

Genetic Structures at the Origin of Acquisition of the β -Lactamase bla_{KPC} Gene[∇]

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Genetic structures surrounding the carbapenem-hydrolyzing Ambler class A bla_{KPC} gene were characterized in several KPC-positive *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains isolated from the United States, Colombia, and Greece. The bla_{KPC} genes were associated in all cases with transposon-related structures. In the *K. pneumoniae* YC isolate from the United States, the β -lactamase bla_{KPC-2} gene was located on a novel Tn3-based transposon, Tn4401. Tn4401 was 10 kb in size, was delimited by two 39-bp imperfect inverted repeat sequences, and harbored, in addition to the β -lactamase bla_{KPC-2} gene, a transposase gene, a resolvase gene, and two novel insertion sequences, ISKpn6 and ISKpn7. Tn4401 has been identified in all isolates. However, two isoforms of this transposon were found: Tn4401a was found in *K. pneumoniae* YC and *K. pneumoniae* GR from the United States and Greece, respectively, and differed by a 100-bp deletion, located just upstream of the bla_{KPC-2} gene, compared to the sequence of Tn4401b, which was found in the Colombian isolates. In all isolates tested, Tn4401 was flanked by a 5-bp target site duplication, the signature of a recent transposition event, and was inserted in different open reading frames located on plasmids that varied in size and nature. Tn4401 is likely at the origin of carbapenem-hydrolyzing β -lactamase KPC mobilization to plasmids and its further insertion into various-sized plasmids identified in nonclonally related *K. pneumoniae* and *P. aeruginosa* isolates.

Carbapenem resistance in *Klebsiella pneumoniae* is mainly related to acquired carbapenem-hydrolyzing β -lactamases (19). These β -lactamases can be either metallo- β -lactamases (IMP and VIM), expanded-spectrum oxacillinases (OXA-48), or Ambler class A enzymes (NMCA, IMI, SME, GES, and KPC) (1, 4, 11, 14, 19, 22, 24). KPC-type enzymes in carbapenem-resistant *K. pneumoniae* strains were first reported in 2001 in North Carolina (33), and until 2005, the geographical distribution of these enzymes in members of the family *Enterobacteriaceae* in general and in *K. pneumoniae* in particular was limited to the eastern part of the United States (2, 24, 27, 32), where KPC-producing *K. pneumoniae* isolates are now frequently identified among nosocomial pathogens (7). Outside of the United States, KPC-producing *K. pneumoniae* isolates have been reported from only a few patients; the first case was reported in 2005 in France and had a U.S. origin (16), and more recently, similar cases have been reported from Colombia, China, and Greece (6, 28, 30). The first outbreak of KPC-producing *K. pneumoniae* outside of the United States was in Israel and has been described extensively (13).

KPC carbapenemases have been observed even more rarely among other gram-negative bacterial species, including *Enterobacter* spp., *Escherichia coli*, and *Serratia marcescens* (3, 8, 14, 34). Outside of the United States, KPC-2 was identified once

from an *S. marcescens* isolate from China (35), from *E. coli* strains from Israel (17), and in a *P. aeruginosa* isolate in Colombia (29).

Whereas detailed crystallographic data have been obtained (9) and the description of this enzyme in novel locations is increasing worldwide, signaling a very active process of spreading, very little information is known about the genetic elements responsible for this rapid spread. The aim of the present work was to characterize the genetic elements involved in bla_{KPC} gene mobilization and diffusion.

MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* YC (16), *K. pneumoniae* GR (6), *K. pneumoniae* KN633 (28), *K. pneumoniae* KN2303 (28), and *P. aeruginosa* 2404 (29) were used in this study. Electrocompetent *E. coli* DH10B (Invitrogen, Eragny, France) and *P. aeruginosa* KG2505, which does not express the naturally and chromosome-encoded AmpC β -lactamase and is deficient for the multidrug efflux system MexAB-OprM (20), were used as recipients in the electroporation experiments. *E. coli* J53 Az^r, which is resistant to sodium azide, and ciprofloxacin-resistant *P. aeruginosa* PU21 (15) were used for the conjugation experiments. *E. coli* 50192 was used as a reference strain for plasmid extraction (21). The plasmid vector pBKCMV, which carries a kanamycin resistance marker, was used for the cloning experiments (21).

Antimicrobial agents and MIC determinations. Antibiograms were determined by the disk diffusion method on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France), and the susceptibility breakpoints were determined as described previously (21) and interpreted as recommended by the Clinical and Laboratory Standards Institute (5). All plates were incubated at 37°C for 18 h. The MICs of the β -lactams were determined by the Etest technique (AES, Bruz, France).

Plasmid content, mating out, and electroporation experiments. The direct transfer of resistance into azide-resistant strain *E. coli* J53 and ciprofloxacin-resistant strain *P. aeruginosa* PU21 was attempted, as reported previously (15).

