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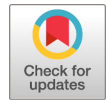


# An IncR Plasmid Harbored by a Hypervirulent Carbapenem-Resistant *Klebsiella pneumoniae* Strain Possesses Five Tandem Repeats of the $bla_{KPC-2}$::$NTE_{KPC}$-Id Fragment

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**ABSTRACT** Completed sequences of three plasmids from a carbapenem-resistant hypervirulent *Klebsiella pneumoniae* isolate, SH9, were obtained. In addition to the pLVPK-like virulence-conferring plasmid (pVir-CR-HvKP_SH9), the two multidrug-resistant plasmids (pKPC-CR-HvKP4_SH9 and pCTX-M-CR-HvKP4_SH9) were predicted to originate from a single pKPC-CR-HvKP4-like multireplicon plasmid through homologous recombination. Interestingly, the $bla_{KPC-2}$ gene was detectable in five tandem repeats exhibiting the format of an $NTE_{KPC}$-Id-like transposon (IS*26*-ΔTn*3*-IS*Kpn8*-$bla_{KPC-2}$-ΔIS*Kpn6*-*korC*-*orf*-IS*26*). The data suggest an important role of DNA recombination in mediating active plasmid evolution.

**KEYWORDS** hypervirulent, *Klebsiella pneumoniae*, $bla_{KPC-2}$, cointegration, tandem repeat

The notorious nosocomial pathogen *Klebsiella pneumoniae* has evolved into two clinically significant clades, namely, hypervirulent *K. pneumoniae* (hvKP) and carbapenem-resistant *K. pneumoniae* (CR-Kp), both of which can cause severe infections (1). Recently, convergence of genetic elements encoding hypervirulence and carbapenem resistance (i.e., CR-hvKP) in a single *K. pneumoniae* strain was reported, suggesting that such strains continue to evolve and pose a serious threat to public health (2–5). Emergence of CR-hvKP was due to acquisition of the virulence plasmid by a carbapenem-resistant strain or acquisition of a carbapenemase-producing plasmid by a hypervirulent strain (2, 3). The KPC-2-encoding gene $bla_{KPC-2}$ normally exists as a single copy in plasmids. However, by using Oxford Nanopore sequencing technology, we detected five copies of $bla_{KPC-2}$ on a single plasmid in an sequence type 11 (ST11) hvKP isolate. This study characterized the virulence potential and antimicrobial susceptibility of this phenotypically convergent superbug and unveiled the genetic basis of phenotypes conferred by the plasmids that this strain harbored.

*K. pneumoniae* SH9, a strain identified from a nationwide surveillance project, was subjected to antimicrobial susceptibility testing with the agar dilution method, and the results were interpreted according to CLSI guidelines (6). SH9 exhibited resistance to cefotaxime (MIC, >128 μg/ml), ceftazidime (MIC, 128 μg/ml), cefepime (MIC, 128 μg/ml), ertapenem (MIC, >128 μg/ml), imipenem (MIC, 16 μg/ml), meropenem (MIC, 128 μg/ml), amikacin (MIC, >256 μg/ml), and ciprofloxacin (MIC, 64 μg/ml). However, the strain remained susceptible to colistin (MIC, ≤0.25 μg/ml). Wax moth larvae were tested for virulence as described previously and SH9 was shown to be hypervirulent (data not shown) (7). MLST with Kleborate and capsular typing with Kaptive indicated that SH9 belonged to ST11 and serotype K47, a dominant clone of KPC-producing *K. pneumoniae* in China (8–11). These findings suggested that *K. pneumoniae* SH9 was an

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ST11 carbapenem-resistant hypervirulent strain that carried a pLVPK-like virulence-conferring plasmid and multidrug-resistant (MDR) plasmids resembling the previously reported ST11 CR-hvKP strains (2, 3).

