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Complete Sequence of a bla_{KPC}-Harboring Cointegrate Plasmid Isolated from *Escherichia coli*

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Horizontal transfer of bla_{KPC}-harboring plasmids contributes significantly to the inter- and intraspecies spread of *Klebsiella pneumoniae* carbapenemase (KPC). Here we report the complete nucleotide sequence of a bla_{KPC}-harboring IncFIA plasmid, pBK32533, from *Escherichia coli*. pBK32533 is a cointegrate plasmid comprising of a 72-kb sequence identical to that of the nonconjugative pBK30661 plasmid plus an additional 170-kb element that harbors the genes for plasmid transfer. pBK32533 demonstrates how bla_{KPC} can be spread from a nonconjugative plasmid through cointegration.

The rapid emergence of carbapenem resistance in pathogenic Gram-negative bacteria (GNB) is a major global concern. The increasing incidence and spread of *Klebsiella pneumoniae* carbapenemase (KPC) have been well documented among nosocomial *K. pneumoniae* infections in the northeastern United States (1, 2). Two scenarios contribute to the spread of these emerging pathogens which challenge the design of effective infection control strategies. First, there is dispersion of KPC-harboring strains from well-established backgrounds—clonal dissemination (1, 2). For example, KPC has successfully disseminated worldwide, and the dissemination is mostly attributed to a *K. pneumoniae* clone, ST258 (2, 3). Second, strains become resistant as a consequence of *de novo* acquisition of bla_{KPC}-harboring plasmids (4). The inter- and intraspecies dissemination of bla_{KPC} is made possible by its presence on various bla_{KPC}-harboring transferable plasmids. Currently, a wide range of KPC plasmids with different incompatibility (Inc) replicon groups, including IncFIA, IncFII, IncN, IncX, IncR, IncI2, IncA/C, ColE1, and IncL/M, have been identified (2). The importance of these varied genetic backbones has been reviewed recently (2).

IncF plasmids are a large family of low-copy-number plasmids and are primarily restricted to the family of *Enterobacteriaceae* (5). Plasmids belonging to IncF family are linked to the emergence of resistance to β-lactam antibiotics worldwide, including carbapenem resistance (5–8). Among them, bla_{KPC}-harboring IncFIA plasmids are widely distributed in parts of the northeastern United States (7). In a recent molecular surveillance study, we found two bla_{KPC}-harboring IncFIA plasmids, pBK30661 and pBK30683, accounting for ~20% of the KPC-producing isolates in 10 hospitals in New York and New Jersey (7). Among them, pBK30661 is nonconjugative and harbors multiple antimicrobial resistance determinants but lacks the plasmid transfer operon (*tra*) and the origin of transfer site (*oriT*) regions (7). In contrast, pBK30683 carries a nearly identical pBK30661-like region as well as an additional 68-kb fragment harboring the *tra* and *oriT* elements (7). To determine the significance of this finding, we designed four duplex PCRs using plasmid markers to detect plasmids similar to pBK30661 and pBK30683 in a collection of KPC-carrying *Enterobacteriaceae* isolates (7). Intriguingly, pBK30661-like plasmids (determined by pBK30661 PCR-specific markers) were found in this study in both *K. pneumoniae* and other non-*K. pneumoniae Enterobacteriaceae* isolates (*Enterobacter cloacae* and *Escherichia coli*), suggesting that an additional mechanism(s) contributes to their transfer (7). In order to further investigate a possible transfer mechanism(s), we completely sequenced the bla_{KPC}-harboring pBK30661-like IncFIA pBK32533 plasmid from the *E. coli* isolate identified in the previous study (7).

E. coli isolate BK32533 was recovered from a urine sample from a patient with a urinary tract infection in a New Jersey hospital in April 2011. Multiplex real-time PCR for bla_{KPC} variants showed that BK32533 harbors bla_{KPC-3} (9). Multilocus sequence typing (MLST) analysis revealed that BK32533 belongs to ST2289 (13-13-5-13-16-10-9) (10). Conjugation experiments were performed to examine the transferability of bla_{KPC}-harboring plasmid from BK32533 using the *E. coli* J53Az^r strain as the recipient as described previously (11). *E. coli* J53 transconjugants were selected on lysogeny broth (LB) agar plates with 100 μg/ml ampicillin and 100 μg/ml sodium azide. Multiplex real-time PCR was used to screen for the presence of the bla_{KPC} gene in *E. coli* J53 transconjugants (9). The bla_{KPC}-bearing plasmid was successfully transferred to *E. coli* J53 by conjugation with an efficiency of 2.1×10^{-7}.