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TABLE 1. Primers used in this study^a

Primer name	No. in Fig. 3	Sequence (5'–3')
KpcA	1	CTGTCTGTCTCTCATGGCC
KpcB	2	CCTCGCTGTGCTTGTATCC
4281	3	GGCACGGCAAATGACTA
4714	4	GAAGATGCCAAGGTCAATGC
SeqRIout	5	ACGACCACGCACGCACAAAC
3'EndYC	6	GCATCAAACGGAAGCAAAAAG
3781L	7	GCTTTCTGTGCTCCGCTGTG
3098U	8	TGACCCTGAGCGGCGAAAGC
905L	9	GCGACCGGTCAGTTCCTTCT
816U	10	CACCTACACCACGACGAACC
141R-6	11	TCACCGGCCCTCACCTTTGG
5'endYC	12	CTTAGCAAATGTGGTGAACG

^a All primers were developed in the present study.

Plasmids were introduced by electroporation into *E. coli* DH10B (21) and *P. aeruginosa* KG2505 (20, 26) by using a Gene Pulser II apparatus (Bio-Rad).

Recombinant plasmid DNAs were extracted with a Qiagen plasmid DNA maxi kit (Courtaboeuf, France) and were analyzed by restriction endonuclease digestions (Amersham Biosciences) and agarose gel electrophoresis (Invitrogen, Paris, France).

Natural plasmids were extracted by the Kieser extraction method (10) or with the Qiagen plasmid DNA maxi kit. Plasmid extracts were subsequently analyzed by electrophoresis on a 0.7% agarose gel.

Hybridization. DNA-DNA hybridizations were performed as described by Sambrook et al. (25) with a Southern transfer of an agarose gel containing total DNA extracted by the Kieser extraction method (10). The probe consisted of a 796-bp PCR-generated fragment from recombinant plasmid pRYC-1 and was internal to the *bla*_{KPC-2} gene. Labeling of the probe and signal detection were carried out by using the ECL nonradioactive labeling and detection kit, according to the manufacturer's instructions (Amersham Biosciences, Orsay, France).

Cloning experiments and analysis of recombinant plasmids. All enzymes for DNA manipulations were used according to the recommendations of the supplier (Amersham Biosciences). Unless specified otherwise, standard molecular techniques were used (25). Whole-cell DNAs were extracted as described previously (21). The cloning procedure consisted of the ligation of either HindIII-, BamHI-, or EcoRI-digested fragments from genomic DNAs from *K. pneumoniae* YC into the HindIII-, BamHI-, or EcoRI-restricted pBKMV vector, respectively (21). Recombinant plasmids were transformed by electroporation, and antibiotic-resistant colonies were selected on Trypticase soy agar plates containing amoxicillin (50 µg/ml) and kanamycin (30 µg/ml).

Genetic environment of *bla*_{KPC-2} gene. Precise determination of the genetic structures surrounding the *bla*_{KPC-2} gene in *K. pneumoniae* YC allowed us to design a series of primers for PCR amplification and mapping of the *bla*_{KPC}-surrounding sequences and the identification of insertion sequence (IS) elements from the other KPC-positive isolates. PCR experiments were performed as described below on an ABI 2700 thermocycler (Applied Biosystems, Les Ulis, France) by using laboratory-designed primers (Table 1). Two microliters of the supernatant from the whole-cell DNA extract was used as the template. PCR experiments with AmpliTaq Gold DNA polymerase (Roche, Meylan, France) were performed with 35 cycles consisting of 45 s of denaturation at 94°C, 45 s of annealing at 55°C, and variable extension times at 72°C, depending on the expected product size (60 s per 1 kb to be amplified). The PCR products were then analyzed on an agarose gel and sequenced.

Biochemical properties. Crude β-lactamase extracts, obtained as described previously (21) from 10-ml cultures of clinical isolates and their *E. coli* transconjugants or electroporants, were subjected to analytical isoelectrofocusing on an ampholine-containing polyacrylamide gel with a pH range of 3.5 to 9.5 (Ampholine PAG plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamases were detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Dardilly, France). The pI values were determined and compared to those of known β-lactamases (21).

DNA sequencing and protein analysis. Both strands of the PCR products, the cloned DNA fragment of recombinant plasmid pRYC-1, and the natural plasmids were sequenced by using laboratory-designed primers with an automated sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide and the de-

duced protein sequences were analyzed with software available at the National Center of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been assigned to the EMBL/GenBank nucleotide database under the accession numbers EU176011 to EU176014. The nucleotide sequences of the ISs reported in this paper have been submitted to the IS Finder website (<http://www-is.biotoul.fr>).

RESULTS

Genetic support of *bla*_{KPC} in the various clinical isolates.