Whole-genome sequencing was conducted to study the complete sequence of plasmids in strain SH9 by using the Illumina NextSeq 500 platform and the long-read MinION sequencer (12). The rapid sequencing kit (SQK-RBK001) and flow cell type R9.4 were used for Nanopore MinION sequencing. Genome assembly was conducted using Illumina reads with SPAdes v.3.11.1 (13). Hybrid assembly of short Illumina reads and long MinION reads was constructed with Unicycler v.0.3.0 (14). The complete circular plasmid sequences were modified using Pilon v.1.22 for several rounds until no change was detected (15). Complete plasmid sequences were annotated by the RAST tool (16).

We recovered three circular plasmids of 188,648 bp (pVir-CR-HvKP_SH9; GenBank accession no. MH255828), 113,941 bp (pKPC-CR-HvKP4_SH9; GenBank accession no. MH255827), and 98,684 bp (pCTX-M-CR-HvKP4_SH9; GenBank accession no. MH255829). A BLASTN search against the NCBI nucleotide database indicated that plasmid pVir-CR-HvKP_SH9 was 99% identical to the pLVPK-like virulence plasmids pVir-CR-HvKP267 (GenBank accession no. MG053312) and pSGH10 (GenBank accession no. CP025081) recovered from clinical *K. pneumoniae* strains, with 97% coverages (17, 18). Plasmid pVir-CR-HvKP_SH9 contains the IncHI1B and IncFIB(K) replicons, exhibited a GC content of 49.9%, and comprised 225 predicted coding sequences. It also harbored virulence-associated genes *rmpA*, *rmpA2*, and *iutAiucABCD* (data not shown). However, a gene cluster encoding salmochelin (*iroBCDN*) in plasmid pLVPK was absent from pVir-CR-HvKP_SH9. By encoding siderophores (aerobactin and/or salmochelin) and regulators of the mucoid phenotype (RmpA and RmpA2), which were restricted to hvKP isolates, pLVPK-like virulence plasmids were found to play a pivotal role in *K. pneumoniae* hypervirulence (19).

The carbapenem resistance-encoding plasmid (pKPC-CR-HvKP4_SH9) harbored by strain SH9 comprises the IncR replicon, exhibited a GC content of 55.0%, and comprised 159 predicted open reading frames (ORFs). This plasmid carried the *catA2* gene and five copies of $bla_{\text{KPC-2}}$, as confirmed by the Nanopore raw reads and assembled sequences. The third plasmid harbored by this strain, pCTX-M-CR-HvKP4_SH9, was found to exhibit a GC content of 51.5% and comprise 139 predicted coding sequences, among which were the IncFII replicon and the $bla_{\text{CTX-M-65}}$, $bla_{\text{TEM-1B}}$, *rmtB*, and *fosA_14* genes bound by various insertion sequences. Interestingly, both pKPC-CR-HvKP4_SH9C and pCTX-M-CR-HvKP4_SH9 exhibited $>$99% identities with the IncFII/R-type conjugative MDR plasmid pKPC-CR-HvKP4 (GenBank accession no. MF437312), previously recovered from an ST11 CR-hvKP isolate (2), at 100% and 92% coverage, respectively (Fig. 1), suggesting that genetic recombination events might be responsible for generation of these plasmids. As many as 12 copies of IS*26* were scattered across the complete sequence of plasmid pKPC-CR-HvKP4; however, target site duplications flanking IS*26* were not observed, indicating that homologous recombination events mediated by two IS*26* elements in plasmids of different replicons rather than replicative transposition among the three plasmids occurred (Fig. 2c) (20, 21). The conjugation experiment was performed using an azide-resistant *Escherichia coli* J53 ($Az^r$) strain as the recipient, and MacConkey agar supplemented with 100 mg/ml sodium azide and 2 mg/ml meropenem was used to select transconjugants (22). A previous study indicated that IncR plasmids did not possess conjugational transfer genes but may be mobilizable, thus broadening their host spectrum by forming multireplicon cointegrates with plasmids of other incompatible types, such as IncA/C and IncN (23). In this study, plasmid pKPC-CR-HvKP4_SH9 was not transferrable to the recipient *E. coli* strain J53 via conjugation. Also, no pKPC-CR-HvKP4-like cointegrate was detected after conjugation, leading us to hypothesize that homologous recombination occurs at a relatively low frequency, thereby limiting the rate of transmission of $bla_{\text{KPC-2}}$ genes located in IncR plasmids.