BK32533 and its *E. coli* J53 transconjugant were subjected to susceptibility testing using broth microdilution in cation-adjusted Mueller-Hinton broth (MHB) and Sensititre GNX2F panels (Thermo Fisher Scientific, Waltham, MA) as described previously (11) and according to Clinical and Laboratory Standards Institute

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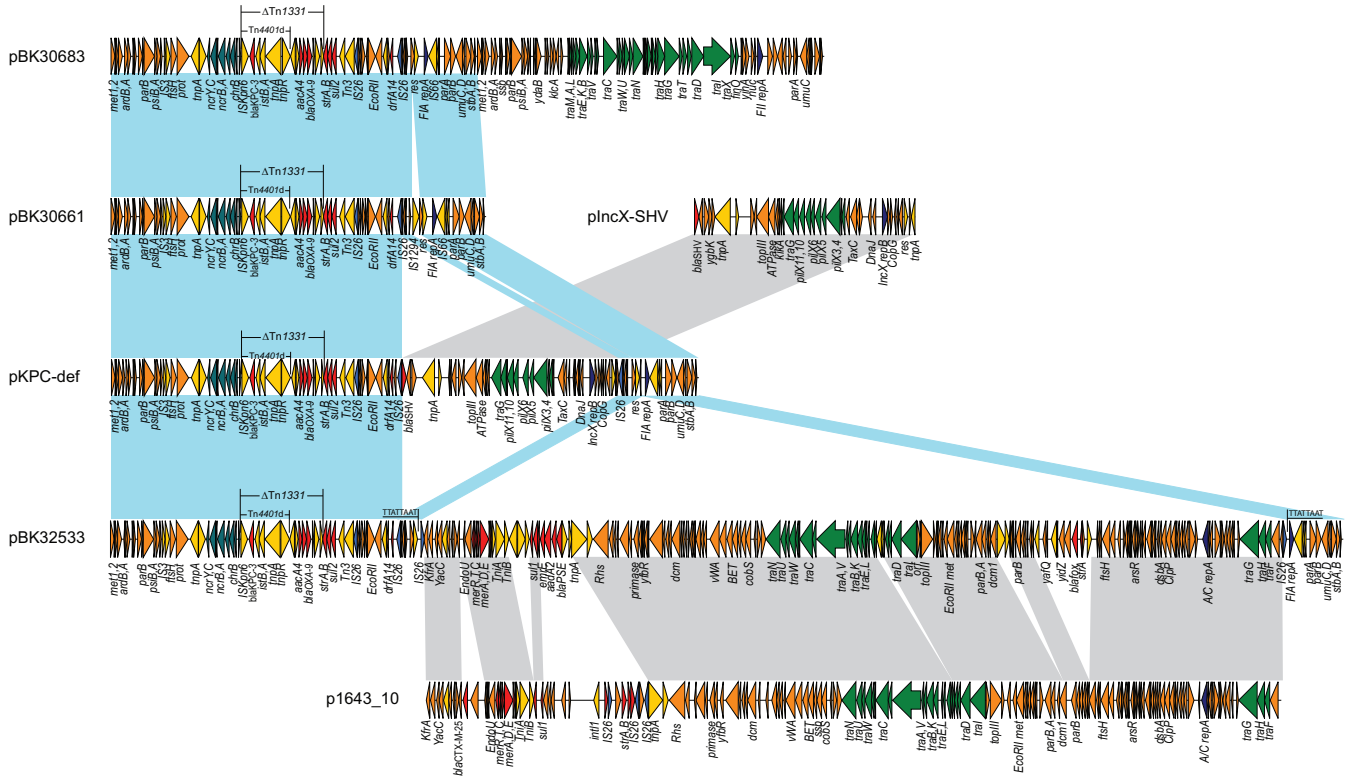