The carbapenem-resistant *K. pneumoniae* isolates contained several plasmids of different sizes, ranging from ca. 10 kb to 170 kb (Fig. 1; Table 2). In each strain, at least one plasmid hybridized with an internal probe for the *bla*_{KPC-2} gene and ranged from 12 to 80 kb in size (Fig. 1; Table 2). For *K. pneumoniae* KN2303, two hybridization signals were observed (35 and 75-kb). The plasmid locations of the *bla*_{KPC} genes were confirmed by electroporation of these plasmids into *E. coli* DH10B and *P. aeruginosa* KG2505. Whereas all plasmids replicated into *E. coli* and yielded electroporants, only plasmid pCOL, isolated from *P. aeruginosa* 2404, was able to be electroporated into *P. aeruginosa* KG2505 (Table 2). The *E. coli* transformants had a β-lactam resistance pattern compatible with the expression of a *bla*_{KPC}-like gene (Table 3). No other antibiotic resistance marker was cotransferred, as the transformants remained susceptible to nalidixic acid, levofloxacin, ciprofloxacin, gentamicin, kanamycin, netilmicin, tobramycin, amikacin, chloramphenicol, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and colistin on a disk diffusion antibiogram. Natural plasmid pCOL conferred a high-level-resistance phenotype to all β-lactams in *P. aeruginosa* KG2505, which is AmpC deficient (Table 3). Similarly, no other resistance marker was phenotypically detected in *P. aeruginosa*.

Mating-out assays revealed that the ca. 75- to 80-kb plasmids

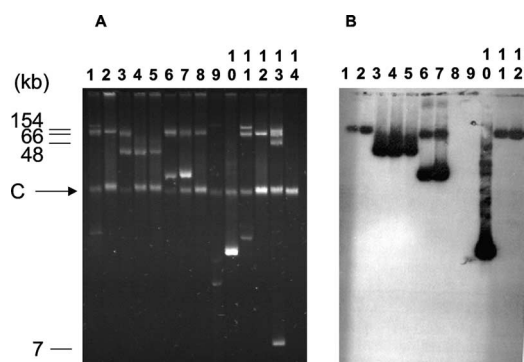


FIG. 1. Plasmid extractions from cultures of the different isolates and their transconjugants or transformants (A) and Southern hybridization carried out with an internal probe for *bla*_{KPC-2} (B). Lanes 1, *K. pneumoniae* YC; lanes 2, *E. coli* J53 transconjugant harboring plasmid pNYC-1; lanes 3, *P. aeruginosa* 2404; lanes 4, *P. aeruginosa* KG2505 transformant harboring plasmid pCOL; lanes 5, *E. coli* J53 transformant harboring plasmid pCOL; lanes 6, *K. pneumoniae* KN2303; lanes 7, *E. coli* J53 transconjugant harboring plasmid pBC2303; lanes 8, *K. pneumoniae* KPC-negative strain; lanes 9, *K. pneumoniae* KN633; lanes 10, *E. coli* J53 electroporant harboring plasmid pBC633; lanes 11, *K. pneumoniae* GR; lanes 12, *E. coli* J53 transconjugant harboring plasmid pNGR-1; lanes 13, *E. coli* 50192 harboring four plasmids (7, 48, 66, and 154 kb); lanes 14, *E. coli* J53 reference strain (only the chromosomal band is visible). C, chromosome.

TABLE 2. Strains and plasmid analysis

Parental strain	Plasmid size (kb)	KPC plasmid ^a size (kb)	Conjugation result		Electroporation result	
			<i>E. coli</i> J53	<i>P. aeruginosa</i> KG	<i>E. coli</i> DH10B	<i>P. aeruginosa</i> KG
<i>K. pneumoniae</i> YC	170, 80, 23	80	+	–	+	–
<i>K. pneumoniae</i> GR	170, 80, 20	80	+	–	+	–
<i>K. pneumoniae</i> 633	12, 10	12	–	–	+	–
<i>K. pneumoniae</i> 2303	75, 35	75, 35	+	–	+	–
<i>P. aeruginosa</i> 2404	70, 45	45	–	–	+	+

^a A plasmid that hybridizes with bla_{KPC} .

pBC2303a, pNYC, and pNGR from *K. pneumoniae* KN2303, *K. pneumoniae* YC, and *K. pneumoniae* GR, respectively, were self-transferable to *E. coli* but not to *P. aeruginosa*, whereas the 12-kb plasmid of *K. pneumoniae* KN663 failed to be transferred to *E. coli* or *P. aeruginosa*. The 35-kb pBC2303b plasmid from *K. pneumoniae* KN2303 was transferred to *E. coli* together with a larger plasmid of 75 kb. The 70-kb plasmid pCOL from *P. aeruginosa* 2404 was able to replicate into *E. coli* and *P. aeruginosa*, given its transfer by electroporation, but attempts to transfer the β -lactam resistance marker into *E. coli* J53 and *P. aeruginosa* PU21 by mating-out assays failed (Table 2).

Southern hybridization of the extracted plasmids revealed strong hybridization signals on plasmids present in both the parents and the transconjugants or transformants. The plasmids harboring bla_{KPC} expressed only this β -lactamase gene when they were tested by isoelectrofocusing, even though the parental strains expressed multiple β -lactamases (data not shown).

