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Novel Genetic Environment of the Carbapenem-Hydrolyzing β-Lactamase KPC-2 among *Enterobacteriaceae* in China[▽]

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Thirty-nine *bla*$_{KPC}$-producing isolates of the family *Enterobacteriaceae* with carbapenem resistance or reduced carbapenem susceptibility were obtained from inpatients from eight hospitals in six cities of three provinces in eastern China. The pulsed-field gel electrophoresis analysis of all 36 *Klebsiella pneumoniae* isolates revealed six major patterns. The resistant plasmids of most isolates were successfully transferred by conjugation and evaluated experimentally to be 40 to 180 kb in size. A 20.2-kb *bla*$_{KPC}$-surrounding nucleotide sequence from plasmid pKP048 has been obtained and contains an integration structure of a Tn*3*-based transposon and partial Tn*4401* segment, with the gene order Tn*3*-transposase, Tn*3*-resolvase, IS*Kpn8*, the *bla*$_{KPC-2}$ gene, and the IS*Kpn6*-like element. The chimera of several transposon-associated elements indicated a novel genetic environment of the *K. pneumoniae* carbapenemase β-lactamase gene in isolates from China.

Carbapenems often are used as the most appropriate agents in the treatment of infections caused by multiresistant gram-negative bacteria. However, reports of carbapenem-hydrolyzing enzymes have become increasingly frequent, and the most common carbapenemases to emerge in recent years have been the *Klebsiella pneumoniae* carbapenemases (KPCs) (8). These plasmid-carried Ambler class A enzymes have since been identified in multiple genera and species of the *Enterobacteriaceae*, including *Klebsiella* spp. (36), *Escherichia coli* (3), *Enterobacter* spp. (14), *Citrobacter* spp. (37), *Salmonella* spp. (18), *Serratia marcescens* (38), and *Proteus mirabilis* (29). The KPC-producing isolates of *Pseudomonas aeruginosa* and *Pseudomonas putida* also were reported recently (1, 30). The geographical distribution of *bla*$_{KPC}$-producing isolates has widened not only within the United States, including the New York City region (2, 4, 5, 33), Pennsylvania, Ohio, Delaware, and Arkansas (8, 24), but also in Israel (22), France (21), Greece (7), Colombia (31), China (32), and recently in Argentina (23), Brazil (19), and the United Kingdom (34).

Whereas the KPC enzymes in novel locations are reported increasingly worldwide, very little information is known about the genetic elements around this resistance gene. Naas et al. characterized a new transposon-related structure named Tn*4401*, which mediated KPC β-lactamase mobilization in several *bla*$_{KPC}$-positive *K. pneumoniae* and *P. aeruginosa* strains isolated from the United States, Colombia, and Greece (20). This transposon seems to be responsible for rapid *bla*$_{KPC}$ spread. Another finding for the *bla*$_{KPC}$-surrounding structure was the KQ element, which is a large composite element consisting of a Tn*1331* backbone and a Tn*4401*-like element and *qnrB19* insertion (25). However, in China, the genetic environment of *bla*$_{KPC}$ genes is unclear. The aim of this study was to characterize the detailed genetic environment surrounding the *bla*$_{KPC}$ gene and report the emergence of *bla*$_{KPC-2}$-producing *Enterobacteriaceae*, including *K. pneumoniae*, *Citrobacter freundii*, *Klebsiella oxytoca*, and *Enterobacter cloacae* isolates from several hospitals in eastern China.

MATERIALS AND METHODS

Bacterial isolates. Thirty-nine nonduplicated isolates of the family *Enterobacteriaceae* (36 *K. pneumoniae*, 1 *C. freundii*, 1 *K. oxytoca*, and 1 *E. cloacae* isolate) with carbapenem resistance or reduced carbapenem susceptibility were obtained from inpatients from eight hospitals in six cities of three provinces in eastern China from March 2006 to December 2007 (Table 1). The species-level identification of these isolates was confirmed by an API 20E system. These isolates were recovered mainly from specimens of sputum and others, including blood, wound swabs, and urine.

