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Complete Nucleotide Sequences of bla_{KPC-4}- and bla_{KPC-5}-Harboring IncN and IncX Plasmids from *Klebsiella pneumoniae* Strains Isolated in New Jersey

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***Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* have emerged as major nosocomial pathogens. bla_{KPC}, commonly located on Tn*4401*, is found in Gram-negative bacterial strains, with the two most common variants, bla_{KPC-2} and bla_{KPC-3}, identified in plasmids with diverse genetic backgrounds. In this study, we examined bla_{KPC-4}- and bla_{KPC-5}-bearing plasmids recovered from two *K. pneumoniae* strains, which were isolated from a single New Jersey hospital in 2005 and 2006, respectively. IncN plasmid pBK31551 is 84 kb in length and harbors bla_{KPC-4}, bla_{TEM-1}, *qnrB2*, *aac*(*3*)-*Ib*, *aph*(*3′*)-*I*, *qacF*, *qacEΔ1*, *sul1*, and *dfrA14*, which confer resistance to β-lactams, quinolones, aminoglycosides, quaternary ammonium compounds, and co-trimoxazole. The conserved regions within pBK31551 are similar to those of other IncN plasmids. Surprisingly, analysis of the Tn*4401* sequence revealed a large IS*110*- and Tn*6901*-carrying element (8.3 kb) inserted into the *istA* gene, encoding glyoxalase/bleomycin resistance, alcohol dehydrogenase, and *S*-formylglutathione hydrolase. Plasmid pBK31567 is 47 kb in length and harbors bla_{KPC-5}, *dfrA5*, *qacEΔ1*, and *sul1*. pBK31567 belongs to a novel IncX subgroup (IncX5) and possesses a highly syntenic plasmid backbone like other IncX plasmids; however, sequence similarity at the nucleotide level is divergent. The bla_{KPC-5} gene is carried on a Tn*4401* element and differs from the genetic environment of bla_{KPC-5} described in *Pseudomonas aeruginosa* strain P28 from Puerto Rico. This study underscores the genetic diversity of multidrug-resistant plasmids involved in the spread of bla_{KPC} genes and highlights the mobility and plasticity of Tn*4401*. Comparative genomic analysis provides new insights into the evolution and dissemination of KPC plasmids belonging to different incompatibility groups.**

Carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as a major cause of nosocomial infections worldwide. The therapeutic challenges associated with these highly resistant strains correlate with higher morbidity and mortality, increased length of hospitalization, and an overall increase in health care costs (1). In the past decade, the spread of *Klebsiella pneumoniae* carbapenemase (KPC), a class A serine β-lactamase, has led to a rapid rise in prevalence of CRE infections in the United States and other global regions (2). Currently, 12 KPC variants (KPC-2 to KPC-13) have been identified since the initial report, with KPC-2 and KPC-3 identified as the most frequent types and *K. pneumoniae* as the most common host species (2, 3). Genotypic studies of emerging KPC-bearing *K. pneumoniae* strains from international sources indicate that the predominant strains are typically multilocus sequence type 258 (ST258), which is suggestive of pandemic spread by a single clone (2, 4–7).

KPC is encoded by the bla_{KPC} gene, which is carried by Tn*4401* or Tn*4401*-like genetic elements, and has been reported in a variety of transferable plasmids (8–14). Tn*4401* is approximately 10 kb in size, is delimited by two 39-bp imperfect inverted repeats, and harbors insertion sequences IS*Kpn6* and IS*Kpn7* in addition to transposase and resolvase genes (15). Tn*4401* and bla_{KPC} genes have been reported in plasmids of various sizes, which differ in their ability to transfer within and between species, harbor different antibiotic resistance elements, and possess different incompatibility (Inc) groups, including IncFII, IncL/M, IncN, IncR, and ColE1 (4, 6, 10, 16).

Whole-plasmid sequencing and comparative genomic analyses have identified common functional modules and unique acquired genes that provide important molecular information for developing epidemiological tools to identify and track the spread of resistance plasmids within and among *Enterobacteriaceae* species (17–20). Various bla_{KPC-2}- and bla_{KPC-3}-harboring plasmids belonging to different Inc groups have been successfully sequenced, including IncN plasmids p9 and p12 (10), IncFII plasmids pKPHS2 (21), pKP048 (19), pKpQIL (20), pKpQIL-IT (18), and pSLMT (HQ589350), and ColE1 plasmid p15S (10). However, the complete sequence analysis of carbapenem resistance plasmids belonging to other Inc groups, or harboring other bla_{KPC} variants, has not been described. Here we report the complete sequences of two plasmid genomes harboring bla_{KPC-4} and bla_{KPC-5}, recovered

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from two *K. pneumoniae* isolates cultured at the same New Jersey hospital. Our analysis shows the genetic diversity of multidrug-resistant plasmids involved in the spread of the bla_{KPC} genes and highlights the mobility and plasticity of Tn*4401*.

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MATERIALS AND METHODS

Retrospective analysis of carbapenem-resistant *K. pneumoniae*. A total of 27 carbapenem-resistant *K. pneumoniae* strains isolated from 2005 to 2010 at a single New Jersey hospital were genetically characterized. Detection of bla_{KPC} genes and identification of the epidemic *K. pneumoniae* ST258 clone were performed using multiplex real-time PCR methods described previously (22, 23), and full-length bla_{KPC} genes were characterized by conventional PCR and DNA sequencing (24). Two KPC-4-harboring strains were detected: BK31551 (CK7) and BK31572 (CK180) were isolated from blood culture samples from two patients with bacteremia in September 2005 and November 2006, respectively. KPC-5-bearing strain BK31567 (CK102) was isolated from a blood culture sample from a patient with urospesis in April 2006.