Cloning of the β -lactamase gene from *K. pneumoniae* YC. Several *E. coli* transformants were obtained for each cloning experiment and were selected on medium supplemented with kanamycin and amoxicillin. The largest recombinant plasmid expressing reduced susceptibility to imipenem, pRYC-1, which had a 22-kb EcoRI insert (Fig. 2), was retained for further analysis. Higher β -lactam MICs were observed when the bla_{KPC} gene was expressed from the multicopy cloning vector than when it was expressed from the natural plasmid (data not shown).

Characterization of genetic environment of the bla_{KPC-2} gene. The nucleotide sequence of the ca. 22-kb insert of plasmid pRYC-1 was determined and revealed several open reading frames (ORFs) (Fig. 2). Several of these ORFs have previously been associated with the bla_{KPC} -like genes in clinical isolates. Detailed analysis of the ORFs allowed identification and description of two novel ISSs, ISKpn6 and ISKpn7 (Fig. 2 and 3). ISKpn6, which belongs to a novel family of ISSs, the IS1182 family (M. Chandler, personal communication), was identified immediately downstream of the bla_{KPC} gene. It was 1,540 bp long, and its putative transposase (439 amino acids) shares 54% identity with the sequence of ISMaq from *Marinobacter aquaeolei* VT8 (GenBank accession no. YP_958264.1). The inverted repeats (IRs) of ISKpn6 are 17 bp long, and its transposition generated a 2-bp TA target site duplication (TSD). Another IS, ISKpn7, a member of the IS21 family, was found upstream of the bla_{KPC} gene. It is 1,956 bp long and encodes two consecutive ORFs: a long upstream frame designated *istA* and a shorter downstream frame, *istB*. *istA* encodes a 341-amino-acid putative transposase that shares 75% amino

acid identity with the amino acid sequence of ISAvi from *Azotobacter vinelandii* AvOP (GenBank accession no. ZP_00415985.1), and *istB* encodes a 259-amino-acid transposition helper protein which shares 83% amino acid identity with the amino acid sequence of the ISAvi transposase in *Azotobacter vinelandii* AvOP (GenBank accession no. ZP_00419950.1). The IRs of ISKpn7 were 28 bp long, and transposition of ISKpn7 generated a 3-bp TSD.

Two additional ORFs, designated *tnpA* and *tnpR*, were identified upstream of ISKpn7. *TnpA* is 3,027 bp long and encodes a transposase of 1,009 amino acids that shares 86% amino acid sequence identity with the amino acid sequence of a transposase of *Ralstonia pickettii* 12J and 84% identity with the amino acid sequence of a transposase found in *Pseudomonas* sp. strain ND6 (unpublished data; GenBank accession nos. ZP_01663250 and NP_943128). *TnpR*, a 1,713-bp-long resolvase gene, encodes a 571-amino-acid protein that shares 69% identity with the site-specific recombinase of *Burkholderia mallei*, *B. vietnamiensis*, and *B. pseudomallei* 305 (GenBank accession no. ZP_01765313.1) (18).

A 39-bp sequence with 92% (36/39) identity with the left IR (IRL) of a putative Tn3-type transposon of *B. vietnamiensis* (18) was identified downstream of the *tnpR* gene. A similar 39-bp sequence in the opposite orientation could not be identified on recombinant plasmid pRYC-1. Thus, sequencing of the natural plasmid pNYC was conducted to search for the right IR (IRR). Two hundred base pairs after the EcoRI site, which was used for cloning purposes, a similar sequence (87% sequence identity) was identified, thus forming a Tn3-like transposon of 10 kb named Tn4401. Tn4401 was bracketed by two 39-bp imperfect IRs. Upon insertion, Tn4401 generated a 5-bp TSD ATTGA sequence, which is a signature of a transposition process. Tn4401 was surrounded by several ORFs found on plasmids pKPN3, pKPN4, and pKPN5, which have recently been sequenced and identified in *K. pneumoniae* MGH 78578. The genetic environment of transposon Tn4401 on plasmid pNYC-1 was made of a mosaic of ORFs found on one of these three plasmids (data not shown). Whereas most of these ORFs are of unknown function, some share high degrees of sequence identity with genes involved in plasmid transformation or plasmid replication (*traI*, *traX*, and *repA*).