Antimicrobial susceptibility testing. MICs of meropenem, imipenem, ertapenem, colistin, tigecycline, ceftazidime, ciprofloxacin, piperacillin-tazobactam, cefoxitin, and amikacin were determined by the Etest technique (AB Biodisk, Sweden) according to the manufacturer's instructions, and the susceptibility breakpoints were interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI) and some previous reports (11, 16). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as quality controls.

PCR for carbapenemase genes. All original isolates and their *E. coli* transconjugants were screened by PCR with primers that are specific for the carbapenem resistance determinant, such as *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SME}$, *bla*$_{IMI}$, and *bla*$_{NMC}$, as described previously (33). All of the positive PCR products were sequenced as described below. All isolates were positive with *bla*$_{KPC}$ primers, confirming the production of a KPC enzyme.

PFGE. Genomic DNA of all *K. pneumoniae* isolates was analyzed by pulsed-field gel electrophoresis (PFGE) after digestion with XbaI (Sangon, China) using the contour-clamped homogenous electric field (CHEF) technique (13). DNA fragments were separated by electrophoresis in 1% agarose III (Sangon, China) in 0.5× Tris-borate-EDTA buffer with a CHEF apparatus (CHEF Mapper XA; Bio-Rad) at 14°C and 6 V/cm and with alternating pulses at a 120° angle in a 2- to 40-s pulse time gradient for 22.5 h. *Salmonella enterica* serotype Braenderup H9812 was used as the size marker (15). Restriction patterns were interpreted by the criteria proposed by Tenover et al. (28).

Plasmid manipulations and Southern hybridization. The conjugation experiment was carried out in mixed-broth cultures. Rifampin (rifampicin)-resistant *E. coli* EC600 (LacZ$^-$ Nal^r Rif^r) was used as the recipient strain. Exponential-phase L broth cultures of donor and recipient were mixed at a volume ratio of 2:1. This

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TABLE 1. MICs of several antimicrobial agents for 39 clinical isolates

Hospital (city, province)	Isolate (*n*)	Clone	Genetic environment of bla_{KPC-2}	MIC[a] (μg/ml)								
				MEM	IPM	ETP	CST	TGC	CAZ	TZP	CIP	FOX	AMK
a (Shanghai, Shanghai)	*K. pneumoniae* (1)	A	Consistent with pKP048	>32	>32	>32	0.25	2	>256	>256	>32	>256	>256
b (Nanjing, Jiangsu)	*K. pneumoniae* (5)	B	Variant 1	>32	16–>32	>32	0.5–1	0.5–2	128–>256	>256	8–>32	128–>256	1–>256
b (Nanjing, Jiangsu)	*K. pneumoniae* (3)	C	Variant 2	>32	>32	>32	0.5–1	0.5–2	>256	>256	>32	128–>256	1–>256
c (Hangzhou, Zhejiang)	*K. pneumoniae* (10)	D	Consistent with pKP048	2–>32	2–>32	16–>32	0.5–1	0.5–1	32–>256	>256	>32	64–>256	>256
c (Hangzhou, Zhejiang)	*K. pneumoniae* (5)	E	Variant 2	16–>32	16–>32	>32	0.5–1	1–1.5	32–>256	>256	>32	32–128	1–>256
c (Hangzhou, Zhejiang)	*C. freundii* (1)		Consistent with pKP048	>32	>32	>32	0.75	2	32	>256	>32	>256	2
d (Hangzhou, Zhejiang)	*K. pneumoniae* (9)	C	Variant 2	>32	>32	>32	0.5–1	0.5–2	>256	>256	>32	128–>256	1–>256
e (Shaoxing, Zhejiang)	*K. pneumoniae* (2)	F	Consistent with pKP048	>32	>32	>32	0.5–2	1	>256	>256	16–>32	>256	4
f (Taizhou, Zhejiang)	*K. pneumoniae* (1)	F	Consistent with pKP048	>32	32	>32	0.75	0.38	>256	>256	12	32	2
g (Taizhou, Zhejiang)	*K. oxytoca* (1)		Consistent with pKP048	>32	>32	16	1	2	128	>256	>32	>256	2
h (Ningbo, Zhejiang)	*E. cloacae* (1)		Consistent with pKP048	>32	>32	>32	0.5	2	>256	>256	>32	>256	>256

[a] MEM, meropenem; IPM, imipenem; ETP, ertapenem; CST, colistin; TGC, tigecycline; CAZ, ceftazidime; TZP, piperacillin-tazobactam; CIP, ciprofloxacin; FOX, cefoxitin; AMK, amikacin.

