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MECHANISMS OF RESISTANCE

Coexistence of Two *bla*$_{NDM-5}$ Genes on an IncF Plasmid as Revealed by Nanopore Sequencing

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ABSTRACT In a carbapenem-resistant *Escherichia coli* clinical isolate of sequence type 167, two copies of *bla*$_{NDM-5}$ were found on a 144,225-bp IncF self-transmissible plasmid of the F36:A4:B$^-$ type. Both *bla*$_{NDM-5}$ genes were located in 11,065-bp regions flanked by two copies of IS*26*. The two regions were identical in sequence but were present at different locations on the plasmid, suggesting a duplication of the same region. This study highlights the complex genetic contexts of *bla*$_{NDM-5}$.

KEYWORDS carbapenem resistance, plasmids, NDM, *Escherichia coli*, carbapenemase, carbapenems

New Delhi metallo-β-lactamase (NDM) is a type of carbapenem-hydrolyzing enzyme (carbapenemase) with the ability to hydrolyze all β-lactams except monobactams (1), and it represents a serious challenge for treatment of bacterial infections, infection control, and public health. To date, there are 21 variants of NDM, including NDM-5, one of the most common variants encountered in the *Enterobacteriaceae* (2–5). The NDM-5-encoding gene, *bla*$_{NDM-5}$, usually exists in a single copy on plasmids. However, we have found the peculiar presence of two copies of *bla*$_{NDM-5}$ on a single plasmid within an *Escherichia coli* clinical isolate, which is reported here.

E. coli strain SCEC020007 was recovered from the urine of a female outpatient with a urinary tract infection in October 2016 in China. The strain was resistant to amikacin (MIC, >512 μg/ml), ceftazidime (>512 μg/ml), ceftazidime-avibactam (>512/4 μg/ml), ciprofloxacin (256 μg/ml), imipenem (64 μg/ml), meropenem (256 μg/ml), piperacillin-tazobactam (>512/4 μg/ml), and trimethoprim-sulfamethoxazole (128/2,432 μg/ml) and intermediate to aztreonam (8 μg/ml) but was susceptible to colistin (2 μg/ml) and tigecycline (0.25 μg/ml), as determined using the broth dilution method of the Clinical and Laboratory Standards Institute (6). As there are no breakpoints of colistin and tigecycline from CLSI, those defined by EUCAST (http://www.eucast.org/) were applied.

A draft genome sequence of the strain was generated on the Illumina HiSeq X10 platform, which generated 5,557,833 clean reads and 1.67 Gb of clean bases. A total of 113 contigs (102 >1,000 bp; N_{50}, 126,680 bp) with a 50.76% GC content were *de novo* assembled using SPAdes (7). Strain SCEC020007 belonged to phylogenetic group A, determined using PCR as described previously (8), and sequence type 167 (ST167) was determined by using the genomic sequence to query the *E. coli* multilocus sequence typing database (http://enterobase.warwick.ac.uk/species/index/ecoli). 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/). Strain SCEC020007 had 9 antimicrobial resistance genes

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mediating resistance to aminoglycosides (*aadA2*, *aadA5*, and *rmtB*), β-lactams (bla_{NDM-5} and bla_{TEM-1}), tetracycline [*tet*(A)], sulfonamides (*sul1*), and trimethoprim (*dfrA12* and *dfrA17*). Plasmid replicon types within strain SCEC020007 were determined using the PlasmidFinder tool (http://genomicepidemiology.org/). Surprisingly, strain SCEC020007 had an IncFIA, an IncFII, and an IncB/O/K/Z replicon, but no IncX3, which is the common replicon type of plasmids associated with bla_{NDM-5}.