Structure of Tn4401 in clinical isolates of various geographical origins. By using primer pairs (Table 1; Fig. 3) specific for the different genes found on Tn4401, fragments of similar sizes were obtained from all the strains, suggesting similar genetic organizations. For only one primer pair, which hybridized to ISKpn7 and the bla_{KPC} gene, a fragment ca. 100 bp shorter than the fragments from isolates from Colombia or from se-

TABLE 3. MICs of β -lactams

β -Lactam(s) ^a	MIC (μ g/ml)													
	<i>K. pneumoniae</i> YC	<i>E. coli</i> pNYC	<i>E. coli</i> pRYC	<i>K. pneumoniae</i> GR	<i>E. coli</i> pNGR	<i>K. pneumoniae</i> 633	<i>E. coli</i> pBC633	<i>K. pneumoniae</i> 2303	<i>E. coli</i> pBC2303	<i>P. aeruginosa</i> 2404	<i>P. aeruginosa</i> KG2505 pCol	<i>E. coli</i> pCOL	<i>E. coli</i> DH10B	<i>P. aeruginosa</i> KG2505
Amoxicillin + CLA	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	2	0.25
Amoxicillin	32	64	48	48	24	32	32	24	24	>256	>256	24	2	0.25
Ticarcillin	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	2	0.38
Ticarcillin + CLA	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	2	0.25
Piperacillin	>256	>256	>256	>256	128	>256	>256	>256	>256	>256	>256	>256	1.5	0.38
Piperacillin + TZB	>256	>256	>256	>256	128	>256	>256	128	128	>256	>256	32	1.5	0.12
Cephalothin	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	2	2	0.25
Cefoxitin	>32	>32	>32	>32	4	>32	>32	8	8	>32	>32	2	2	0.25
Cefotaxime	>32	>32	>32	>32	4	>32	>32	16	16	>32	>32	3	0.05	0.25
Cefotaxime + CLA	16	2	32	32	0.38	>32	>32	0.38	0.38	>32	>32	0.25	0.05	0.19
Ceftazidime	64	4	>256	>256	4	32	32	256	256	>256	>256	8	0.19	1.0
Aztreonam	256	16	>256	>256	16	>256	>256	192	192	>256	>256	24	0.03	0.19
Aztreonam + CLA	256	8	>256	>256	1.5	>256	>256	0.32	0.32	>256	>256	1.5	0.02	0.19
Cefepime	16	1	32	>32	1.5	4	4	2	2	>256	>256	1.0	0.02	0.50
Imipenem	4	8	12	12	1.5	6	6	1.0	1.0	>32	>32	1.5	0.19	0.19
Imipenem + CLA	2	2	0.75	0.75	0.25	0.75	0.75	0.25	0.25	>32	>32	0.38	0.1	0.19
Meropenem	2	2	3	6	0.38	2	2	0.38	0.38	>32	>32	0.01	0.01	0.05
Meropenem + CLA	1	0.25	2	3	0.01	0.38	0.38	0.12	0.12	>32	>32	0.1	0.002	0.01
Ertapenem	24	0.50	4	12	0.38	2	2	0.25	0.25	>32	>32	0.25	0.004	0.25
Ertapenem + CLA	2	0.05	1.5	6	0.01	0.19	0.19	0.02	0.02	>32	>32	0.05	0.002	0.03

^a CLA, clavulanic acid at a fixed concentration of 2 μ g/ml; TZB, tazobactam at a fixed concentration of 4 μ g/ml.

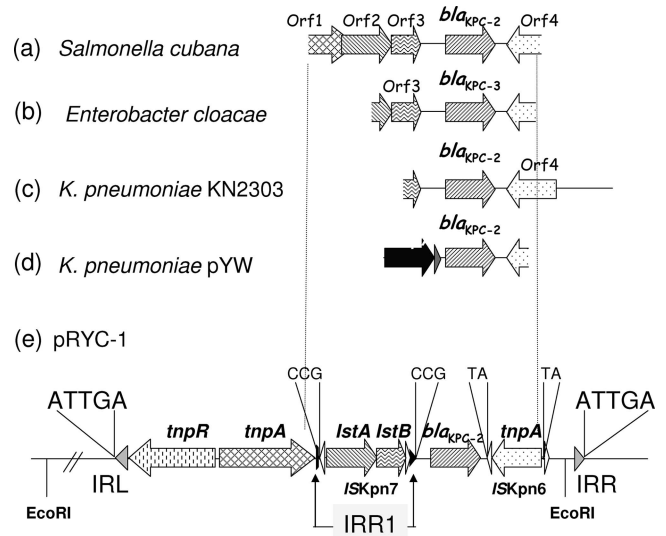


FIG. 2. Schematic representation of *bla*_{KPC}-positive structures identified in enterobacterial isolates. (a) *Salmonella enterica* serovar Cubana (*Salmonella cubana*) (14); (b) *Enterobacter cloacae* (unpublished data; GenBank accession no. AM774409); (c) *K. pneumoniae* KN2303 (28); (d) *K. pneumoniae* pYW (30); (e) recombinant clone pRYC-1 containing the *bla*_{KPC-2}-coding region from *K. pneumoniae* YC (16). The vertical dotted lines indicate the largest known structure of the *bla*_{KPC} genetic environment. Genes and their corresponding transcription orientations are indicated by horizontal arrows. Grey triangles represent IRL and IRR of Tn4401. IRR1 represents another IRR (black triangle) that is disrupted by the *ISKpn7* insertion. Small and empty triangles represent the inverted repeats of *ISKpn6* and *ISKpn7*. TSDs are indicated above the sequence.

quences obtained from nucleotide databases (14) was found upstream of the *bla*_{KPC} gene (Fig. 4) in *K. pneumoniae* YC and *K. pneumoniae* GR, from Paris, France, and Greece, respectively. Sequencing of the entire Tn4401 revealed very high degrees of nucleotide sequence identity (99.9%) and confirmed the presence of a 100-bp deletion.