Species were identified using the Vitek 2 system (bioMérieux), and MICs were determined by broth microdilution in cation-adjusted Mueller-Hinton broth (MHB) according to Clinical and Laboratory Standards Institute methods (25), using Sensititre GNX2F trays (Thermo Fisher Scientific, Waltham, MA). Carbapenem resistance was defined by MICs of higher than the 2012 CLSI breakpoints for one or more of the following carbapenem agents: imipenem, ≥4 μg/ml; meropenem, ≥4 μg/ml; ertapenem, ≥2 μg/ml; and doripenem, ≥4 μg/ml (25).

Characterization of bla_{KPC-4}- and bla_{KPC-5}-harboring plasmids and strains. Plasmid DNA was extracted using a Qiagen plasmid Maxikit (Qiagen, Valencia, CA), followed by electroporation into *Escherichia coli* DH10B (Invitrogen) using a Gene Pulser II instrument (Bio-Rad Laboratories). *E. coli* DH10B transformants were selected on Luria-Bertani (LB) agar plates containing 100 μg/ml ampicillin or 1 μg/ml imipenem and then screened by multiplex real-time PCR for the presence of bla_{KPC} genes (23). Plasmid size was estimated by S1 nuclease digestion of plasmid DNA, followed by pulsed-field gel electrophoresis (PFGE) using a Bio-Rad CHEF-DR III variable-angle system (26). Transformants with a single plasmid were then selected and subjected to susceptibility testing as described above. Plasmids isolated from *E. coli* DH10B transformants were digested with restriction endonuclease EcoRV (New England BioLabs, Boston, MA), and their restriction patterns were compared. Transferability of bla_{KPC-4}- and bla_{KPC-5}-bearing plasmids was examined by conjugation experiments using three clinical isolates (BK31551, BK31572, and BK31567) as donors and *E. coli* J53Az^r as the recipient as described previously (27). *E. coli* J53 transconjugants with KPC-encoding plasmids were selected on LB plates containing 50 μg/ml sodium azide and 100 μg/ml ampicillin. The presence of the bla_{KPC-4} or bla_{KPC-5} gene in *E. coli* J53 transconjugants was confirmed by the aforementioned multiplex real-time PCR (23).

Plasmid incompatibility groups were determined using the multiplex PCR method described by Carattoli et al. (28). β-Lactamase genes, including bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{GES}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}, bla_{ACT-1}, bla_{ACC}, bla_{BIL-1}, bla_{CMY}, bla_{DHA}, bla_{FOX}, bla_{LAT}, bla_{MIR-1}, and bla_{MOX}, were investigated by PCR using methods described elsewhere (29, 30). Multilocus sequence typing (MLST) of the parental strains (31) and plasmid MLST for IncN group plasmids (32) were performed using previously described methods. OmpK35 and OmpK36 porins were analyzed by SDS-PAGE, and their genes were amplified by PCR followed by DNA sequencing (33).

Plasmid sequencing and bioinformatics. Plasmid DNA from *E. coli* DH10B transformants of BK31551 (KPC-4) and BK31567 (KPC-5) were extracted as described above using a Qiagen plasmid Maxikit (Qiagen, Valencia, CA). The plasmid DNA was fragmented, and genomic libraries were prepared and sequenced using a Roche 454 GS-FLX system. Sequencing reads were assembled into consensus *de novo* assembly contigs using the Roche genome sequencer FLX software GSA assembler, version 2.5.3. Gaps between contigs were closed by PCR with standard Sanger sequencing.

Open reading frames (ORFs) were predicted and annotated using the RAST (rast.nmpdr.org) server (34), followed by manual comparative curation and sequence similarity searches directed against the NCBI (www.ncbi.nlm.nih.gov/BLAST) and IS Finder (www-is.biotoul.fr) databases. MEGA 5.01 was used for sequences comparison and alignment (35). Mauve 2.3.1 was used to perform comparative genome alignment for different plasmids (36).

Nucleotide sequence accession numbers. The complete nucleotide sequences of pBK31551 and pBK31567 have been deposited in GenBank under accession numbers JX193301 and JX193302, respectively.

RESULTS

Characteristics of clinical isolates. Genotyping of the 27 carbapenem-resistant isolates indicated that they all possessed the bla_{KPC} genes, including bla_{KPC-3} ($n = 22$; 81.5%) and bla_{KPC-2} ($n = 2$; 7.4%), while three strains were unexpectedly identified with bla_{KPC-4} ($n = 2$) and bla_{KPC-5} ($n = 1$). With the exception of one isolate, all bla_{KPC-2}- and bla_{KPC-3}-harboring strains belonged to the epidemic *K. pneumoniae* ST258 clone (22); however, neither the bla_{KPC-4}- nor the bla_{KPC-5}-carrying strains were ST258. Multilocus sequence typing (31) indicated that the three non-ST258 isolates belonged to uncommon sequence types (STs): ST834 (2-1-2-1-7-1-25), strain BK31551; ST964 (41-1-1-1-7-4-87), strain BK31572; and ST429 (2-1-2-1-9-1-116), strain BK31567. The differing PFGE profiles of these three strains also indicated they were not genetically related (data not shown). S1-PFGE on different *E. coli* DH10B transformants revealed that each strain carried a single plasmid ranging in size from 49 to 84 kb (49 kb for pBK31567, 75 kb for pBK31572, and 84 kb for pBK31551). Conjugation experiments showed that three bla_{KPC-4}- and bla_{KPC-5}-harboring plasmids could be successfully transferred to *E. coli* J53 recipients. In addition, SDS-PAGE and DNA sequence analysis showed no evidence of porin loss of OmpK35 and OmpK36 in three parental strains (data not shown).