FIG 1 Plasmid structures of pBK32533 (KP345882), pBK30661 (KF954759), pBK30683 (KF954760), pKPC-def (CP009776), pIncX-SHV (JN247852), and p1643_10 (KF056330). Light-blue shading denotes regions of IncFIA plasmid backbone shared among four plasmids, pBK30661, pBK30683, pKPC-def, and pBK32533. Light-gray shading denotes homologous regions acquired from other plasmids (pIncX-SHV and p1643_10). Open reading frames (ORFs) are represented by arrows and colored based on predicted gene function. Plasmid scaffold regions are indicated by orange arrows. Green arrows indicate genes associated with the *tra* locus, and dark blue arrows indicate replication-associated genes. Antimicrobial genes are represented by red arrows, while the accessory genes are indicated by yellow arrows. IS*26* elements are indicated by light-blue arrows. The 8-bp target sequences duplicated by IS*26* integration were underlined.

(CLSI) methods and interpretations (12, 13). BK32533 exhibited resistance to imipenem (MIC > 4 μg/ml), ertapenem (MIC > 4 μg/ml), meropenem (MIC > 8 μg/ml), doripenem (MIC > 2 μg/ml), cefotaxime (MIC > 32 μg/ml), ceftazidime (MIC > 16 μg/ml), cefepime (MIC > 16 μg/ml), aztreonam (MIC > 16 μg/ml), ticarcillin-clavulanate (MIC > 128/2 μg/ml), piperacillin-tazobactam (MIC > 64/4 μg/ml), amikacin (MIC > 32 μg/ml), tobramycin (MIC > 8 μg/ml), and tigecycline (MIC > 128 μg/ml), but it was susceptible to gentamicin (MIC ≤ 2 μg/ml), ciprofloxacin (MIC ≤ 0.25 μg/ml), levofloxacin (MIC ≤ 1 μg/ml), doxycycline (MIC ≤ 2 μg/ml), minocycline (MIC ≤ 2 μg/ml), colistin (MIC ≤ 0.5 μg/ml), and polymyxin B (MIC ≤ 0.5 μg/ml). A representative *E. coli* J53 transconjugant also displayed similar resistant profiles but showed lower resistance to carbapenems imipenem (MIC = 2 μg/ml), meropenem (MIC = 2 μg/ml), ertapenem (MIC = 1 μg/ml), and doripenem (MIC = 1 μg/ml) and was intermediate against cefepime (MIC = 8 μg/ml).

The plasmid in BK32533 and that in the transconjugant were sized by S1 nuclease digestion of whole-cell DNA (chromosome and plasmids), followed by pulsed-field gel electrophoresis (S1-PFGE) (14). BK32533 and the transconjugant each carried a single bla_{KPC}-harboring plasmid (pBK32533) with a molecular weight of approximately 240 kb. This plasmid was ~170 kb larger than IncFIA plasmid, pBK30661 (68kb), indicating that pBK32533 contains additional genetic material. Plasmid DNA from *E. coli* J53 transconjugant was extracted using a Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA) and sequenced using an Illumina MiSeq system. The sequencing reads were *de novo* assembled, gaps were closed, and annotations were performed as described previously (15).

Plasmid pBK32533 is 241,963 bp in length with an average G+C content of 53.1%, harboring 297 predicted open reading frames (ORFs) (Fig. 1). The upstream 72-kb sequence in pBK32533 is almost identical (>99.9% nucleotide identity) to that of pBK30661, except for an extra IS*1294* sequence found in pBK30661 (Fig. 1). Similar to pBK30661, pBK32533 also carries the IncFIA *repA* gene, the *nic* operon, and the truncated Tn*1331*-Tn*4401d* nested transposon (7). However, pBK32533 carries an additional ~170-kb sequence that is unrelated to pBK30661 (Fig. 1). This 170-kb sequence in pBK32533 carries genes for plasmid replication (IncA/C *repA*), conjugation (*tra* clusters), and antibiotic resistance (*sul1*, *aadA2*, *cmlA1*, *emrE*, bla_{FOX}, bla_{PSE}, and *strA*), mercury resistance operons that confer resistance to heavy metals (*merRTPCADE*), and stability genes (*parA*, *parB*). Unlike pBK30661, pBK32533 carries four *tra* clusters encoding the type IV secretion system (T4SS) as well as the *oriT* region, which is essential for plasmid conjugation and can explain the conjugative ability of pBK32533. The results of the sequencing confirmed that pBK32533 is a cointegrate plasmid made up of pBK30661 with an additional ~170-kb genetic element. In addition, the S1-PFGE (described above) showed that parental *E. coli* BK32533 and its J53 transformant carried the same size pBK32533, evidence that the

cointegrate plasmid was established in the parental *E. coli* strain before the conjugation experiment was performed. The source of pBK32533 remains an unresolved issue.