Tn4401 insertion sites. In order to investigate the flanking sequence of Tn4401, PCR primers that were specific for a location within Tn4401 and in the flanking sequence and derived from *K. pneumoniae* YC were used. PCR products of the expected size on the basis of the sizes of the fragments from *K. pneumoniae* YC from Paris were obtained only from *K. pneumoniae* isolate GR from Greece, thus indicating that the genetic backgrounds of the other strains might be different.

The natural plasmids were extracted from the transconjugants and/or from the electroporants and were directly sequenced by using outwards-directed primers specific for locations next to the IRs of Tn4401. Except for Tn4401, found on plasmid pNGR, the genetic environment was different. Thus, Tn4401, found on plasmid pBC2303, was inserted into an ORF of 345 bp encoding a 114-amino-acid putative protein of unknown function. Upon insertion, an ATTAC target site was duplicated (Fig. 3). This ORF belongs to the left end side of Tn5708, a Tn3-based transposon (GenBank accession no. AJ010745). The IRL of Tn5708 was found immediately upstream of the Tn4401 IRL. On the other side of Tn4401, this ORF was itself interrupted by a miniature IR transposable element sequence, which contained two 39-bp IR sequences

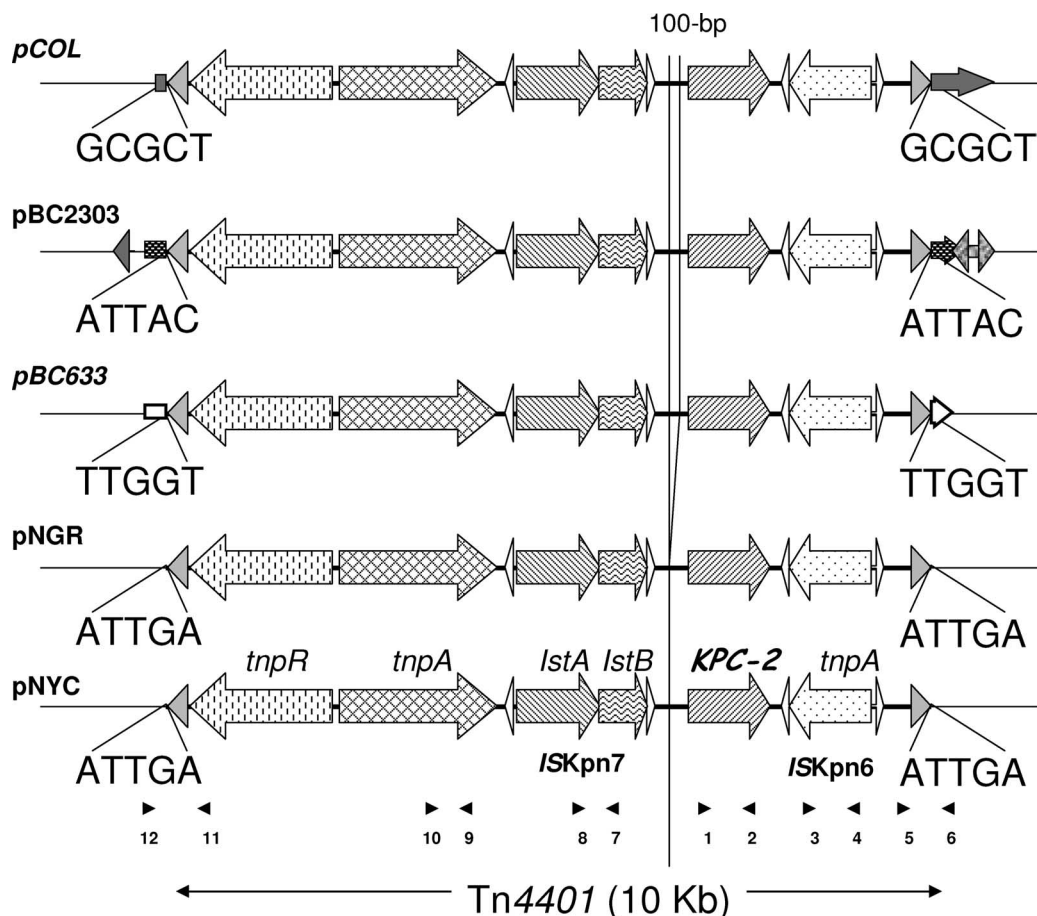


FIG. 3. Schematic representation of Tn4401 structures identified on naturally occurring plasmids pCOL from *P. aeruginosa* 2404 (29), pBC2303 from *K. pneumoniae* (28), pBC633 from *K. pneumoniae* (28), pNGR from *K. pneumoniae* (6), and pNYC-1 from *K. pneumoniae* (16). Genes and their corresponding transcription orientations are indicated by horizontal arrows. Tn4401 is delimited by two IR sequences (grey triangles). Small and empty triangles represent the IRs of ISKpn6 and ISKpn7. Tn4401 target site duplications are indicated. The location of a 100-bp deletion in pNGR and pNYC-1 is indicated by vertical lines. The disrupted ORFs resulting from the Tn4401 insertion are indicated. In the case of pBC2303, Tn4401 inserted into an ORF that is located at the left end of another transposon. This ORF was also disrupted by a 220-bp miniature IR element. Small arrowheads with numbers indicate the primers listed in Table 1 and used for PCR mapping.

separated by 180 bp (Fig. 3). On plasmid pBC633, the insertion occurred in an ORF of 291 nucleotides coding for a 97-amino-acid putative membrane protein with 75% identity at the nucleotide sequence level and 81% identity at the amino acid sequence level with the protein of *Erwinia carotovora* subsp. *atroseptica* SCRI1043 (GenBank accession no. NC_004547). Upon insertion, a 5-bp TTGGT TSD was generated. On plasmid pCOL, the insertion occurred in an ORF of 297 bp, a 99-

amino-acid hypothetical protein found on plasmid pRSB105, a plasmid of 57,137 bp found in an uncultured bacterium from a sewage plant in Germany. Upon insertion, Tn4401 generated a 5-bp GCGCT TSD.

DISCUSSION