Five copies of $bla_{\text{KPC-2}}$ genes were identified on pKPC-CR-HvKP4_SH9, each in one of five identical 5,699-bp regions linked to each other in tandem, suggesting that this region exhibits a high degree of mobility and has been heavily duplicated (Fig. 2). The

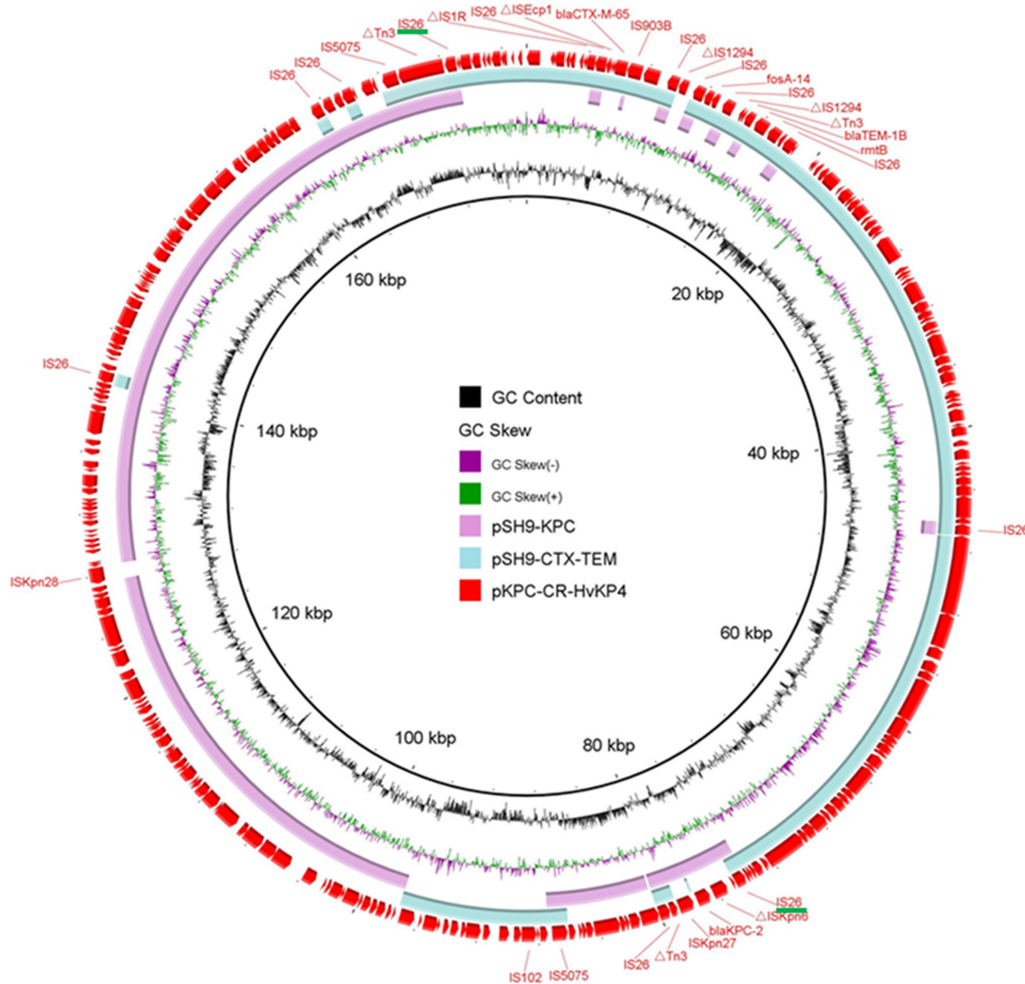


**FIG 1** Circular maps of two MDR plasmids recovered from *K. pneumoniae* SH9 and a similar plasmid pKPC-CR-HvKP4 recorded in the NCBI database. The two IS*26* elements responsible for the homologous recombination are underlined in green. This figure was constructed with BRIG (33).