A list of antimicrobial agents and their MICs against KPC-4- or -5-producing clinical isolates (and their transformants) is shown in Table 1. The parental strains were resistant to aztreonam, cefotaxime, ceftazidime, ticarcillin/clavulanate, and trimethoprim-sulfamethoxazole, to imipenem (BK31572), and to ertapenem (BK31572 and BK31567) and exhibited reduced susceptibility to piperacillin-tazobactam. In addition, BK31551 and BK31567 displayed intermediate resistance to imipenem (MIC 2 μg/ml), while BK31572 displayed intermediate resistance to doripenem (MIC 2 μg/ml) (25). The *E. coli* DH10B transformants displayed antimicrobial susceptibility profiles similar to those of their parental strains but were not as resistant to imipenem, ertapenem, and doripenem (Table 1). Notably, although some of the MIC values in Table 1 fall within the susceptibility range for carbapenems (≤1 μg/ml for imipenem, meropenem, and doripenem and ≤0.5 μg/ml for ertapenem), they are nevertheless higher than those reported previously for wild-type *K. pneumoniae* strains (without carbapenemase or porin loss) (37).

Structure of bla_{KPC-4}-harboring plasmid pBK31551. Plasmid pBK31551 is a circular molecule of 83,712 bp belonging to incompatibility group N, with an average GC content of 53.4%, and harboring 80 predicted ORFs (Fig. 1). The plasmid com-

TABLE 1 Characteristics of KPC-4- and KPC-5-producing *K. pneumoniae* strains and their *E. coli* DH10B transformants

Isolate[a]	β-Lactamase(s)	MIC (μg/ml)[b]																		
		IMP	MER	ERT	DOR	CAZ	CTX	CEF	ATM	TIC-CLAV	PIP-TAZ	AMI	TOB	DOX	CIP	LEV	SXT	TGC	COL	PLB
BK31551	TEM-1, SHV-11, KPC-4	2	0.5	1	1	**>16**	**>32**	4	**>16**	**128/2**	64/4	≤4	≤1	**>16**	1	2	**>4/76**	1	0.5	1
T-BK31551	TEM-1, KPC-4	1	0.5	0.5	0.5	**>16**	**16**	8	**>16**	**>128/2**	>64/4	≤4	4	≤2	≤0.25	≤1	**>4/76**	≤0.25	≤0.25	0.5
BK31572	TEM-1, SHV-11, KPC-4	**4**	1	**4**	2	**>16**	**>32**	4	**>16**	**>128/2**	>64/4	≤4	≤1	≤2	≤0.25	≤1	**>4/76**	≤0.25	0.5	1
T-BK31572	TEM-1, KPC-4	2	1	1	0.5	**>16**	**>32**	8	**>16**	**>128/2**	64/4	≤4	2	2	≤0.25	≤1	**>4/76**	≤0.25	≤0.25	0.5
BK31567	SHV-1, KPC-5	2	1	**4**	0.5	**>16**	**32**	≤2	**>16**	**>128/2**	>64/4	≤4	≤1	4	>2	≤1	**>4/76**	1	0.5	1
T-BK31567	KPC-5	1	0.5	0.5	0.5	**>16**	**4**	≤2	**>16**	**>128/2**	16/4	≤4	≤1	≤2	≤0.25	≤1	**>4/76**	≤0.25	≤0.25	0.5

[a] T-, *E. coli* DH10B transformant.

[b] MICs were determined using broth microdilution; resistance is indicated in boldface. IMP, imipenem; MER, meropenem; ERT, ertapenem; DOR, doripenem; CAZ, ceftazidime; CTX, cefotaxime; CEF, cefepime; ATM, aztreonam; TIC-CLAV, ticarcillin-clavulanate; PIP-TAZ, piperacillin-tazobactam; AMI, amikacin; TOB, tobramycin; DOX, doxycycline; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; COL, colistin; PLB, polymyxin B. The 2012 CLSI breakpoints were used to interpret the MIC results for carbapenem-susceptible (S), -intermediate (I), and -resistant (R) isolates, as follows (S/I/R): imipenem, ≤1/2/≥4 μg/ml; meropenem, ≤1/2/≥4 μg/ml; ertapenem, ≤0.5/1/≥2 μg/ml; and doripenem ≤1/2/≥4 μg/ml (25).

prises a 37,343-bp core region and two discontinuous acquired regions (42,924 and 3,445 bp). The core region defines plasmid "housekeeping genes," including a replication module, two transfer (*tra*) systems, a stability operon, and an antirestriction system (Fig. 1). The two discontinuous acquired regions are composed of numerous resistance genes, insertion sequences, and transposons (Fig. 1).

