genomicepidemiology.org/).

2.3. Nucleotide sequence accession numbers

The complete sequence of plasmids p1_020002, p2_020002 and pKPC2_020002 as well as the chromosome of strain WCHKP2 have been deposited in GenBank under the accession nos. CP028539–CP028542. The short reads of strain WCHKP2 (SCKP020002) have been deposited into the SRA database under the no. SRR6955424.

2.4. Mating experiments

Conjugation experiments were carried out in broth and on filters using azide-resistant *Escherichia coli* strain J53 as recipient strain at 25 °C and 37 °C. Potential transconjugants were selected on Luria-

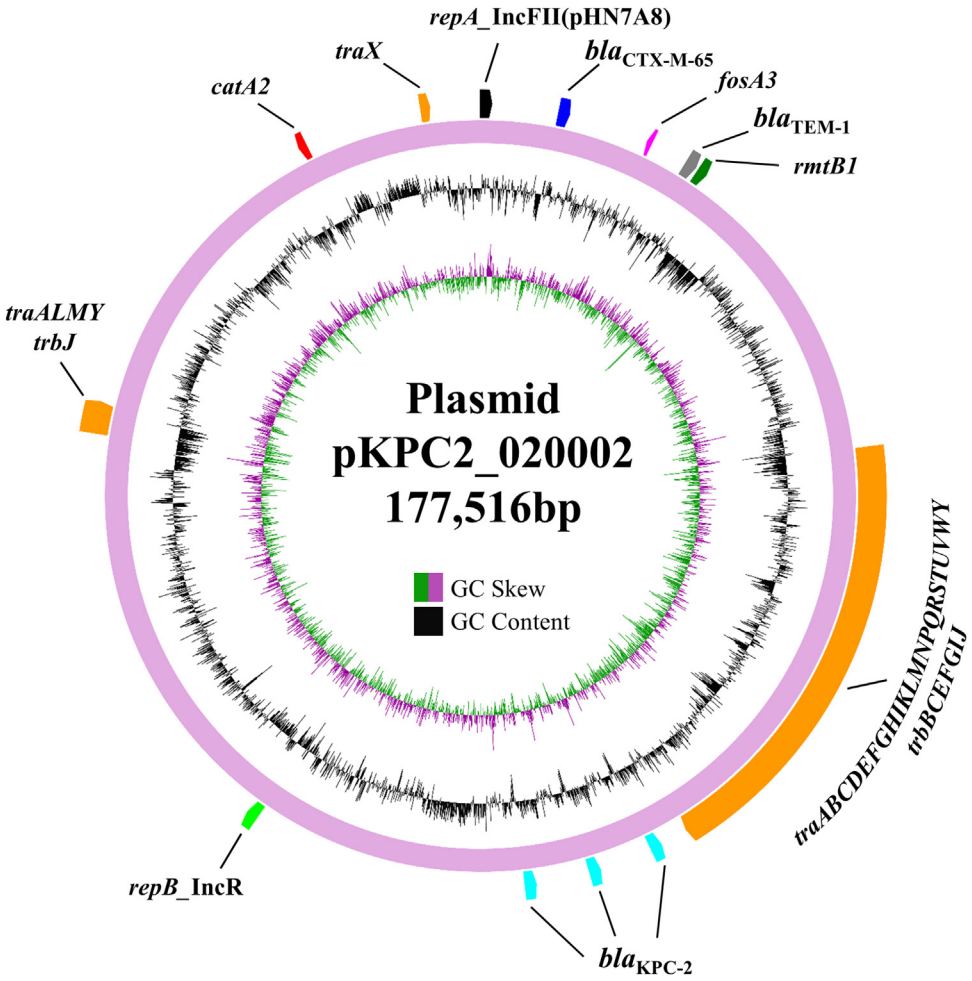

Fig. 1. Circular map of plasmid pKPC2_020002. The locations of antimicrobial resistance genes, plasmid replicon genes and genes encoding conjugation (*tra* and *trb*) are shown.

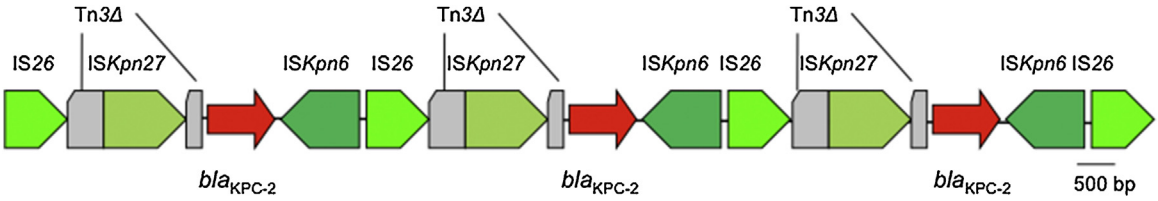

Fig. 2. Genetic context of bla_{KPC-2} on plasmid pKPC2_020002. Three copies of the IS*26*–Tn*3*Δ–IS*Kpn27*–Tn*3*Δ–bla_{KPC-2}–IS*Kpn6*–IS*26* unit are arranged in tandem.