Previous studies on the genetic environment of *bla*_{KPC} have identified several ORFs encoding putative transposases located upstream and downstream of the *bla*_{KPC} genes. In the present work, we have further characterized the genetic environment of the *bla*_{KPC} gene by detailed analysis of a 22-kb insert derived from the natural plasmid pNYC-1 containing the *bla*_{KPC} gene from *K. pneumoniae* isolate YC from Paris but with a U.S. origin (16) and by analysis of *bla*_{KPC}-containing natural plasmids from isolates from Greece (6) and Colombia (28, 29) and *bla*_{KPC}-containing sequences available in the GenBank database. We were able to identify a novel Tn3-based transposon, Tn4401, which is at the origin of *bla*_{KPC}-like gene acquisition and dissemination. In addition to the *tnpA*

A

IRL	GGGGTTCTAAGCGGGAATCCAGAAAATCCGTCATTCC
IRR	GGGGGGGTAAAGCGGGAACCCAGAAAATCCGCCATTCC
	**** *

B

IRL	GGGGTTCTAAGCGGGAATCCAGAAAATCCGTCATTCC
IRR1	GAGGG. .TACGCCGGAACCGCTGAAAATCCGTCATTCC
	* *

FIG. 4. Alignment of the 39-bp Tn4401 IRs. (A) IRL and IRR; (B) IRL with a reconstructed IRR1. The underlined nucleotides correspond to the nucleotides duplicated after ISKpn7 insertion. Identical positions are indicated by asterisks.

transposase, Tn4401 possesses the resolvase *tnpR*, the *bla*_{KPC} gene, and two ISs, ISKpn6 and ISKpn7. These ISs must have inserted into the parental transposon, since both ISs are flanked by target site duplications, signaling a recent transposition event of each IS that occurred independently. Thus, *bla*_{KPC} is likely not part of a composite transposon made of two different ISs, as shown for the *bla*_{PER-1} gene (23). In the case of the *bla*_{PER-1} gene, it is located on a composite transposon, Tn1213, bracketed by two different ISs, ISPa12 and ISPa13 (23).

The identification of this transposon, which was inserted at different loci on different plasmids and which was flanked by different 5-bp target site duplications, indicated a frequent and dynamic process. Tn4401 was present in all the strains tested. Similarly, parts of this transposon have been identified in every sequence of *bla*_{KPC}-like genes submitted to the GenBank database. The overall structure of Tn4401 seemed to be conserved except in *K. pneumoniae* GR and *K. pneumoniae* YC, from Greece and Paris, respectively, for which a 100-bp deletion was observed upstream of the *bla*_{KPC} gene compared to the sequence found in *K. pneumoniae* KN2303 and *P. aeruginosa* 2404 from Colombia. Thus, we have characterized two isoforms of Tn4401 that differ by 100 bp and that are currently spreading in different geographical locations. The 100 bp, which is absent from the Tn4401 transposon found in *K. pneumoniae* GR and *K. pneumoniae* YC, are present in most of the *bla*_{KPC}-containing sequences released to the GenBank database. However, in one sequence recently released to the GenBank database, a 200-bp deletion has been described at the same genetic location (GenBank accession no. DQ989640), suggesting that this region might be highly polymorphic or genetically unstable. Another description of the genetic environment of the *bla*_{KPC} gene on plasmid pYW in a Chinese *K. pneumoniae* isolate (30) revealed the presence of another IS 50 bp upstream of *bla*_{KPC-2}. From the available sequence released to the GenBank database, it was not possible to test whether this IS had inserted into Tn4401 structures or whether the overall sequence located upstream is different. Nevertheless, the sequence located downstream of the *bla*_{KPC} gene perfectly matched that of Tn4401. ISs may play important roles in the evolution of the Tn4401 backbone, as reported, for example, for *vanA*-containing transposon Tn1546 (31).

