Functional Characterization of Tn*4401*, a Tn*3*-Based Transposon Involved in *bla*$_{KPC}$ Gene Mobilization[▽]

Gaelle Cuzon, Thierry Naas,* and Patrice Nordmann

*Service de Bactériologie-Virologie, INSERM U914: Emerging Resistance to Antibiotics, Hôpital de Bicêtre,
Assistance Publique-Hôpitaux de Paris, 94275 Le Kremlin-Bicêtre,
Faculté de Médecine, Université Paris-Sud, France*

The carbapenemase gene *bla*$_{KPC}$, which is rapidly spreading worldwide, is located on a Tn*3*-based transposon, Tn*4401*. In a transposition-conjugation assay, Tn*4401* was able to mobilize *bla*$_{KPC-2}$ gene at a frequency of 4.4×10^{-6}/recipient cell. A 5-bp target site duplication was evidenced upon each insertion without target site specificity. This study demonstrated that Tn*4401* is an active transposon capable of mobilizing *bla*$_{KPC}$ genes at high frequency.

By far, the most frequent class A carbapenemases are the KPC enzymes (*Klebsiella pneumoniae* carbapenemases), with the KPC-2 variant being most common (15). These carbapenemases were identified first in 2001 in North Carolina (26), and until 2005, the geographical distribution of these enzymes in the *Enterobacteriaceae*, including *Klebsiella pneumoniae*, was limited to the eastern part of the United States (3, 26). KPC producers are now reported to be found worldwide, mainly in the *Enterobacteriaceae* but also in *Pseudomonas aeruginosa* (24) and *Acinetobacter* sp. (20). Possible explanations for this rapid dissemination are that *bla*$_{KPC}$ genes are present in an epidemic *K. pneumoniae* strain ST258 (4) and also that they are present on a wide variety of plasmids that vary in size, nature, and structure (4, 6, 11). In most cases, these plasmids are self-transferable at least to *Escherichia coli*. Studies of the genetic structure surrounding *bla*$_{KPC-2}$ genes have identified a Tn*3*-based transposon, Tn*4401* (13). Tn*4401* is 10 kb, is delimited by two 39-bp imperfect inverted repeat sequences, and harbors transposase and resolvase genes and two insertion sequences, IS*Kpn6* and IS*Kpn7*, in addition to *bla*$_{KPC-2}$ (Fig. 1). Three isoforms of Tn*4401*, differing by a 100- to 200-bp sequence upstream of *bla*$_{KPC-2}$, are currently known (isoforms a, b, and c). A few descriptions of different genetic environments of *bla*$_{KPC}$ genes have been published, reporting the presence of other insertion sequences upstream of the *bla*$_{KPC}$ gene (21, 25) but with downstream sequences similar to those of Tn*4401*, suggesting that these ISs have been inserted in Tn*4401*. Here, we investigated the functional role of Tn*4401* in mobilization and diffusion of the *bla*$_{KPC-2}$ gene.

Transposition experiments were performed with the natural nonconjugative plasmid pBC633 (13), which was electroporated into the *recA Escherichia coli* strain RZ211 harboring the plasmid pOX38-Gen for transposition experiments (2, 9). A 24-h broth was used as the donor for subsequent mating-out assays with the azide-resistant *E. coli* strain J53 as the recipient, as previously described (2, 9, 13). pOX38 transconjugants harboring Tn*4401* insertions (pOX38-Gen::Tn*4401*) were selected on ticarcillin (Tic) (100 µg/ml) plus azide (Az) (100 µg/ml) as Tic^r Gen^r Az^r colonies. The plasmid content of *E. coli* J53(pOX38-Gen::Tn*4401*) was investigated by the Kieser extraction method (2, 10). The presence of the *bla*$_{KPC}$ gene in transconjugants was confirmed by PCR, and the location of the *bla*$_{KPC}$ gene was determined by Southern hybridization with a specific and internal probe (13). Transposition experiments with the natural plasmid pBC633 showed that, along with pOX38-Gen, pBC633 was also transferred to *E. coli* J53 Az^r in mating-out assays, suggesting comobilization of pBC633 by pOX38-Gen (Fig. 2A). The presence of the two plasmids in the same transconjugants made further analysis of transposition events difficult. Nevertheless, true transposition events were also evidenced; a unique plasmid (pOX38-Gen::Tn*4401*) from two transconjugants could be extracted, and direct sequencing revealed insertion of Tn*4401* in pOX38-Gen (Fig. 2A and 3B).

