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Partial Excision of bla_{KPC} from Tn*4401* in Carbapenem-Resistant *Klebsiella pneumoniae*

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We describe a novel Tn*4401* variant (Tn*4401d*) in epidemic *Klebsiella pneumoniae* clone ST258, from which a partial bla_{KPC} fragment has been excised along with IS*Kpn7* and a partial *tnpA* fragment. Nested-PCR experiments confirmed that this region can be removed from distinct Tn*4401* isoforms in both *K. pneumoniae* and *Escherichia coli*. This study highlights that the region surrounding bla_{KPC} is undergoing recombination and that Tn*4401* itself is heterogeneous and highly plastic.

Klebsiella pneumoniae carbapenemase (KPC) is a molecular class A serine β-lactamase belonging to functional group 2f, which hydrolyzes β-lactams of several different classes, including carbapenems (1). The enzyme is encoded by the bla_{KPC} gene, which maps to a transposon (Tn*4401*) that has been reported in a variety of transferable plasmids (10, 17). Tn*4401* is approximately 10 kb in size, is delimited by two 39-bp imperfect inverted repeat sequences, and harbors insertion sequences IS*Kpn6* and IS*Kpn7* in addition to transposase and resolvase genes (Fig. 1) (5). Three isoforms (a, b, and c) of Tn*4401* have been described, differing by a 100- to 200-bp sequence upstream of bla_{KPC} (6, 17), while isoforms with 68-bp (13), 215-bp (GenBank accession no. DQ989640), and 255-bp (14) deletions were recently reported. Several descriptions of heterogeneous genetic environments have been reported for bla_{KPC}, including the presence of other insertion sequences upstream of the bla_{KPC} gene (7, 19, 20), suggesting that the region upstream of bla_{KPC} is variable (5). Here we describe a novel truncated Tn*4401* structure, tentatively named Tn*4401d*, with a 5.3-kb deletion encompassing more than half of the bla_{KPC} gene, along with IS*Kpn7* and a partial *tnpA* gene fragment located upstream of Tn*4401* (Fig. 1).

K. pneumoniae isolate BK32529 was recovered from a urine culture from a patient with a urinary tract infection in a New York City hospital. Multilocus sequence typing (MLST) confirmed that the isolate belonged to the epidemic *K. pneumoniae* ST258 clone (9). Species identification and antibiotic susceptibility were initially determined using a Vitek-2 instrument (bioMérieux, Lyon, France). The isolate exhibited resistance to amikacin, aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, gentamicin, levofloxacin, piperacillin, tigecycline, tobramycin, and trimethoprim-sulfamethoxazole but was susceptible to imipenem and meropenem. Additional susceptibility testing by broth microdilution (4) demonstrated that the isolate was resistant to ertapenem (MIC $>$ 4 mg/liter), meropenem (MIC = 2 mg/liter), and doripenem (MIC = 2 mg/liter) but not imipenem (MIC $\leq$ 1 mg/liter) (Table 1).

A previously described molecular-beacon-based multiplex real-time PCR assay (3) used in routine surveillance of suspected carbapenem-resistant *Enterobacteriaceae* displayed an anomalous result suggestive of a novel bla_{KPC} variant. Specifically, isolate BK32529 was positive only for the 716T target and negative for the other five single nucleotide polymorphisms (SNPs) associated with known bla_{KPC} variants (147A, 308T, 308C, 716C, and 814C). However, we were unable to amplify the entire bla_{KPC} region using previously published primers (16), suggesting the presence of a potential deletion or insertion within the bla_{KPC} gene. The entire Tn*4401* region was therefore amplified using long-range PCR with primers 4401-it-F1 (ACGTCGTGGCGATCGACGCA) and 4401-it-R2 (TTCCAGGTCCGCAATAGTTC), followed by DNA sequencing using primer walking. The results revealed the presence of a 5.3-kb deletion within Tn*4401*, from nucleotide (nt) 537 in *tnpA* to nt 553 in bla_{KPC}, thereby encompassing all of IS*Kpn7* (*istA* and *istB*), 80% of *tnpA*, and 60% of bla_{KPC} (Fig. 1). This truncated Tn*4401* structure has been tentatively named Tn*4401d* (Tn*4401* deletion). Further inspection of the deletion sites in Tn*4401* indicated that the deleted region is bracketed by two 9-bp imperfectly matched direct repeats (DRs) in *tnpA* (CGCCGAGCG) and bla_{KPC} (CGCCGCGCG), respectively (the mismatched nucleotides are underlined) (Fig. 1), suggesting that the deletion in Tn*4401d* may have arisen through DR-mediated slippage.