The ISKpn6 and ISKpn7 ISs have likely contributed to the genesis of Tn4401. In fact, the genesis of this transposon might be responsible for mobilization of the *bla*_{KPC} gene, as illustrated in Fig. 5. Detailed analysis of the sequences located on both sides of the ISKpn7 insertion revealed the presence a second 39-bp IR (termed IRR1) that has been interrupted by the ISKpn7 insertion (Fig. 1). The sequence of IRR1 is 80% identical to that of IRL, and the sequences of IRL and IRR are also 80% identical (Fig. 4). Thus, we postulate that a transposon, made of *tnpA* and *tnpR* might have been inserted upstream of the *bla*_{KPC} gene. Subsequently, ISKpn6 and ISKpn7 have inserted downstream and upstream of the *bla*_{KPC} gene, respectively. The ISKpn7 insertion led to the disruption of the IRR (IRR1) of the transposon, thus forcing the transposase to recognize a second right inverted repeat (IRR) located farther downstream of the *bla*_{KPC} gene. The novel transposon formed may be able to move the *bla*_{KPC} gene from its initial position to various plasmid locations. A similar strategy has been demon-

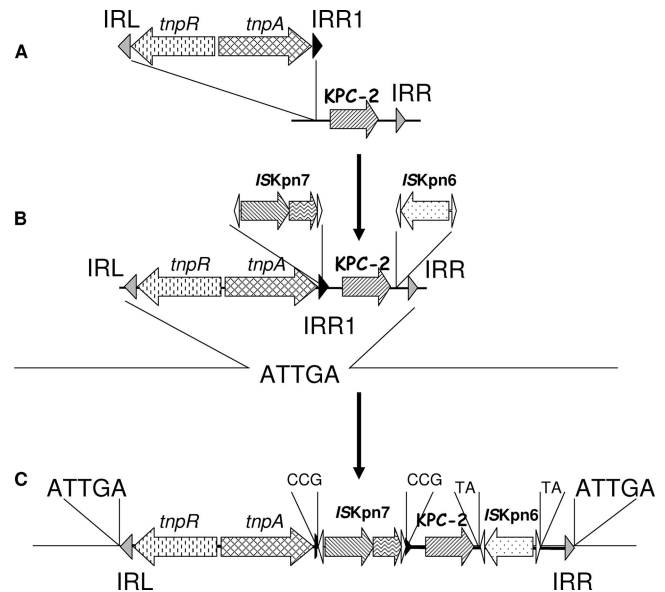


FIG. 5. Genesis of Tn4401 and origin of *bla*_{KPC} mobilization. Three steps might have been necessary for the genesis of Tn4401. (A) Insertion of a Tn3-based transposon delimited by IRL and IRR1, upstream of *bla*_{KPC}; (B) insertion of ISKpn6 and ISKpn7, which disrupted IRR1; (C) another IRR located just downstream of *bla*_{KPC} and IRL are recognized by the transposase, leading to the excision of Tn4401, which can then insert into a novel target sequence.

strated for ISEcp1 and *bla*_{CTX-M} gene mobilization (12). Further experiments will be necessary to validate this model.

In clinical isolates of the family *Enterobacteriaceae*, as well as in *E. coli* transconjugants and transformants, the presence of KPC does not always result in frank resistance to carbapenems in vitro. Instead, the MICs, even though they are high, may still remain in the susceptibility range. For *P. aeruginosa*, the situation is quite different, even in AmpC-deficient strains. Indeed, once the *bla*_{KPC} gene transferred into reference strain *P. aeruginosa* KG2505, from which AmpC is deleted, it conferred high levels of resistance to most β -lactam antibiotics. Plasmids from *K. pneumoniae* could not be transferred to *P. aeruginosa*, suggesting that horizontal transfer between these species is not so easy. However, given its transposition properties, it is likely that Tn4401 might be found on broad-host-range plasmids that could easily be transferred to *P. aeruginosa* or even *Acinetobacter baumannii*.

In conclusion, our analysis of several *K. pneumoniae* and *P. aeruginosa* isolates of different geographical origins revealed an identical genetic structure, Tn4401, which sustained the acquisition of *bla*_{KPC}, which could likely be at the origin of the worldwide spread of this emerging resistance gene.

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