A plasmid comparison based on a full-plasmid BLAST query against NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the extra 170-kb sequence in pBK32533 is closely related to the p1643_10 plasmid in *Salmonella enterica* subsp. *enterica*, with 86% query coverage and an overall 99% nucleotide identity at a cutoff value of 1.097e + 05 (Fig. 1). *S. enterica* subsp. *enterica* serovar (*S.*) Kentucky strain 1643-2010 was isolated from a turkey flock from a farm in Poland and harbored an IncA/C plasmid (1643_10) which is 167 kb in size (16). Close inspection of the pBK32533 sequence revealed that the 170-kb integrated DNA is flanked by two identical copies of IS*26* in the same orientation. IS*26* is a member of a family of insertion elements that are 820 bp long with 14-bp perfect terminal inverted repeats (17). Upon integration, IS*26* generated an 8-bp duplication (TTATTAAT) of its target sequence (Fig. 1). The presented data provide evidence that IS*26* facilitates horizontal gene transfer between microorganisms with a wide host range and lends credence to the hypothesis that IS*26* is involved in the evolution of pBK32533 (17).

Subsequent BLAST searches against NCBI GenBank revealed additional IncFIA pBK30661-like plasmid backbones. An example is the above-described cointegrate plasmid, pBK30683, which carries the same IncFIA pBK30661 "core" plasmid structure along with an additional 68-kb integrated sequence which harbors the full machinery of genes responsible for plasmid replication, stability, and conjugation (Fig. 1) (7). However, unlike pBK32533, where we propose that cointegration was mediated by IS*26*, the cointegration in pBK30683 was created by homologous recombination facilitated by a 4-kb region of homology (7). Another example is the recently described cointegrate-like plasmid pKPC-def (18), isolated from ST258 *K. pneumoniae* strain KPNIH32, which bears an ~70-kb plasmid backbone homologous to pBK30661 (97.9% identity) plus an ~43-kb element with 99.9% identity to plasmid pIncX-SHV (18, 19). In similarity to our findings with pBK32533, the ~43-kb pIncX-SHV-like element was flanked by two IS*26* sequences but at a different location. pIncX-SHV was initially identified in a *K. pneumoniae* ST258 strain (19) that harbors the *tra* operon. It is probable that pKPC-def evolved by the cointegration of a pBK30661-like plasmid and a pIncX-SHV-like plasmid within the same isolate.

The pBK32533 plasmid reported in this study is the third cointegrate identified with a common IncFIA pBK30661 background suggestive of its genomic plasticity and its compatibility with other elements. The finding leads us to hypothesize that IncFIA pBK30661 is prone to forming cointegrates in order to facilitate their transfer and to increase the spread of resistance. Additionally, the creation of a multireplicon plasmid broadens its host range by overcoming the incompatibility due to the presence of a single resident plasmid (5). Our study and data from others suggest that plasmid cointegration may significantly facilitate the spread of antimicrobial resistance genes (7, 20, 21) as well as of other determinants that have clinical impact.

In summary, we describe here the complete sequence of a novel IncFIA cointegrate plasmid structure from an *E. coli* isolate and its possible spread from a KPC-producing *K. pneumoniae* isolate by IS*26*-mediated acquisition of a 170-kb genetic element, thereby enhancing its ability to transfer by conjugation. Plasmid cointegration may play an important role in facilitating the spread of carbapenem resistance in different species.

Nucleotide sequence accession number. The complete nucleotide sequence of pBK32533 was deposited as GenBank accession no. KP345882.

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B.N.K. discloses that he holds two patents that focus on using DNA sequencing to identify bacterial pathogens.

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