As shown in Fig. 1, only 40% of the bla_{KPC} gene is still present in Tn*4401d.* This finding calls into question the functionality of truncated bla_{KPC}, as the clinical isolate is still resistant to three carbapenems (ertapenem, meropenem, and doripenem) (Table 1). In order to transfer the Tn*4401*-harboring plasmid into a different genetic background, plasmid DNA was extracted from BK32529 using a Qiagen plasmid maxi kit (Qiagen, Valencia, CA), followed by electroporation into *Escherichia coli* DH10B using a Gene Pulser II instrument (Bio-Rad Laboratories). Potential transformants were selected on LB agar plates containing 100 μg/ml ampicillin and then screened by multiplex real-time PCR

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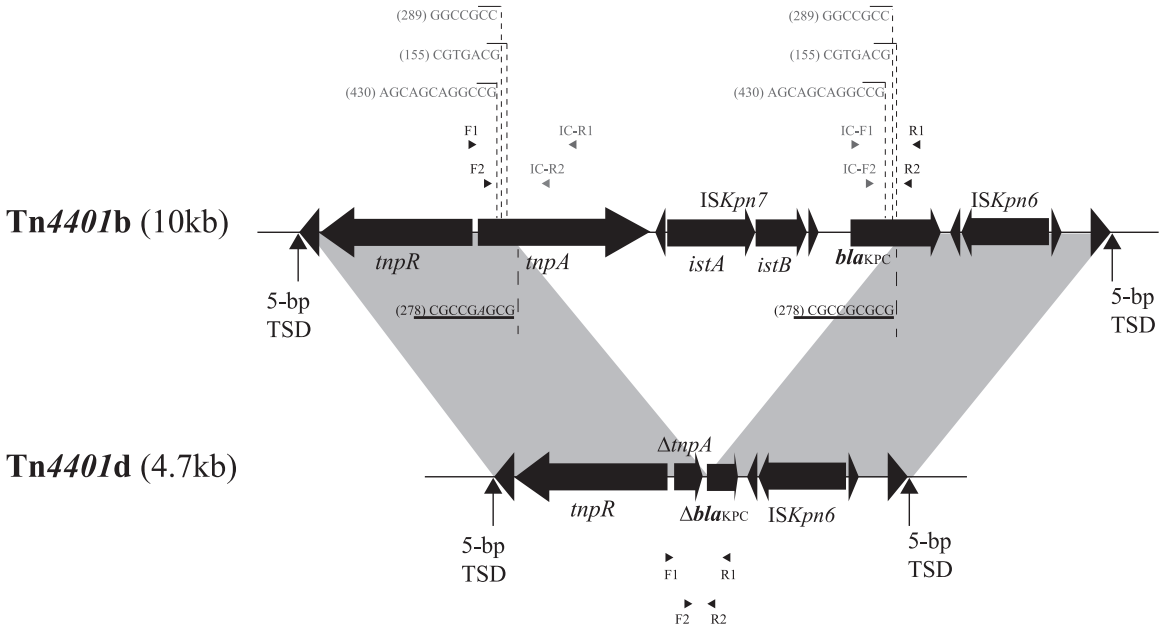

FIG 1 Structure of truncated Tn*4401* variant Tn*4401*d. Gray shading denotes regions of homology shared by Tn*4401*b and Tn*4401*d. Open reading frames (ORFs) are portrayed as large black arrows. Excision sites found in BK32529 are indicated by dotted lines beneath Tn*4401*b, with characteristic direct repeat (DR) sequences underlined. Imperfectly homologous sequences (with mismatches) within the two DRs are shown in italics. Numbers within parentheses denote the nested-PCR product sizes listed in Table 2. Additional potential excision sites determined by nested PCR are indicated by dotted lines above Tn*4401*b, with the corresponding direct repeat sequences shown in gray. Small black arrowheads represent the locations of primers used for nested PCR; small gray arrowheads indicate the locations of primers used for outward-directed nested PCR. Primers used for nested PCR were as follows: F1 (AATGCCCCATGTTTCTACGA), R1 (GGTCGTGTTTCCCTTTAGCC), F2 (TCACCAAGCATGAACGCTAC), and R2 (GCAGAGCCCAGTGTCAGTTT). Primers used for outward-directed nested PCR were as follows: IC-F1 (ATCGCCGTCTAGTTCTGCTG), IC-R1 (CAGCAGGTAGAGTTGGGTCTG), IC-F2 (CAGCTCATTCAAGGGCTTTC), and IC-R2 (CGTCGAGTTTAGGCAGCAGT). TSD, target site duplication.