To untangle the genetic context of bla_{NDM-5}, strain SCEC020007 was subjected to sequencing using the long-read MinION Sequencer (Nanopore, Oxford, UK). MinION sequencing generated 66,354 reads and 524,460,566 nucleotides (mean size, 7,905 bp; N_{50}, 12,429 bp; mean read quality, 9). A *de novo* hybrid assembly of both short Illumina reads and long MinION reads was constructed using Unicycler v0.4.3 (9) in conservative mode for increased accuracy. The complete circular contigs generated were then corrected using Plion v1.22 (10) with Illumina reads for several rounds until no change was detected. The hybrid assembly of Illumina and MinION reads revealed that strain SCEC020007 had a 4.8-Mb circular chromosome, a 144,225-bp plasmid (designated pNDM5_020007) containing IncFIA and FII replicons, and an 84,952-bp plasmid with an IncB/O/K/Z replicon (designated pBOKZ_020007). Surprisingly, there were two copies of bla_{NDM-5} in strain SCEC020007, both of which were present on pNDM5_020007. Both bla_{NDM-5} genes were located in 11,065-bp regions flanked by two copies of IS*26*. The two regions were identical in sequence but were present at different locations on pNDM5_020007 (Fig. 1), suggesting that the 11,065-bp region is duplicated. The presence of the two bla_{NDM-5} genes and their locations on pNDM5_020007 were confirmed by PCR. The 11,065-bp region contained a complex class 1 integron with a *dfrA17-aadA5* cassette array and IS*CR1* (insertion sequence common region 1), which is truncated by IS*26* at its 5′ conserved segment; a 69-bp remnant of *cutA1* (encoding an ion-tolerant protein); *dsbC* (encoding an oxidoreductase); *trpF* (encoding a phosphoribosyl anthranilate isomerase); *ble* (mediating bleomycin resistance); bla_{NDM-5}, a truncated IS*Aba125*; and a truncated IS*Ecp1*/IS*Ec9* element (Fig. 1). The coexistence of two bla_{NDM-5} genes has not been reported before, but the coexistence of two bla_{NDM-1} genes has been described previously (11, 12). Two tandem copies of bla_{NDM-1} genes have been found in the chromosomes of an ST167 *E. coli* in China (11) and a *Pseudomonas aeruginosa* strain in Serbia (12). In both cases, the tandem copies of bla_{NDM-1} are associated with IS*CR1* but not with IS*26*. It is known that IS*CR1* uses the rolling circle mechanism for transposition and may generate tandem duplication of its mobilized sequence via homologous recombination (13). However, the duplication of the 11,065-bp region carrying bla_{NDM-5} on pNDM5_020007 is not tandem, suggesting that the duplication might not result from the action of IS*CR1*, but could be mediated by IS*26*. Two copies of IS*26* could form a composite transposon able to mobilize the intervening genetic components. However, no 8-bp direct target repeats, which are characteristics of IS*26* transposition, were present flanking any of the IS*26*-bracketed regions (Fig. 1), suggesting that homologous recombination had occurred. The exact mechanism for the duplication of such a large region warrants further studies.

Assembly based on Illumina reads alone generated only a single contig containing bla_{NDM-5} and was unable to reveal that there were actually two identical copies of the same contig. This imposes difficulties for completing the bla_{NDM-5}-carrying plasmid sequence by conventional methods, including by PCR and Sanger sequencing to close gaps between contigs. In contrast, MinION sequencing was able to resolve the copy numbers of genes and contigs and their exact position on the plasmid relative to each other.

Plasmid multilocus sequence typing (pMLST) was performed using the pMLST tool (https://cge.cbs.dtu.dk/services/pMLST/). pNDM5_020007 belongs to the F36:A4:B$^-$ type. pNDM5_020007 has the closest similarity (97% coverage and 99% identity) to a 149.5-kb unnamed plasmid (GenBank accession no. CP023871) from *E. coli* strain FDAARGOS_434, which was recovered from a human rectal swab in British Colombia, Canada, in 2014. This unnamed plasmid also carries bla_{NDM-5} (a single copy) and belongs to the F36:A4:B$^-$ type. Backbones of pNDM5_020007 and the unnamed