The core genes in pBK31551 and other IncN plasmids are clustered into different functional modules (Fig. 1). The replication region includes a 720-bp replication initiation protein gene (*repA*), flanked by direct repeat sequences and adjacent to a resolvase gene (*uvp1* or *resP*) required for resolving cointegrates at the end of replication (38). pBK31551 also possesses two modules that are predicted to be involved in the conjugative transfer of plasmid DNA. The first module consists of 11 genes (*traL*, *traM*, *traA*, *traB*, *traC*, *traD*, *traN*, *traE*, *traO*, *traF*, and *traG*) flanked by a group of plasmid core genes involved in regulating transfer and replication (*korA* and *korB*) (39), excluding DNA entry (*eex*) (40), DNA degradation (*nuc*) (41), and killing of *Klebsiella* (*kikA*) (42, 43) (Fig. 1). The second module includes three genes (*traI*, *traJ*, and *traK*) that also function in replication and DNA transfer, while the *fipA* gene, located upstream of *traI*, functions in fertility inhibition of IncP plasmids (44).

Additional core genes common to IncN plasmids and found on pBK31551 contribute to plasmid stability. The restriction/antirestriction system encoded by the *EcoRII* and *EcoRII met* genes prob-

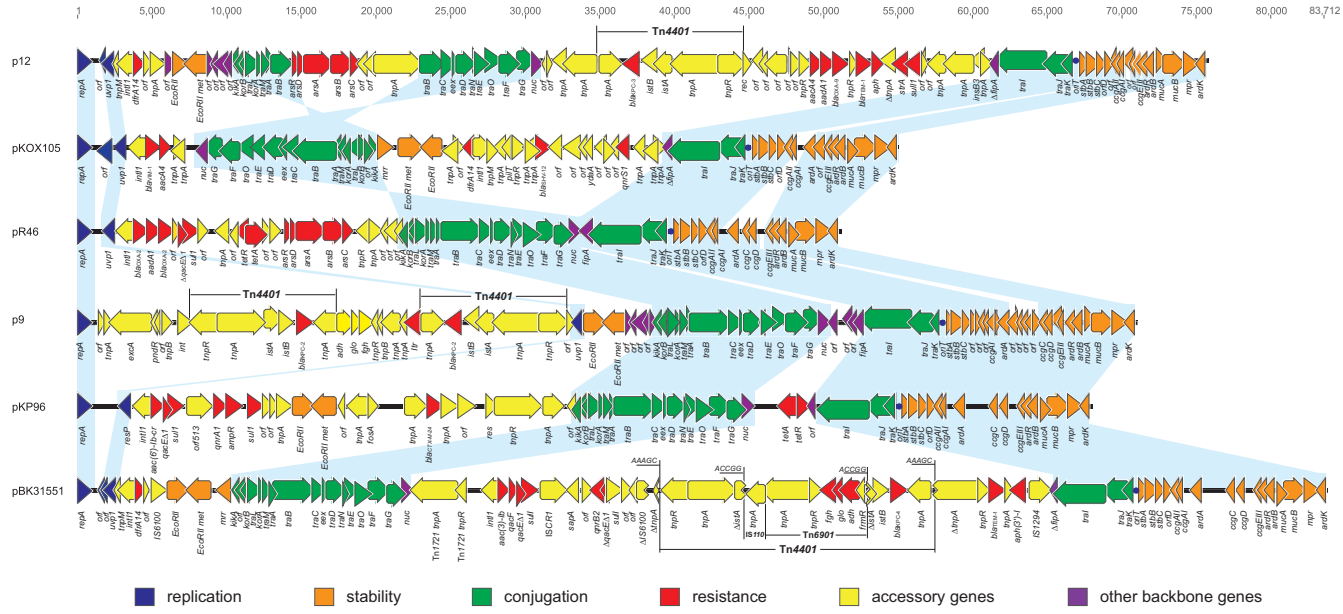

FIG 1 Comparative analysis of IncN plasmids pBK31551 (JX193301), pKP96 (EU195449), p9 (FJ223607), pR46 (AY046276), pKOX105 (HM126012), and p12 (FJ223605). Light blue shading denotes shared regions of homology. It is noted that the region between *kikA* and *nuc* is inverted in pKOX105 relative to the other plasmids and is split into two regions in p12 due to the insertion of the *ars* operon. Open reading frames (ORFs) are portrayed by arrows and colored based on predicted gene function. Resistance genes are indicated in red arrows and include aminoglycoside resistance genes [*aph* (3′)-*I*, *aac*(3)-*Ib*, *aac*(6′)-*Ib-cr*, *aph*, *aadA1*, and *aacA4*], β-lactamase genes (bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-4}, bla_{KPC-5}, bla_{VIM-1}, $bla_{CTX-M-24}$, bla_{OXA-2}, bla_{OXA-9}, bla_{TEM-1}, bla_{SHV-12}, and *ampR*), quinolone resistance genes (*qnrA1*, *qnrB2*, and *qnrS1*), arsenic resistance genes (*arsA*, *arsB*, *arsC*, *arsD*, and *arsR*), tetracycline resistance genes (*tetA* and *tetR*), and quaternary compound, trimethoprim, and sulfonamide resistance genes (*sul1*, *qacF*, *qacEΔ1*, and *dfrA14*), as well as glyoxalase/bleomycin resistance gene *glo*, alcohol dehydrogenase gene *adh*, and *S*-formylglutathione hydrolase gene *fgh*.

ably functions as a toxin/antitoxin system to lyse cells which have lost the plasmid (45). Genes located downstream of the *traIJK* modules for conjugative transfer were also predicted to play a role in plasmid stability. Specifically, the proteins encoded by the *stbABC* operon are believed to stabilize single-stranded DNA during conjugation (46), while *ardAB* and *ardR* encode proteins involved in regulating antirestriction functions (47, 48). pBK31551 also possesses *ccg* genes (cup-controlled genes), which encode products involved in the protection of plasmid DNA from type I restriction enzymes (48).