for the presence of truncated bla_{KPC} genes (positive only for target 716T, as described above) (3). Plasmid size and copy number were 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. Transformants with single plasmids ~70 kb in size were then selected and subjected to susceptibility testing according to CLSI guidelines (4).

In contrast to the parental strain, the transformant was susceptible to all four carbapenems tested (MICs were ≤1 mg/liter for imipenem and meropenem and ≤0.25 and ≤0.12 mg/liter for ertapenem and doripenem, respectively) but resistant to aztreonam, ceftazidime, tobramycin, and trimethoprim-sulfamethoxazole, suggesting that the truncated bla_{KPC} enzyme is not functional. Further genotyping failed to identify additional carbapenem resistance determinants (bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{OXA-48}, etc.) or AmpC β-lactamase genes within the parental isolate (Table 1), while the modified Hodge test was negative for both the parental strain and the corresponding DH10B transformant (15). Porin genes *ompK35* and *ompK36* were then investigated using a PCR assay described elsewhere (12), followed by DNA sequencing. The results revealed an additional guanine (G) insertion at nt 121 within *ompK35*, resulting in a premature stop codon at amino acid position 89. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of outer membrane proteins (OMP) (12) from BK32529 confirmed the loss of OmpK35 (data not shown). Several nonsynonymous mutations were identified within the sequence of *ompK36*, but SDS-PAGE indicated that the full-length protein was still expressed (data not shown). The apparent loss of porin OmpK35, as well as the presence of other β-lactamases, such as TEM-1 and SHV-12 (Table 1), may contribute to the carbapenem resistance observed within strain BK32529, as suggested previously (11).

TABLE 1 Characteristics of *K. pneumoniae* strain BK32529 and the Tn*4401*d-harboring *E. coli* DH10 transformant

Isolate	InC type(s)[a]	β-Lactamases[b]	MIC (mg/liter)[c]																	
			IMP	MER	ERT	DOR	CAZ	CTX	CEF	ATM	AMI	GEN	TOB	TIC-CLAV	PIP-TAZ	CIP	LEV	SXT	TGC	COL	PLB
BK32529	A/C, FIIs	TEM-1, SHV-12	≤1	**2**	**>4**	**2**	**>16**	**>32**	**>16**	**>16**	**>32**	**>8**	**>8**	**>128/2**	**>64/4**	**>2**	**>8**	**>4/76**	1	**>4**	**>4**
DH10B transformant	A/C	TEM-1, SHV-12	≤1	≤1	≤0.25	≤0.12	**>16**	4	≤2	**>16**	**>32**	8	**>8**	64/2	<8/4	≤0.25	≤1	**>4/76**	0.5	0.5	0.5

[a] Plasmid incompatibility (InC) groups were determined using the multiplex-PCR method described by Carattoli et al. (2).

[b] bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{GES}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}, bla_{BIC}, bla_{SPM}, bla_{GIM}, bla_{AIM}, bla_{SIM}, and bla_{DIM}, as well as AmpC β-lactamases bla_{ACT-1}, bla_{ACC}, bla_{BIL-1}, bla_{CMY}, bla_{DHA}, bla_{FOX}, bla_{LAT}, bla_{MIR-1}, and bla_{MOX}, were identified by PCR using methods described elsewhere (8, 18).

[c] 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; AMI, amikacin; GEN, gentamicin; TOB, tobramycin; TIC-CLAV, ticarcillin-clavulanate; PIP-TAZ, piperacillin-tazobactam; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; COL, colistin; PLB, polymyxin B.