In order to address whether the comobilization of pBC633, along with pOX38-Gen, was due to comobilization or cointegration events followed by subsequent resolution in the recipient strain, plasmid (pACYC184), which is known for not being comobilizable and which carries a chloramphenicol (Cm) resistance gene, was used to obtain a recombinant plasmid, pTn*4401*. The latter was constructed by inserting an 11.4-kb PsiI-HpaI fragment of pBC633 containing Tn*4401* into an EcoRV-restricted pACYC184 vector. Recombinant plasmid pTn*4401* was electroporated into *E. coli* DH10B, and transformants expressing reduced susceptibility to imipenem and resistance to chloramphenicol were retained for further analysis. Transposition experiments were performed as described for pBC633. Transconjugants

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* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre cedex, France. Phone: 33-1-45-21-29-86. Fax: 33-1-45-21-63-40. E-mail: thierry.naas@bct.aphp.fr.

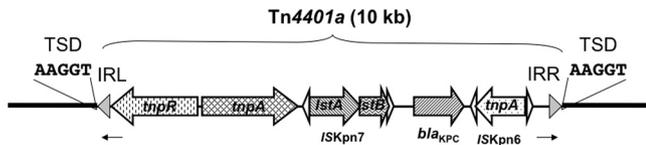▽ Published ahead of print on 15 August 2011.

FIG. 1. Schematic representation of Tn4401a inserted into pOX38-Gen (insertion I1 in Fig. 3). Genes and their corresponding transcription orientations are indicated by horizontal arrows. Tn4401a (isoform a [100-bp deletion as described in reference 13]) is delimited by two inverted repeat sequences, IRR and IRL (grey triangles). Small open triangles represent the inverted repeats of ISKpn6 and ISKpn7. Small arrows below the sequence represent primers used for direct sequencing of the insertion sites of Tn4401. The thick line corresponds to the pOX38-GEN DNA sequence.

were additionally screened for resistance to chloramphenicol. Screening for chloramphenicol resistance together with plasmid extraction revealed that in up to 20% of cases, pTn4401 was also transferred to *E. coli* J53 Az^r, resulting in Tic^r Gen^r Az^r Cm^r colonies (Table 1). Transfer of both plasmids, pTn4401 and pOX38-Gen, suggests cointegration events that occur during replicative transposition, the transposition mechanism used by Tn3-like transposons (23). To detect true transposition events, Cm-susceptible Tic^r Gen^r Az^r colonies were searched for (Table 1). Analysis of their plasmid content revealed a unique plasmid with a size compatible with the presence of pOX38-Gen::Tn4401 (Fig. 2B). In order to study more putative transposants and their target site insertion, *E. coli* J53 transconjugants harboring pTn4401 and exhibiting a band corresponding in size to insertion of pTn4401 in pOX38-Gen were used for a second mating-out experiment to transfer pOX38-Gen::Tn4401 from

E. coli J53 to *E. coli* DH10B Str^r (Fig. 2B). Transconjugants were successfully obtained, and pOX38-Gen::Tn4401 transposants were further characterized. Transposition experiments were then performed in triplicate. Results of screening for Cm resistance and calculated frequencies of transposition events are indicated in Table 1. Overall, Tn4401 was capable of true transposition events (80% of events), in addition to cointegration (20% of events), which could subsequently be resolved in the recipient cells. Transposition of Tn4401 and conjugation of pOX38-Gen::Tn4401 occurred at high frequency (4.4×10^{-6}/recipient cell) (Table 1). Given that the conjugation frequency of pOX38-Gen ranged from 10^{-2} to 10^{-3}/recipient cell, the true transposition frequency is around 10^{-3} to 10^{-4}/recipient plasmid.