TABLE 2. Primers for PCR amplification of the bla_{KPC}-surrounding sequences

Primer name	No. in Fig. 3	Sequence (5′–3′)
For1231	1	TCCTCTGCGTGAGCTACACT
Re4510	2	TTCTGACCACTGAGCAGACT
For4100	3	CAGGACGTTCGTTGCTTATC
Re5198	4	GGCAATACTGAGCTGATGAG
For4710	5	GTCTCAACCAGCCAGCAGTC
Re5712	6	TTACGTAGATCCGAGACACC
For5451	7	TGGCCAGGATGTACAACGTC
Re6282	8	CATTCCTTGAGCGCCTGAAC
For5958	9	TCAAGCTTCTGACCGACAAC
Re6838	10	CCTTGAATGAGCTGCACAGT
Kpc-up	11	GCTACACCTAGCTCCACCTTC
Kpc-dw	12	ACAGTGGTTGGTAATCCATGC
For7085	13	GCGATACCACGTTCCGTCTG
Re8069	14	TCCGTAGTGAGGCTGTTCTG
For7755	15	ACAGATACGCCATTCGCCTC
Re8728	16	CGAACATAAGGCCGAACGTG
KPC-A		TGTAAGTTACCGCGCTGAGG
KPC-B		CCAGACGACGGCATAGTCAT

mixture was incubated for 4 h at 35°C. The transconjugants were selected on Mueller-Hinton agar containing imipenem (1 μg/ml) or ampicillin (50 μg/ml) and rifampin (700 μg/ml). The selected transconjugants were identified by an API 20E system. The Qiagen Plasmid Midi kit (Qiagen, Germany) was used to extract plasmids according to the manufacturer's instructions. *E. coli* V517 (54, 5.6, 5.1, 3.9, 3.0, 2.7, and 2.1 kb), *E. coli* R1 (92 kb), and *E. coli* R27 (182 kb) were used as the standards for plasmid size analysis. For Southern blot hybridization, plasmids were transferred from electrophoresis gels to nylon membranes (Bio-Rad) and hybridized with [α-^{33}P]dCTP (DuPont)-labeled bla_{KPC-2}-specific probes. Probes were generated by PCR with primers KPC-A and KPC-B (Table 2). After being washed, the membrane was compressed with a storage phosphor screen (Kodak, Japan) for 48 h and then scanned for hybridization signals.

Shotgun sequencing of plasmid pKP048. The plasmid pKP048 from a transconjugant of *K. pneumoniae* strain KP048 in Hangzhou, Zhejiang, China, was sequenced by using a whole-genome shotgun approach (27). DNA for plasmid pKP048 was randomly sheared by ultrasonication, and the 1.5- to 3.0-kb fragments were cloned into vector pUC18 (Takara Bio, Japan). End sequencing was performed by using BigDye Terminator 3.1 chemistry and a 3730XL sequencer (Applied Biosystems). Sequence gaps were filled by primer walking on linking clones and by the sequencing of the PCR products from the plasmid DNA. The resulting sequence data were assembled by using the Phred/Phrap/Consed software suite from the University of Washington, Seattle (9, 10, 12). GeneMark.hmm 2.4 was used to identify putative open reading frames (ORFs) (17). Nucleotide and amino acid sequences were analyzed and compared by use of the BLAST program (http://www.ncbi.nlm.nih.gov).