–Bertani agar plates containing 4 μg/mL meropenem and 150 μg/mL sodium azide.

3. Results and discussion

Strain WCHKP2 was resistant to amikacin (MIC > 512 μg/mL), aztreonam (>512 μg/mL), ceftazidime (256 μg/mL), ciprofloxacin (256 μg/mL), imipenem (64 μg/mL), meropenem (128 μg/mL) and piperacillin/tazobactam (>512/4 μg/mL) but was susceptible to aztreonam/avibactam (1/4 μg/mL), ceftazidime/avibactam (2/4 μg/mL) colistin (2 μg/mL), tigecycline (0.5 μg/mL) and trimethoprim/sulfamethoxazole (<0.5/9.5 μg/mL).

Hybrid assembly of the short and long reads revealed that strain WCHKP2 had a 5 477 148-bp circular chromosome, two small ColRNAI-like plasmids (5596 bp and 10 060 bp, designated p1_020002 and p2_020002), and one large plasmid (177 516 bp, designated pKPC2_020002) containing an IncR and an IncFII (pHN7A8) replicon. Strain WCHKP2 belongs to ST11, the predominant ST of carbapenem-resistant *K. pneumoniae* in China [8], and capsular type KL64. Strain WCHKP2 was not hypermucous in the string test. With respect to virulence, strain WCHKP2 harboured genes encoding yersiniabactin (*fyuA*, *irp1*, *irp2* and *ybtAEPQSTUX*) and type 3 fimbriae (*mrkABCDFHIJ*) but had no *rmpA* or *rmpA2* genes, both of which encode the hypermucous phenotype and serve as a marker for hypervirulence. All of the virulence factors were located on the chromosome of strain WCHKP2.

Strain WCHKP2 had 13 types of antimicrobial resistance genes mediating resistance to aminoglycosides [*ant(2″)-Ia*, *aadA2*, *rmtB*), β-lactams ($bla_{CTX-M-65}$, $bla_{SHV-158}$, bla_{KPC-2}, bla_{TEM-1b}), fosfomycin (*fosA3*, *fosA6*), quinolones (*oqxA*, *oqxB*), rifampicin (*catA2*) and sulphonamides (*sul1*). Among these resistance genes, $bla_{CTX-M-65}$, bla_{KPC-2}, bla_{TEM-1b}, *fosA3*, *rmtB* and *catA2* were carried by the large plasmid pKPC2_020002 (Fig. 1), whilst the remaining genes were located on the chromosome. Surprisingly, there were three copies of bla_{KPC-2} on pKPC2_020002. Coverage of bla_{KPC-2} was 690×, whilst that of *rmtB* and $bla_{CTX-M-65}$, both of which were carried on pKPC2_020002, was 230× and 243×, respectively. This verified that there were three copies of bla_{KPC-2}. The co-existence of two copies of bla_{KPC-3} on a single plasmid has been reported previously [9] but not three copies of the same bla_{KPC} gene. As we demonstrated recently, Illumina sequencing alone was unable to reliably reveal the presence of multiple copies of the same gene, whereas combination with MinION sequencing is able to resolve the copy number of genes and therefore untangle the complicated genetic context of antimicrobial resistance genes.

Each of the bla_{KPC-2} genes was located in the same genetic context with insertion sequence IS*Kpn27* upstream and IS*Kpn6* downstream and bracketed by IS*26* (Fig. 2). The three copies of the IS*26*–IS*Kpn27*–bla_{KPC-2}–IS*Kpn6*–IS*26* unit were present in tandem. Insertion of IS*26* could generate 9-bp direct target repeats (DR). However, there were no 9-bp DR present around any copy of IS*26* or between any two copies of this insertion sequence. This suggests that the tandem multiplication of IS*26*–IS*Kpn27*–bla_{KPC-2}–IS*Kpn6* was due to homologous recombination. It is well known that two copies of IS*26* can form a composite transposon, which may be excised from plasmids to form a circular intermediate [10,11]. IS*26* provides a region for homologous recombination and therefore could serve as a Trojan horse [12]. In the presence of IS*26* on a plasmid, the intermediate may be integrated into the plasmid via homologous recombination to generate tandem repeats. Previous studies have shown that IS*26* is involved in the dissemination and amplification of bla_{KPC} [13], although the co-existence of three copies of bla_{KPC} has not been seen before.

No transconjugants were obtained despite repeated mating attempts, suggesting that pKPC2_020002 was not self-transmissible. Although all conjugative genes were present on pKPC2_020002, *traI*, which encodes the TraI DNA relaxase and is required for conjugative transfer [14], was truncated by the insertion of IS*26*, explaining why pKPC2_020002 was not self-transmissible.

4. Conclusion

Here we report the co-existence of three copies of bla_{KPC-2} on an IncR/IncF plasmid, which was due to the action of IS*26*. Multiple copies of IS*26* could mediate the multiplication of resistance genes to generate complicated genetic context of antimicrobial resistance genes.

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Competing interests

None declared.

Ethical approval

Not required.

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