To determine whether Tn4401 had a target site preference, several pOX38-Gen::Tn4401 plasmids isolated from independent experiments were extracted and analyzed by direct sequencing of the ends of Tn4401 using laboratory-designed primers (13). As previously shown with natural plasmids (4, 13), Tn4401 insertions occurred at different sites (Fig. 3C). Alignment of these insertion site sequences did not reveal conserved motifs. Nevertheless, a 5-bp target site duplication, consistent with a transposition event, was observed in all the pOX38-Gen^r::Tn4401 plasmids studied (Fig. 3B and C).

Analysis of the genetic elements associated with resistance genes give information about their means of acquisition and diffusion. In Gram-negative rods, many ß-lactamase genes have been described as gene cassettes inserted into class 1 integrons, such as bla_{VEB} (14), bla_{VIM} (7), bla_{CARB-9} (16), and bla_{GES} (5), or surrounded by insertion sequences forming composite transposons, such as bla_{OXA-48}
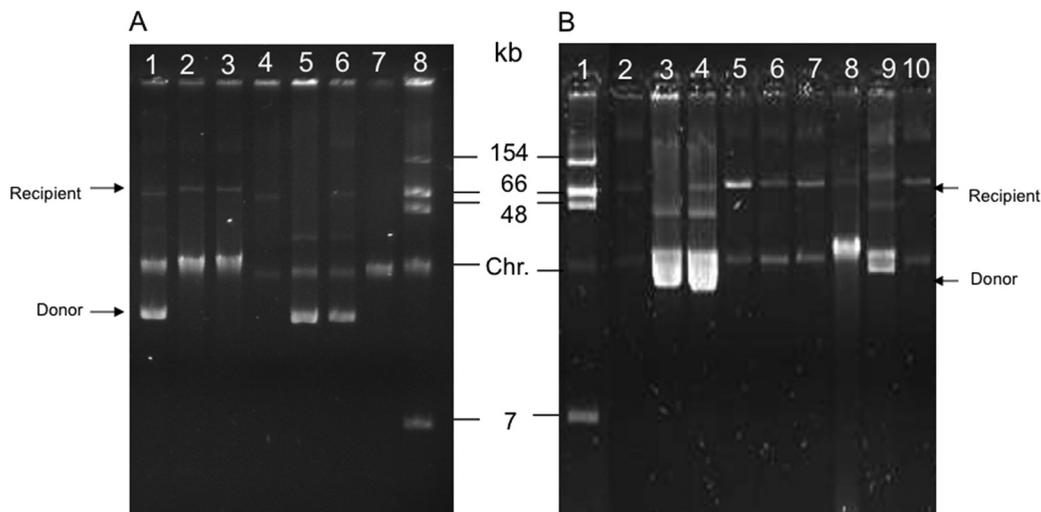