TABLE 2 bla_{KPC} excision patterns in additional Tn*4401*-bearing strains

Strain	Species	InC type(s)[a]	MLST[b]	KPC	Tn*4401* variant[c]	Size(s) of Tn*4401* deletion pattern(s) (bp)[d]
1	*K. pneumoniae*	N, FIIs	63	KPC-4	b	160, 278, 430
2	*K. pneumoniae*	Unk	76	KPC-3	a	155, 217
3	*K. pneumoniae*	FIIs	234	KPC-2	a	289
4	*K. pneumoniae*	FIIs	258	KPC-2	a	278, 430
5	*K. pneumoniae*	FIIs	258	KPC-3	b	186, 278, 430
6	*K. pneumoniae*	N, A/C	258	KPC-2	a	215, 278, 430
7	*K. pneumoniae*	Unk	258	KPC-2	a	191, 278, 430
8	*K. pneumoniae*	FIIs	258	KPC-3	b	278, 430
9	*K. pneumoniae*	FIIs	258	KPC-3	−68	255
10	*K. pneumoniae*	A/C	486	KPC-2	b	278, 430
11	*E. coli*	L1, N, Y, FIA, FIB, FIIs	2	KPC-2	b	278, 430
12	*E. coli*	L1, N, Y, FIA, FIB, FIIs	2	KPC-3	b	278, 430
13	*E. coli*	L1, FIA, FIB, FIIs	43	KPC-2	a	278, 430
14	*E. coli*	FIB, A/C, Frep	33	KPC-2	a	278, 430
15	*E. coli*	A/C	35	KPC-3	b	278

[a] Plasmid replicon typing was performed using a method previously described by Carattoli et al. (2). Unk, unknown.
[b] MLST for *K. pneumoniae* was performed using the method described by Diancourt et al. (9); MLST for *E. coli* was performed using the Institut Pasteur MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html).
[c] Tn*4401* variants were identified by PCR (6), followed by DNA sequencing. "−68" indicates the previously described variant with a 68-bp deletion upstream of bla_{KPC} (13).
[d] Tn*4401* deletion patterns were identified by nested PCR and are expressed as the PCR product length resulting from amplification with primer set F2/R2.

In order to investigate whether the deleted region (from *tnpA* to bla_{KPC}) could be excised from Tn*4401*, nested PCR was performed using primer sets F1/R1 and F2/R2 as outer and inner primers, respectively (Fig. 1). In addition, an outward-directed nested PCR was designed to identify the extrachromosomal circular intermediate (CI) structure resulting from excision of Tn*4401*, using primer sets IC-F1/R1 and IC-F2/R2 as outer and inner primers, respectively.

For nested-PCR amplification, the initial PCR products from primers F1/R1 and IC-F1/R1 were diluted 1:1,000 with ultrapure water and then subjected to a second round of amplification with primers F2/R2 and IC-F2/R2. Plasmids from 10 *K. pneumoniae* and five *E. coli* isolates with different genotypic backgrounds and harboring different Tn*4401* and bla_{KPC} variants were subjected to nested and outward-directed PCR (Table 2). Several deletion structures, in addition to the deletion pattern found in strain BK32529 (corresponding to a 278-bp PCR product generated by primer set F2/R2), were identified by nested PCR (Table 2). Meanwhile, outward-directed nested PCR confirmed the existence of circular intermediates among these structures. Further sequence analysis revealed several potential spontaneous excision sites, including several DRs within *tnpA* and bla_{KPC}, three of which are depicted in Fig. 1. However, no DRs were identified in some of the novel deletion structures (corresponding to 191-, 215-, 217-, and 255-bp PCR products generated by primer set F2/R2) (Table 2), indicating that there may be other deletion mechanisms besides DR-mediated slippage. No apparent associations were observed between the Tn*4401* excision pattern and host species, MLST genotype, Tn*4401* isoform, or bla_{KPC} variant (Table 2).

Taken together, these results demonstrate that bla_{KPC} can be partially excised from Tn*4401*, thereby causing a loss of carbapenemase activity. The observation of different excision patterns highlights the notion that the region surrounding bla_{KPC} is undergoing recombination. Further study of the mechanisms underlying excision may therefore be helpful in controlling the spread of bla_{KPC} in *K. pneumoniae* and other *Enterobacteriaceae*.

Nucleotide sequence accession number. The nucleotide sequence of Tn*4401*d from *K. pneumoniae* strain BK32529 was deposited in GenBank as accession no. JN974188.

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