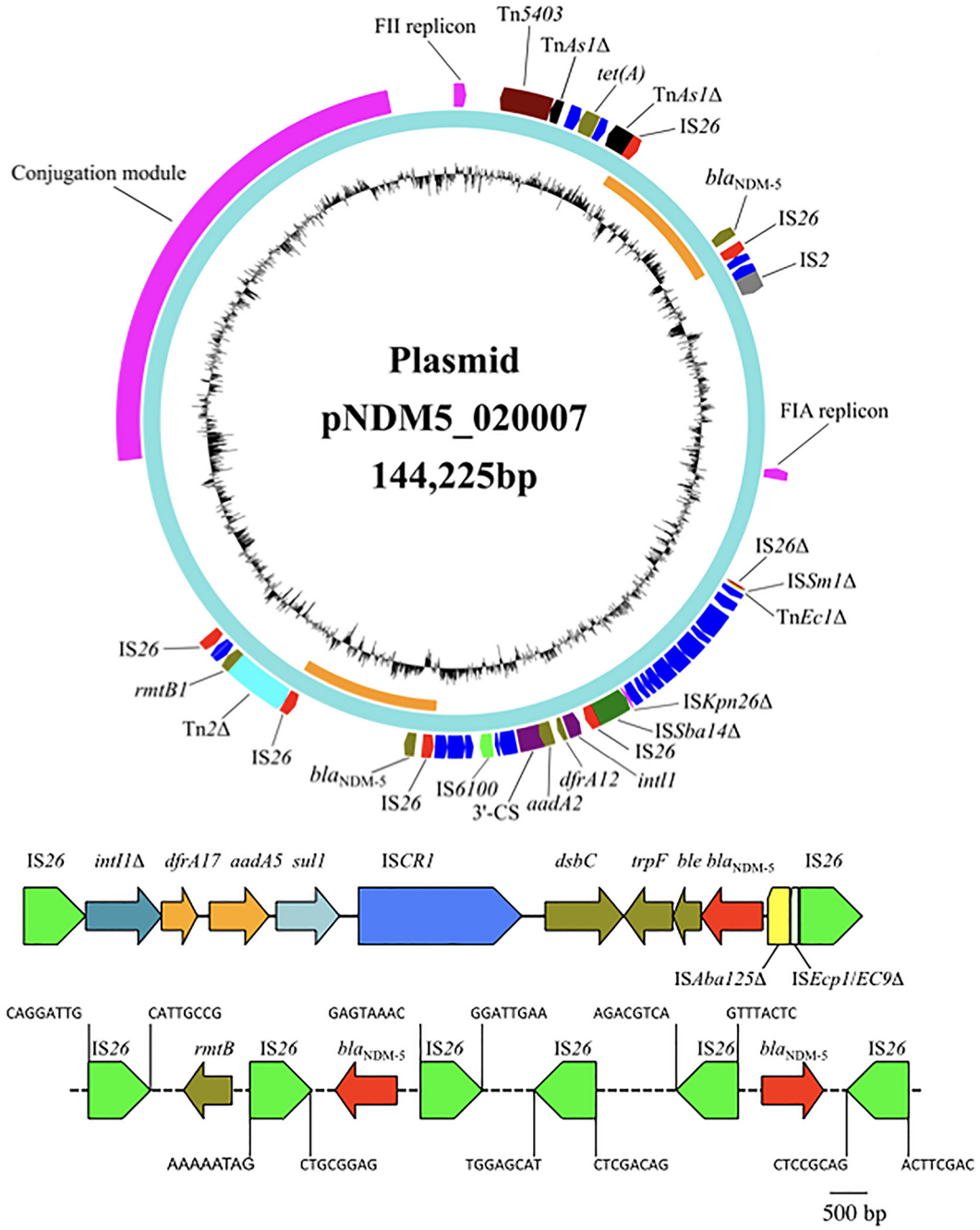

FIG 1 pNDM5_020007 and the genetic context of bla_{NDM-5}. The two 11,065-bp bla_{NDM-5}-containing regions bracketed by IS*26* are indicated by orange circles in the map of pNDM5_020007 and are shown in detail in the middle. Δ represents truncated genes or mobile genetic elements. The 8-bp target sequences flanking IS*26* are shown at bottom.

plasmid of strain FDAARGOS_434 are almost identical, suggesting that they might have originated from a common plasmid. Conjugation experiments were carried out in broth and on filters, with the azide-resistant *E. coli* strain J53 as the recipient. pNDM5_020007 could be transferred by conjugation, suggesting that it is self-transmissible. MICs of imipenem and carbapenem against the *E. coli* J53 transconjugant carrying pNDM5_020007 were 8 and 16 μg/ml, respectively, which were identical to those against the *E. coli* J53 transconjugant carrying an IncX3 plasmid with a single copy of bla_{NDM-5} from a local strain (14). Although the two plasmids belonged to different replicon types, it appears that the two copies of bla_{NDM-5} did not alter the activity of carbapenems compared with that of a plasmid with a single copy.

In conclusion, we identified the presence of two bla_{NDM-5} genes on an F36:A4:B$^-$ self-transmissible plasmid. The coexistence of two bla_{NDM-5} genes was due to the duplication of an IS*26*-bracketed region containing IS*CR1*.

Accession number(s). The complete sequences of pBOKZ_020007 and pNDM5_020007 and the chromosomal sequence of strain SCEC020007 have been deposited in GenBank under the accession no. CP025625, CP025626, and CP025627, respectively.

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