The pBK31551 plasmid also has two distinct regions harboring antibiotic resistance genes. A 3,445-bp region downstream of the *uvp1* gene includes a class 1 integron with a 3′ conserved sequence (3′ CS) truncated by insertion of an IS*6100* element. This integron contains the *dfrA14* gene cassette, which confers trimethoprim resistance.

The primary cluster of resistance genes in pBK31551 is located in a 42,924-bp region between genes *fipA* and *nuc* and includes β-lactamase genes bla_{KPC-4} and bla_{TEM-1}, aminoglycoside resistance genes *aph*(*3′*)-*I* and *aac* (*3*)-*1b*, quinolone resistance gene *qnrB2*, quaternary compound resistance genes *qacF* and *qacEΔ1*, sulfonamide resistance gene *sul1*, glyoxalase/bleomycin resistance gene *glo*, alcohol dehydrogenase gene *adh*, and *S*-formylglutathione hydrolase gene *fgh*.

Within this 42,924-bp region, an 18,282-bp fragment contains the Tn*4401* element harboring the bla_{KPC-4} gene, which encodes the carbapenemase. The insertion of the element, which disrupted the Tn*3* transposase gene, generated a 5-bp (AAAGC) target sequence duplication (TSD) at each end of Tn*4401* (Fig. 1). Surprisingly, sequencing of the Tn*4401* element identified a unique 8,271-bp insertion which disrupted the *istA* gene and generated a 5-bp TSD (ACCGG). This insertion contains two discrete elements: a 1,395-bp insertion sequence that belongs to the IS*110* family and is 91% similar to an insertion sequence in *Yersinia pestis* plasmid pMT (www.ncbi.nlm.nih.gov/GenBank) and a 6,876-bp region that is 99.7% similar to the Tn*3* family transposon Tn*6901* (49). Tn*6901* carries 6 genes, including a transposase gene, a resolvase gene, an alcohol dehydrogenase gene (*adh*), a glyoxalase/bleomycin resistance gene (*glo*), an *S*-formylglutathione hydrolase gene (*fgh*), and a regulator protein gene (*frmR*). The bla_{KPC-4} gene is located downstream of Tn*6901* and displays 100% identity to previously reported bla_{KPC-4} sequences from *Enterobacter cancerogenus* strain E624 (FJ473382) and *K. pneumoniae* strain KpAM1(EU447304).

There are two additional genetic elements located upstream of Tn*4401* (Fig. 1). Transposon Tn*1721* is flanked by a class I integron, consisting of *aac*(*3*)-*Ib*, *qacF*, *qacEΔ1*, and *sul1*. The next element consists of a truncated class I integron containing *sapA*, *qnrB2*, Δ*qacEΔ1*, *sul1*, and two unknown ORFs, as well as an ISCR1 element inserted within the 5′ conserved sequence (5′ CS). Acquired genes located downstream of Tn*4401* include IS*1294*, *aph*(*3′*)-*I*, bla_{TEM-1}, and the transposase (*tnpA*) and resolvase (*tnp*) genes of Tn*3*.

Comparison of pBK31551 with IncN plasmids. pBK31551 was compared to IncN prototype plasmid pR46 (AY046276) as well as several other *bla* gene-carrying IncN plasmids, including pKP96 (EU195449), p9 (FJ223607), pKOX105 (HM126012), and p12 (FJ223605) (Fig. 1). All plasmids shared similarities in core genes responsible for plasmid maintenance, including replication, transfer, and stability systems (Fig. 1). Plasmids p12 and pKOX105 lack the antirestriction genes *ccgC*, *ccgD*, and *ardA*, while p12 has a nonfunctional transfer region due to insertion of an arsenic resistance operon (*ars*) between *traB* and *traA*. In pKOX105, moreover, the region containing the *traAB* operon (from *nuc* to *kikA*) is inverted relative to that in other IncN plasmids.

The major differences among these plasmids are related to the number of acquired genes (Fig. 1). Interestingly, these plasmids possess similar integration sites. For example, with the exception of plasmid p9, the IncN plasmids all share integration of a class I integron downstream of the *uvp1* gene (Fig. 1). Similarly, the 42-kb antibiotic resistance region inserted between the *fipA* and *nuc* genes in pBK31551 closely resembles the acquired resistance genes in p12 (also located between *fipA* and *nuc*), pKOX105 (between EcoRII and *fipA*), and pKP96 (between *nuc* and *traI*). Thus, although pBK31551 and other Inc N plasmids carry different antibiotic resistance genes, they share similar integration sites for the acquisition of variable resistance determinants, which may represent entry sites or "hot spots" for integration of transposable elements within the IncN plasmid scaffold (17).

Characterization of pBK31572. Given the unusual Tn*6901* and IS*110* insertions within Tn*4401* in pBK31551, we investigated additional insertions within other KPC-harboring isolates, including another KPC-4-producing strain (BK31572) isolated in 2006. PCR assays targeting both insertion junctions as well as *istA* showed negative results for all bla_{KPC-2}- or bla_{KPC-3}-harboring strains (data not shown). Interestingly, pBK31572 possesses the same Tn*6901* and IS*110* insertions as pBK31551, as demonstrated by PCR (data not shown). Comparative restriction enzyme digests of pBK31551 and pBK31572 using EcoRV displayed similar profiles, suggesting genetic relatedness (data not shown). Plasmid MLST (32) results also showed that both pBK31551 and pBK31572 belong to IncN ST6 (2-4-2), the same genotype described previously for KPC-3-producing strain p12, isolated in 2005 (10). Additional PCR assays used to detect bla_{TEM-1} (29), *aac*(*3*)-*Ib* (50), *qnrB2* (51), and *aph*(*3′*)-*I* (52) in pBK31551 showed that pBK31572 was negative for *qnrB2* but positive for other three targets. Nevertheless, in addition to Tn*4401*, pBK31572 and pBK31551 appear to share similar backbone genes, with the observed size differences likely due to variable content within acquired genetic elements.