Genetic environment analysis of bla_{KPC} gene. A PCR mapping approach was carried out to compare the genetic context of the bla_{KPC} gene in other isolates with that found in plasmid pKP048. A series of primers were designed at the base of bla_{KPC}-surrounding sequences (Table 2). PCR experiments were performed according to standard conditions with an annealing temperature of 58°C. The obtained amplification products were sequenced.

Nucleotide sequence accession number. A 20,158-bp sequence surrounding the bla_{KPC} gene has been submitted to the GenBank nucleotide sequence database under accession number FJ628167.

RESULTS

Antimicrobial susceptibility testing. The MICs of a variety of antimicrobial agents tested against all isolates are shown in Table 1. All isolates were resistant to ertapenem, and most of them were resistant to meropenem and imipenem, except for five isolates from Hangzhou, for which the MICs were 2 μg/ml. All isolates also were resistant to ceftazidime, piperacillin-tazobactam, ciprofloxacin, and cefoxitin. These isolates were

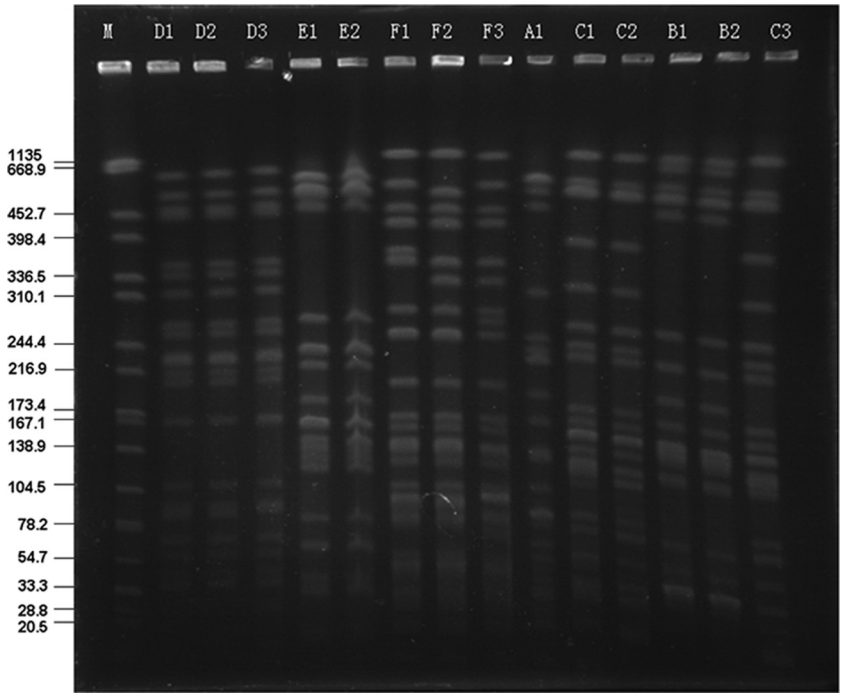

FIG. 1. PFGE analysis of XbaI-digested genomic DNA from isolates of *K. pneumoniae*. M, marker (*Salmonella enterica* serotype Braenderup H9812); A1 was from hospital a in Shanghai; B1, B2, and, C3 were from hospital b in Nanjing; C1 and C2 were from hospital d in Hangzhou; D1, D2, D3, E1, and E2 were from hospital c in Hangzhou; F1 and F2 were from hospital e in Shaoxing; and F3 was from hospital f in Taizhou.

susceptible to colistin (MIC $\leq$ 2 μg/ml) and tigecycline (MIC $\leq$ 2 μg/ml). Of note, MICs of amikacin for these isolates were extremely variable. Seventeen isolates, including 15 *K. pneumoniae* isolates, 1 *C. freundii* isolate, and 1 *K. oxytoca* isolate, were susceptible to amikacin (MIC $\leq$ 4 μg/ml); in contrast, the rest were resistant (MIC $\geq$ 256 μg/ml).