5,699-bp region was located in a non-Tn*4401* element homologous to the $NTE_{KPC}$-Id fragment in plasmid pKPC-LKEc (GenBank accession no. KC788405), with the structure of IS*26*-ΔTn*3*-IS*Kpn8*-$bla_{KPC-2}$-ΔIS*Kpn6*-*korC*-*orf* (Fig. 2) (24, 25). The first copy of the 5,699-bp $NTE_{KPC}$-Id-like region was located directly upstream of an IS*26* element, generating a 6.5-kb fragment bordered by two IS*26*s. Such a 6.5-kb element may effectively act as a composite transposon that can mobilize the intervening genetic components. Detailed sequence analysis of the $bla_{KPC-2}$ region enabled us to predict the duplication mechanism that creates the tandem repeats. First, the 5.6-kb translocatable unit (TU), a circular form of the $NTE_{KPC}$-Id-like fragment, was generated via excision from a preexisting IS*26*-bound transposon via homologous recombination. The TU was then incorporated into an existing IS*26* by using the conservative Tnp26-catalyzed mechanism or homologous recombination (less frequently). Last, repetition of the incorporation process with the same TU should lead to formation of the $NTE_{KPC}$-Id-like tandem repeats in pKPC-CR-HvKP4_SH9 (21). To our knowledge, coexistence of five $bla_{KPC-2}$ genes with the $NTE_{KPC}$-Id structure within a single plasmid was not reported previously, but carriage of multiple copies of $bla_{KPC}$ on Tn*4401* has been described (26–32). Whether the carriage of multiple copies of $bla_{KPC-2}$ genes further enhances carbapenem resistance level in the host strain needs further investigation.

Here, we reported an ST11 CR-hvKP isolate carrying three plasmids, one pLVPK-like virulence plasmid and two MDR plasmids, predicted to originate from a single pKPC-