Structure of bla_{KPC-5}-harboring plasmid pBK31567. Plasmid pBK31567 is a circular molecule of 47,387 bp, with an average GC content of 49.0%, and harboring 54 predicted ORFs (Fig. 2A). Sequence analysis of pBK31567 classified it within plasmid incompatibility group X, and we have further typed it as a novel IncX subgroup (IncX5) (described below). The IncX group of plasmids are narrow-host-range plasmids common in *Enterobacteriaceae*, and they exist even in isolates from the preantibiotic era (53). They are known to encode type IV fimbriae enabling their own conjugative transfer, and they provide accessory functions to their host bacteria such as biofilm formation and resistance to antimicrobial agents (54). IncX plasmids possess highly syntenic plasmid backbones but are quite divergent with respect to nucleotide and amino acid similarity (54). Based on phylogenetic comparisons of sequenced IncX plasmids, the IncX plasmid group has been divided into at least four subgroups, IncX1 to IncX4 (54).

Previous studies indicated that the characteristic structure of the IncX plasmid group includes a set of core genes positioned in the following order: *pir-bis-par-hns-topB-pilX-actX-taxCA* (54,

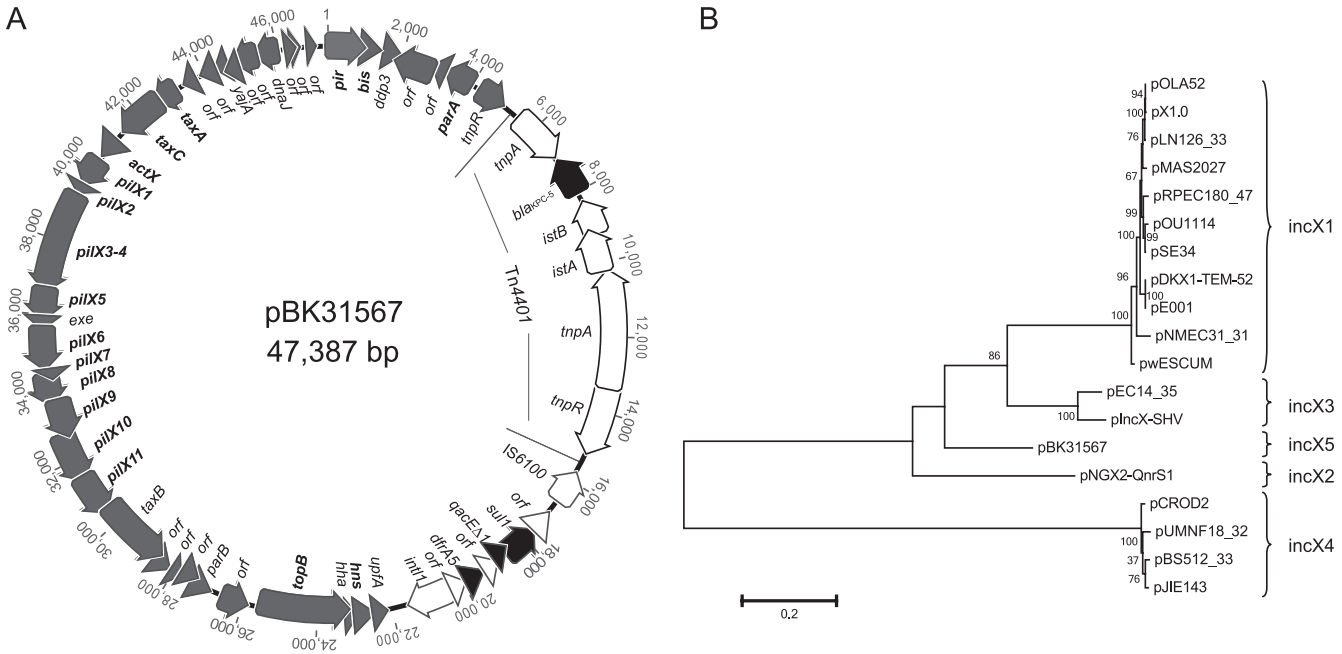

FIG 2 (A) Structure of plasmid pBK31567. Open reading frames (ORFs) are portrayed by arrows, with backbone region ORFs in dark gray and acquired region ORFs in white. Core backbone genes in pBK31567 are shown in boldface. Resistance genes are depicted by black arrows, including β-lactamase genes (bla_{KPC-5}) and quaternary compound, trimethoprim, and sulfonamide resistance genes (*qacF*, *qacEΔ1*, *dfrA5*, and *sul1*). (B) Phylogenetic maximum-likelihood tree of single nucleotide polymorphisms (SNPs) from backbone sequences of 19 IncX plasmids, including (GenBank accession numbers are shown in parentheses) pBK31567 (JX193302), p2ESCUM (CU928149), pBS512_33 (CP001059), pCROD2 (FN543504), pDKX1-TEM-52 (JQ269336), pE001 (JF776874), pEC14_35 (JN935899), pIncX-SHV (JN247852), pJIE143 (JN194214), pLN126_33 (HE578058), pMAS2027 (FJ666132), pNGX2_QnrS1(JQ269335), pNMEC31_31 (JN935897), pOLA52 (EU370913), pOU1114 (DQ115387), pRPEC180_47 (JN935898), pSE34 (EU219533), pUMNF18_32 (AGTD1000006), and pX1.0 (HM114226). All plasmids from a previous study (54), as well as pBK31567, have been included for phylogenetic analysis.