PCR for carbapenemase genes. The bla_{KPC} gene was detected by PCR in all of the isolates, and sequencing results revealed that they belonged to the same KPC-2 allele as that reported several times already in China (32, 38). No metallocarbapenemase gene (bla_{IMP} and bla_{VIM}) or other class A enzyme gene (bla_{SME}, bla_{IMI}, and bla_{NMC}) was detected.

PFGE analysis. According to the datum-interpreting criteria described by Tenover et al. (28), the 36 clinical isolates of *K. pneumoniae* were grouped into six clonal patterns by PFGE, designated patterns A to F (Fig. 1). Pattern A was represented by one isolate that was from a hospital in Shanghai, and pattern B was represented by five isolates that were from a hospital in Nanjing. Twelve isolates representing pattern C were from the hospital of Nanjing (three isolates) and a hospital in Hangzhou (nine isolates). Another hospital in Hangzhou had 15 total isolates; 10 of them exhibited pattern D, and the other 5 represented pattern E. Pattern F, with three subtypes by three isolates, occurred in a hospital of Shaoxing (two isolates) and a hospital of Taizhou (one isolate) (Fig. 1 and Table 1). The four bla_{KPC}-positive clones were observed in the unique region, including patterns A, B, D, and E, while the remaining clones were detected in separate regions (patterns C and F).

Plasmid manipulations and analysis. Except for 5 isolates of PFGE pattern B from Nanjing and 7 isolates of pattern C (3 isolates in Nanjing and 4 isolates in Hangzhou), plasmids in 27 isolates successfully transferred carbapenem resistance to *E. coli* by conjugation. Transconjugants exhibited a phenotype of resistance or reduced susceptibility to carbapenem that was similar to that of their parent isolates. The plasmids of clinical isolates and their transconjugants that possess the bla_{KPC-2} gene were extracted and experimentally evaluated to be 40 to 180 kb in size, displaying the diversity of their genetic contents. Electrophoretic profiles of a subset of these plasmids and hybridization with a bla_{KPC-2}-specific probe are shown in Fig. 2.

Characterization of the genetic environment of the bla_{KPC-2} gene. pKP048, a bla_{KPC-2}-positive plasmid of ~150 kb in size and 10^{-9} transconjugants/donor in frequency of conjugation, was selected for complete sequencing to explore the genetic environment of the bla_{KPC}-surrounding sequence. While this project was being conducted, a nucleotide sequence of 20,158 bp surrounding the bla_{KPC-2} gene was obtained. The annotation of this sequence revealed several ORFs, and some of these have been associated with the bla_{KPC-2} gene. The immediate environment surrounding bla_{KPC-2} in pKP048 from China is composed of a partial Tn*4401* structure and a Tn*3*-based element (Fig. 3). Tn*4401*, which was characterized as a bla_{KPC} mobilization element in several bla_{KPC}-producing isolates from the United States, Colombia, and Greece, consists of a transposase, a resolvase, a bla_{KPC} gene, and two putative insertion sequence (IS) elements, IS*Kpn6* and IS*Kpn7*. In pKP048, only a 2,070-bp region is identical to Tn*4401*, including the bla_{KPC-2} gene and an IS*Kpn6*-like ORF (Fig. 3). An IS*Kpn6*-like element downstream of the bla_{KPC-2} gene shared the same 287-amino-acid fragment in its C terminus with IS*Kpn6* in Tn*4401* (a total of 439 amino acids). The inverted repeats (IRs) of