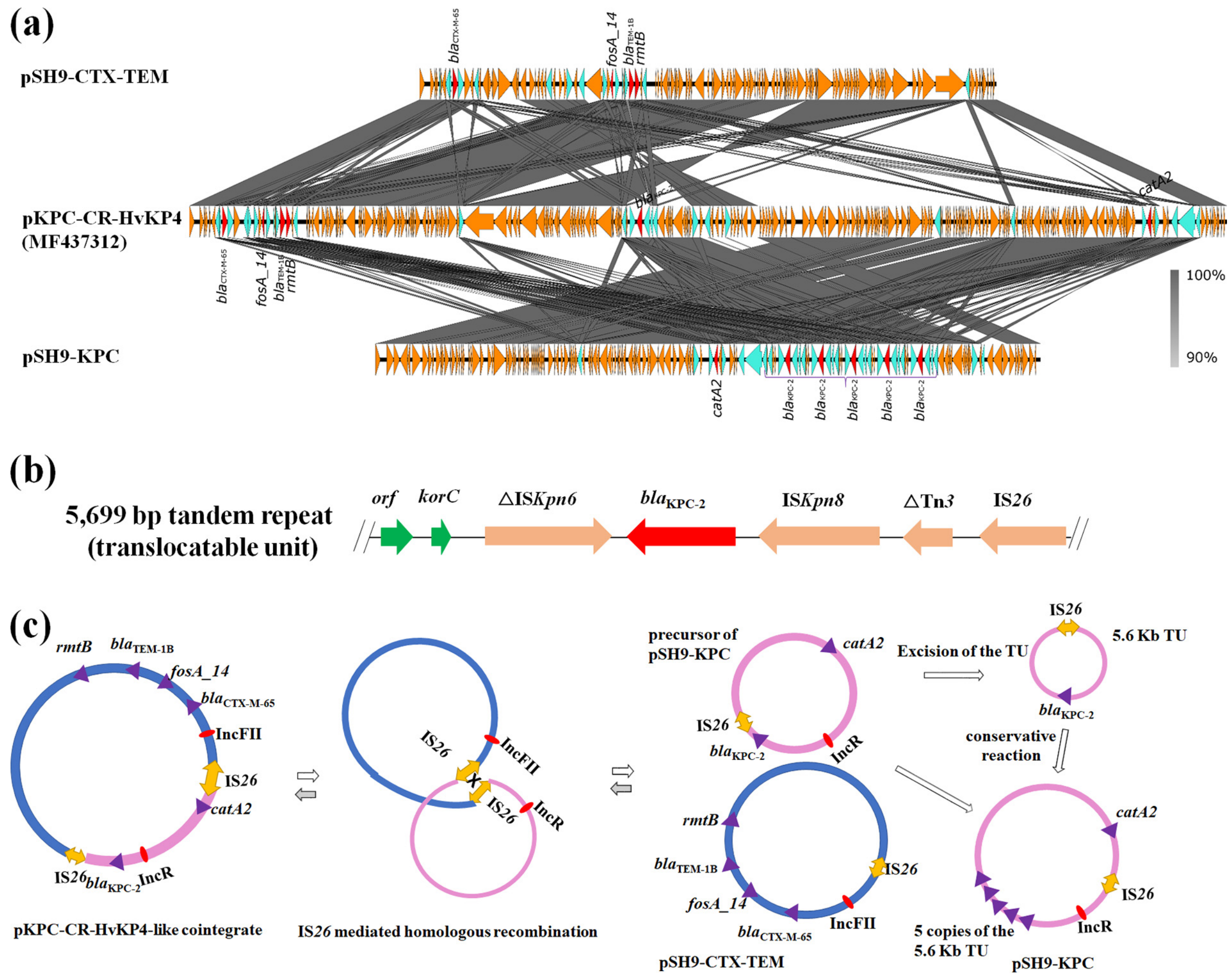


**FIG 2** Mechanisms of plasmid recombination. (a) Structure alignment of the three plasmids using EasyFig (34). Yellow, blue, and red triangles indicate ORFs, insertion sequences, and antimicrobial resistance genes, respectively. (b) Genetic composition of the 5,699-bp *bla*$_{KPC-2}$-bearing tandem repeat region that formed the translocatable unit (TU) that was excised from the precursor of plasmid pKPC-CR-HvKP4_SH9. (c) Mechanisms of plasmid recombination. IS*26* mediated the homologous recombination leads to generation of plasmids pKPC-CR-HvKP4-like cointegrate, pKPC-CR-HvKP4_SH9, and pCTX-M-CR-HvKP4_SH9. Five tandem repeats of *bla*$_{KPC-2}$-bearing fragments were generated via Tnp26-catalyzed conservative reaction, which incorporated the 5.6-kb TU next to a preexisting IS*26*. pSH9-KPC represents pKPC-CR-HvKP4_SH9, and pSH9-CTX-TEM represents pCTX-M-CR-HvKP4_SH9 in the figure.

CR-HvKP4-like multireplicon plasmid through homologous recombination. Five copies of *bla*$_{KPC-2}$ genes were tandemly located on a nonconjugative plasmid with the NTE$_{KPC}$-Id-like structure (IS*26*-ΔTn*3*-IS*Kpn8*-*bla*$_{KPC-2}$-ΔIS*Kpn6*-*korC*-*orf*-IS*26*), which was presumably created as a result of Tnp26-catalyzed conservative reaction through activity of TUs. Findings in this study indicate that plasmids in the ST11 CR-hvKP clone can undergo active genetic recombination events, the evolution trends of which should be closely monitored.

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We have no conflicts of interest to declare.

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