55). These same core genes are found in the backbone region of pBK31567 that is involved in plasmid replication, conjugation and DNA transfer, and partitioning (Fig. 2A). A BLASTn comparison against the NCBI database revealed that the putative backbone region of pBK31567 is closely related to InX3 plasmids pEC14_35 (JN935899) (54) and pIncX-SHV (JN247852) (18), displaying approximately 80% nucleotide similarity.

The core region of pBK31567, which is common to other IncX plasmids, includes a 1.35-kb replicon fragment encoding the essential replication initiator (Pir) and the accessory protein (Bis), as well as the partition-determining proteins ParG and ParF, which are involved with plasmid segregation and stability. The other conserved modules involved in stabilizing horizontal transfer of plasmid DNA include the gene expression modulation (*gem*) region, which contains putative transcriptional regulator (*hha*) and DNA-binding protein (*hns*) genes, as well as the type IA topoisomerase gene (*topB*) (55). The putative *tra* region of pBK31567 is divided into two regions: mating pair formation (Mpf) genes and DNA transfer and replication (Dtr) genes. The Mpf region includes 10 *pilx* gene members (*pilx1* to *pilx11*), while the Dtr complex consists of three relaxase genes (*taxA*, *-B*, and *-C*), which nick the plasmid DNA at the *oriT* site and initiate the formation of the transfer DNA strand (Fig. 2A).

Based on phylogenetic analysis of SNPs from conserved regions, Johnson et al. (54) divided 18 completely sequenced IncX plasmids into 4 subgroups (IncX1 to IncX4). *taxC*, one of the aforementioned Dtr genes, is conserved among different IncX plasmids, and comparative sequence analysis has been used to identify and group IncX plasmids into subgroups (IncX1 to IncX4) (28, 54). The *taxC* genes of plasmids from different incompatibility subgroups share between 37% and 92% nucleotide similarity, whereas those within a given incompatibility subgroup (e.g., IncX1 to IncX4) share between 92% and 99% nucleotide similarity (54). A comparison of the *taxC* gene in pBK31567 to those of the IncX plasmids depicted in Fig. 2B showed 82%, 78%, 73%, and 46% nucleotide similarity with the IncX3 (pIncX-SHV and pEC14-35), IncX1 (e.g., pEC14-35), IncX2 (pNGX2-QnrS1), and IncX4 (e.g., pJIE143) subgroups, respectively, effectively relegating pBK31567 to a novel IncX subgroup. Phylogenetic analysis of single nucleotide polymorphisms (SNPs) from the conserved regions of 19 plasmids was further used to explore the relationship between pBK31567 and other IncX plasmids (Fig. 2B). The results divided the IncX plasmids into five subgroups, with pBK31567 clustering separately from the aforementioned subgroups IncX1 to -4 (54), in agreement with the results of the *taxC* gene comparisons; pBK31567 is therefore best classified as a plasmid subgroup of IncX5.

Also common to IncX plasmids is a conserved integration region for acquired elements, located downstream of the serine resolvase gene, *tnpR* (nucleotides [nt] 4265 to 4912 in Fig. 2A). Several resistance genes have been observed directly downstream of the resolvase gene in variable IncX plasmids, including β-lactamase genes (bla_{TEM-52}, bla_{TEM-1}, bla_{SHV-11}, and $bla_{CTX-M-15}$), as well as genes encoding quinolone resistance (*qnrS1* and *oqxAB*), aminoglycoside resistance (*aphA1*), and bleomycin resistance (*blmS*) (54). Similarly, a 16,727-bp acquired region, which includes a bla_{KPC-5}-harboring Tn*4401* element and a class I integron, has been found inserted downstream of the serine resolvase gene

in pBK31567. The Tn*4401* region of pBK31567 displayed 99.9% similarity with the sequences of previously described Tn*4401* elements from p9 and p12 (10), with the only observed sequence variation associated with their respective bla_{KPC} variants (bla_{KPC-5} versus $bla_{KPC-2/-3}$). An intact class I integron, located downstream of the Tn*4401* and IS*6100* elements, harbors the trimethoprim resistance gene *dfrA5*, the quaternary compound resistance gene *qacE*Δ*1*, and the sulfonamide resistance gene *sul1*. To the best of our knowledge, this is the first description of a carbapenemase gene (bla_{KPC}) and a class I integron carried on the same IncX plasmid.

DISCUSSION

Currently, KPC-2 and KPC-3 carbapenemases have been described in multiple Gram-negative species, including various *Enterobacteriaceae*, and several bla_{KPC-2}- and bla_{KPC-3}-harboring plasmids have been fully sequenced (2, 4, 6, 10, 16). In contrast, strains harboring bla_{KPC-4}- and bla_{KPC-5}-carrying plasmids are rarely reported, and these plasmids have not been completely analyzed. As of this writing, bla_{KPC-4} has been reported in three isolates: *E. cancerogenus* strain E624 from Scotland, isolated in 2003 (56), and *K. pneumoniae* strain KpAM1 (57) and *Acinetobacter* sp. strain M2AC9-31 (58), isolated in Puerto Rico in 2006 and 2009, respectively. Similarly, bla_{KPC-5} has been found only in *Pseudomonas aeruginosa* strain PS28 (PR280) and in isolates in Puerto Rico between October 2006 and March 2007 (14, 59).