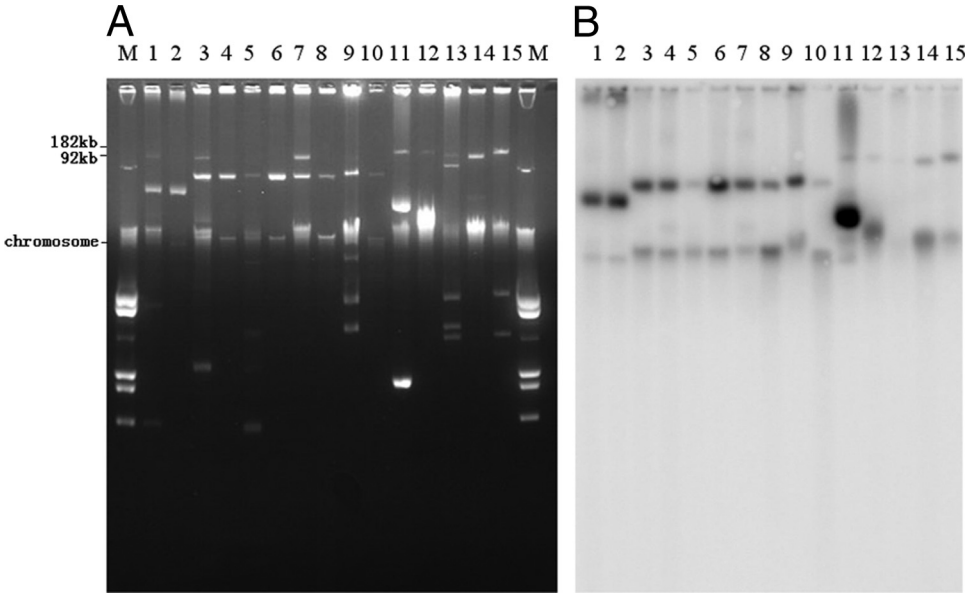

FIG. 2. Electrophoretic profiles of plasmids (A) and hybridization with a bla_{KPC-2}-specific probe (B). M, marker (*E. coli* V517); lanes 1 and 2, *K. oxytoca* from hospital g of Taizhou and its transconjugant; lanes 3 and 4, *K. pneumoniae* from hospital e of Shaoxing and its transconjugant; lanes 5 and 6, *K. pneumoniae* from hospital a of Shanghai and its transconjugant; lanes 7 and 8, *E. cloacae* from hospital h of Ningbo and its transconjugant; lanes 9 and 10, *C. freundii* from hospital c of Hangzhou and its transconjugant; lanes 11 and 12, *K. pneumoniae* from hospital c of Hangzhou harboring plasmid pKP048 and its transconjugant; lanes 13 and 14, *K. pneumoniae* from hospital d of Hangzhou and its transconjugant; and lane 15, *K. pneumoniae* from hospital b of Nanjing.

IS*Kpn6* and its transposition-generated target site duplications (TSDs) are absent.

Upstream of the bla_{KPC-2} gene is located a Tn*3*-based transposon, including its transposase and resolvase genes (Fig. 3). The Tn*3*-based element is flanked by two 3-bp TSDs (TAA) and 38-bp complete IRs, indicating the occurrence of a transposition event. The 3′ segment of the Tn*3* transposon is disrupted by a novel putative IS element, designated IS*Kpn8*, which encodes a putative transposase (326 amino acids) belonging to the IS*481* family and is 57% similar in amino acids to a transposase from *Pelobacter carbinolicus* (NC_007498). Upon the breaking sites, IS*Kpn8* was found to be bracketed by two 6-bp TSDs (ATAGGT) and two incomplete 28-bp IRs. To explore both sides of the Tn*4401*-Tn*3* integration region, we found a segment of the Tn*1721* transposon. The segment of Tn*1721* itself has inserted on the plasmid with a GAATTC TSD. It is split by a Tn*4401*-Tn*3* integration region and a ca. 3-kb sequence that identified with plasmid pFBAOT6 (NC_006143) in *Aeromonas punctata* and plasmid pRA3 (DQ401103) in *Aeromonas hydrophila* (Fig. 3).

By using a PCR mapping approach, fragments of partial structures containing IS*Kpn8*, bla_{KPC-2}, and IS*Kpn6*-like elements were obtained from all isolates, including three non-*Klebsiella* isolates. Seventeen isolates are consistent with the genetic environment in pKP048 completely. Moreover, there are another two variants in 22 isolates of our research. In five isolates of PFGE pattern B, a truncated bla_{TEM} gene also was a part of the Tn*3* transposon located between IS*Kpn8* and the bla_{KPC-2} gene. The 17 isolates belong to PFGE patterns C and E only obtained four PCR fragments lacking the Tn*3*-based transposase and its resolvase, suggesting the diversity of the genetic environment surrounding this region in bla_{KPC}-producing isolates from China. Neither of the two variants has any detected segments of Tn*1721*. Sequences upstream and downstream of these two variants remain unclear.

DISCUSSION

In the last 7 years, several case and outbreak reports describing the presence of KPC β-lactamases have been published (26, 33, 35). The KPC-2 or KPC-3 allele has expanded rapidly across species and geographic regions because of clonal dissemination as well as horizontal gene transfer (8). Here, we describe the appearance of the KPC-2 enzyme in strains of the family *Enterobacteriaceae* with carbapenem resistance or reduced carbapenem susceptibility from eight hospitals in six cities of three provinces in eastern China.

Antimicrobial susceptibility testing showed that carbapenem nonsusceptibility strains also were resistant to many other antimicrobial agents, such as ceftazidime, piperacillin-tazobactam, ciprofloxacin, and cefoxitin. These isolates were susceptible to colistin and tigecycline as described before (6), suggesting a choice for clinical therapy. The susceptibility to amikacin in these isolates was variable, because the 16S rRNA methylase gene *armA* was detected by PCR-based assays in all 22 isolates for which the MIC was ≥256 μg/ml. The nosocomial infection of bla_{KPC}-producing isolates was due mainly to clonal dissemination, because each hospital possessed only one or two pulse clones. The interhospital clonal spread among separate geographic regions also has happened because isolates from two cities shared the same pattern.

Most isolates can easily transfer carbapenem resistance to *E. coli* by conjugation, indicating that the resistant plasmids possessed their own transfer-associated gene clusters. The sizes of