In this study, we characterized complete sequences of bla_{KPC-4}- and bla_{KPC-5}-harboring plasmids pBK31551 and pBK31567, isolated from a single hospital in New Jersey, revealing a number of unusual findings. First, a large element carrying both IS*110* and Tn*6901* (8.3 kb) was found inserted within Tn*4401* in pBK31551 (Fig. 1), and the same element was also identified in plasmid pBK31572. Both plasmids were also shown to have the same plasmid MLST profile, supporting their genetic relatedness, although pBK31572 is smaller and lacks certain antibiotic resistance genes present in pBK31551. Insertions and genetic alterations have been previously described in Tn*4401* adjacent to the bla_{KPC} genes (9, 13, 14), including a previous study in which we described a 5.3-kb deletion within Tn*4401* encompassing all of IS*Kpn7* (*istA* and *istB*), 80% of *tnpA*, and 60% of bla_{KPC} (33). The genetic diversity associated with Tn*4401* underlines the notion that the region surrounding bla_{KPC} undergoes significant recombination.

The insertion of Tn*6901* and IS*110* upstream of bla_{KPC} raises the possibility that they may alter gene expression and the level of carbapenem resistance. In fact, both KPC-4-producing *K. pneumoniae* strains displayed low-level resistance to carbapenems, with MIC values of 1 to 4 μg/ml, 2 to 4 μg/ml, and 1 to 2 μg/ml for ertapenem, imipenem, and doripenem, respectively, and total susceptibility to meropenem (Table 1). In addition, the *E. coli* DH10B bla_{KPC-4} transformants were even more susceptible than the parental strains, providing further evidence that the host influences plasmid gene expression levels. In contrast, the KPC-4 resistance reported in *E. cancerogenus* strain E624 exhibited higher MIC levels against imipenem (>32 μg/ml), meropenem (>32 μg/ml), and ertapenem (>16 μg/ml) (14). Significantly, sequence analysis of the 363-bp region upstream of bla_{KPC-4} in pBK31551, including the putative promoter region (60), showed that these upstream sequences are identical to that found in the *E. cancerogenus* strain E624 plasmid, raising speculation that the difference in MICs could be related to the IS*110* and Tn*6901* insertions in Tn*4401* or else to other factors such as a combination of porin loss/alteration and β-lactamases (extended-spectrum β-lactamases or AmpC enzymes) in *E. cancerogenus* strain E624.

The second unusual finding was the identification of a KPC-5-producing *K. pneumoniae* strain in which the bla_{KPC-5}-harboring Tn*4401* element was located on a plasmid belonging to a novel IncX subgroup (IncX5). IncX plasmids have been implicated in the acquisition and dissemination of transferrable drug resistance in different *Enterobacteriaceae* spp. (54) and have been shown to be transferrable from *E. coli* to *P. aeruginosa* (61). Although various resistance determinants have been described in IncX plasmids, carbapenemases have not been identified previously. It is therefore likely that pBK31567 originated from an IncX5 plasmid which acquired Tn*4401* and the class I integron by horizontal transfer. Interestingly, the bla_{KPC-5} gene was initially described in *P. aeruginosa* strain PS28 and notably was not located on Tn*4401* (14). Instead, the carbapenemase gene was located within a novel genetic environment, with Tn*5563*- and IS*6100*-related elements inserted upstream of bla_{KPC-5}. The differences in the flanking regions of bla_{KPC-5} observed between PS28 and BK31567 indicate that bla_{KPC-5} is not associated with the unique genetic environment described in strain *P. aeruginosa* strain PS28. It is interesting that strain BK31567 was isolated in the same year (2006) as strain PS28 (14, 59); however, data on patient demographics are unknown for this isolate (PS28). Likewise, neither the complete plasmid sequence nor the Inc group of the PS28 plasmid is available, precluding direct sequence comparisons between these two plasmids. Consequently, the relationship between these two bla_{KPC-5}-harboring plasmids remains unclear.

Previous sequence comparison between different bla_{KPC} variants showed that bla_{KPC-3}, bla_{KPC-5}, and bla_{KPC-6} each differ from bla_{KPC-2} by a single nucleotide change, while bla_{KPC-5} and bla_{KPC-6} also differ from bla_{KPC-4} by a single nucleotide (14, 23). Accordingly, it was hypothesized that bla_{KPC-4} may have emerged through a sequential process, wherein bla_{KPC-5} and/or bla_{KPC-6} represents an intermediate step (14, 23). Interestingly, KPC-4 and -5 were both identified in the same hospital; nevertheless, evidence that the bla_{KPC-4} gene in pBK31551 or pBK31572 evolved directly from bla_{KPC-5} in pBK31567 is lacking, as these genes were found in distinct plasmids from diverse genetic backgrounds and different time periods.

In this study, we have described in detail the complete sequences of IncN and IncX plasmids harboring bla_{KPC-4} and bla_{KPC-5} carbapenemase genes. Comparative genomic analyses provide new insights into the evolution and dissemination of KPC-producing carbapenem resistance plasmids belonging to different incompatibility groups. Our studies and previous investigations highlight the essential roles of Tn*4401* in KPC dissemination and evolution, including large-scale deletions, insertions, and replacements, as well as carriage of different bla_{KPC} variants and horizontal transfer between different plasmids and host strains.

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