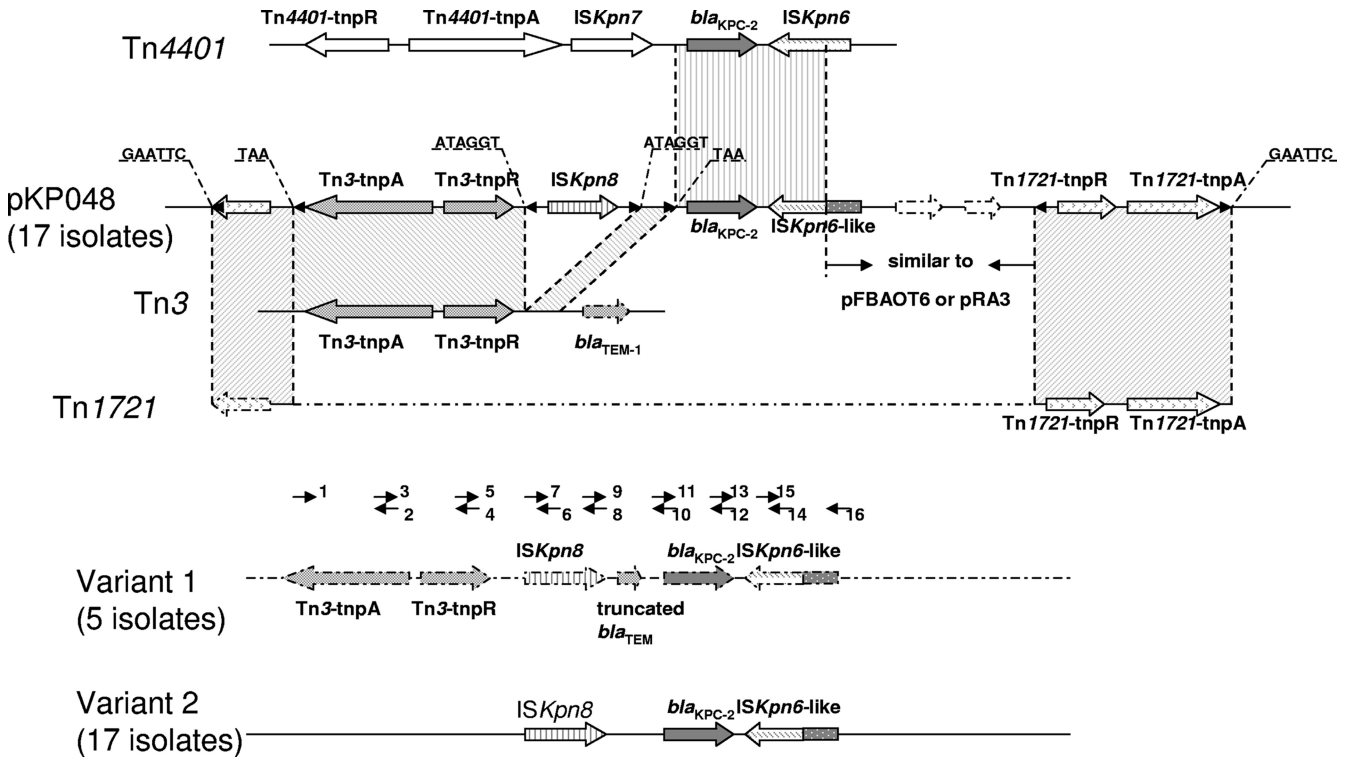

FIG. 3. Schematic representation of the novel genetic structure involved in the bla_{KPC-2} gene in China. ORFs are shown, and their directions of transcription are presented as broad arrows. Inverted repeats of the respective mobile elements are shown as small black triangles. Nucleotide letters above the sequence of pKP048 with underlining represent target site duplications. The gray-shaded area delimited by two dotted lines indicates identical regions. Short arrowheads with numbers show the primers listed in Table 2 and were used for PCR mapping.

these plasmids vary across a broad range, and the plasmid incompatibility group dissimilarity (data not show) exhibited different plasmid profiles, although their host shared the same PFGE pattern. Horizontal gene transfer and transposition events likely modified the genetic contents of resistant plasmids.

Previous studies of the genetic environment of the bla_{KPC} gene had characterized a transposon-associated element, Tn*4401*, from *K. pneumoniae* isolate YC with a U.S. origin (20). The bla_{KPC}-containing plasmids of isolates from Greece and Colombia also were analyzed. Tn*4401* was considered the origin of bla_{KPC}-like gene acquisition and dissemination. However, in our present work, the genetic environment of the bla_{KPC} gene from isolates in China was distinct. The genetic environment surrounding the bla_{KPC-2} gene in most isolates from China is considered the integration of a Tn*3*-based transposon and a partial Tn*4401* structure, with the ORF order of Tn*3*-transposase, Tn*3*-resolvase, IS*Kpn8*, the bla_{KPC-2} gene, and the IS*Kpn6*-like element. Part of Tn*1721* (5′ segment) is interrupted by the overall integration region and itself is inserted with the duplication of 6-bp target site, suggesting the cotransposition of these transposon elements. The detailed analysis of all TSD and IR sequences revealed that several transposition events must be happening. We speculated that Tn*1721* inserted first on the plasmid pKP048 containing the bla_{KPC-2} gene, the IS*Kpn6*-like element, and another 3-kb sequence. Part of the Tn*3*-based transposon then inserted upstream of the bla_{KPC-2} gene. Subsequently, IS*Kpn8* was inserted downstream of the Tn*3*-resolvase gene independently because it possessed its own TSD and IR sequences. The hypothesis must be validated by further experiments.

Some isolates in our study also present diversity in this genetic structure, such as 17 isolates not having the Tn*3*-transposase and its resolvase and 5 isolates having a partial bla_{TEM} gene fragment. The truncated bla_{TEM} gene fragment belonging to the representative structure of the Tn*3* transposon indicates the variety of the length of insertion segments. The sequences of the unknown regions in these isolates need to be explored in depth.

In conclusion, our study of 39 isolates of the family *Enterobacteriaceae* in China characterized the novel genetic environment of the bla_{KPC}-surrounding sequence. The integration structure of the Tn*3*-based transposon and the partial Tn*4401* segment indicated the diversity of the genetic environment harboring this widespread carbapenem-hydrolyzing β-lactamase.

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