FIG. 2. Plasmid extraction by the Kieser method. (A) transposition experiments performed with the natural plasmid pBC633. Lanes 1 to 3, transconjugants in *E. coli* J53 Az^r after a transposition assay. *E. coli* J53 transconjugant 1 (lane 1) possesses both pOX38Gen and pBC633, due to cointegration events, whereas *E. coli* J53 transconjugants 2 and 3 (lanes 2 and 3) harbor only pOX38Gen::Tn4401, due to true transposition events. Lane 4, *E. coli* RZ211 harboring pOX38Gen; lane 5, *K. pneumoniae* KPN633 harboring the natural plasmid pBC633 (11); lane 6, *E. coli* RZ211 harboring both pOX38Gen and pBC633; lane 7, *E. coli* J53 Az^r (only the chromosomal band [Chr] is visible); lane 8, *E. coli* 50192 harboring four plasmids (7, 48, 66, and 154 kb). (B) Transposition experiments performed with the recombinant plasmid pTn4401. Lane 1, *E. coli* 50192 harboring four plasmids (7, 48, 66, and 154 kb); lane 2, *E. coli* RZ211 harboring pOX38Gen; lane 3, *E. coli* DH10B harboring pTn4401; lane 4, *E. coli* J53 transconjugant possessing both plasmids pOX38Gen and pTn4401 due to a cointegration event; lanes 5 to 8, *E. coli* J53 transconjugants harboring pOX38Gen::Tn4401, selected as Cm^s colonies; lane 9, *E. coli* RZ211 harboring both pOX38Gen and pTn4401 (Cm^r); lane 10, subsequent transconjugant from strain 9 to *E. coli* DH10B Str^r.
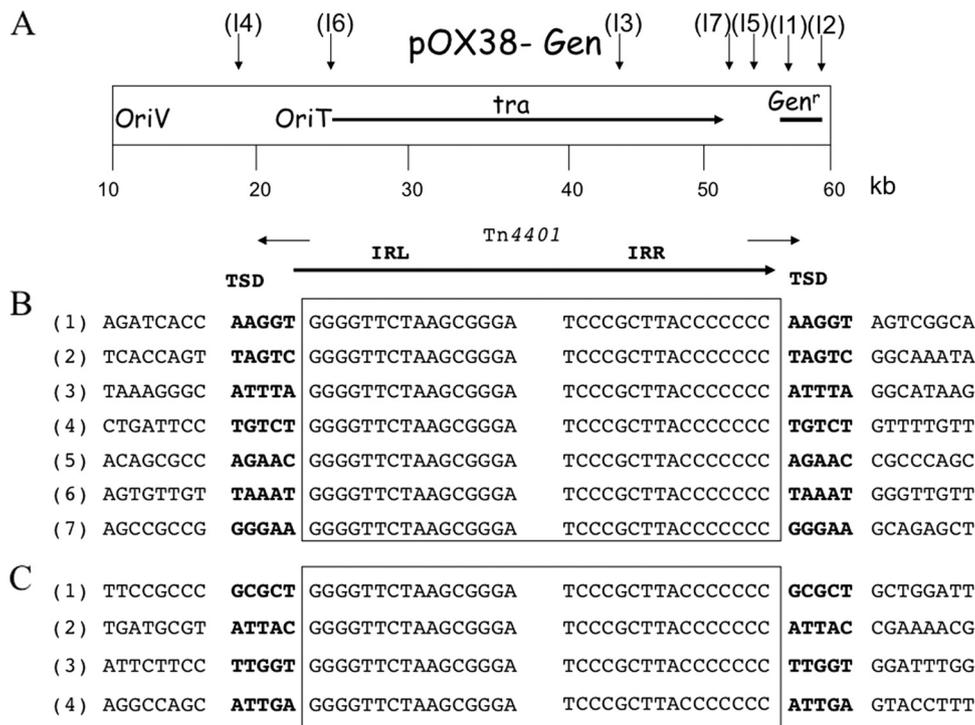
FIG. 3. Target sites of Tn*4401* insertions. (A) Map positions of Tn*4401* insertions in plasmid pOX38-Gen. Insertions of the tagged transposon (I1 to I7) are indicated by vertical arrows. The origin of replication (*oriV*), the origin of transfer (*oriT*), the *tra* genes required for plasmid transfer, and the gentamicin resistance gene (Gen^r) are indicated. (B and C) Nucleotide sequence alignments of Tn*4401* in pOX38-Gen. (B) Seven transconjugants were analyzed from a transposition assay with pBC633 or pTn*4401*. (C) Tn*4401* insertions in natural plasmids. 1, pCOL from *P. aeruginosa* 2404 (13); 2, pBC2303 from *K. pneumoniae* KPN2303 (13); 3, pBC633 from *K. pneumoniae* KPN633 (13); 4, pYC from *K. pneumoniae* YC (13). Nucleotide sequences of the end regions of Tn*4401* are boxed. Target site sequences duplicated after transposition are indicated by boldface letters. The small arrows above the schematic representation of Tn*4401* represent primers used for direct sequencing of pOX38Gen::Tn*4401* transposants.

(2) and bla_{PER} (17). Tn*3*-based transposons have been reported to be associated with bla_{TEM} genes (19), which encode the most widespread ß-lactamase in the *Enterobacteriaceae*. Since the first description of an *E. coli* isolate resistant to aminopenicillins due to the production of TEM-1 ß-lactamase in the 1960s, a rapid increase in the prevalence of TEM-1-producing isolates has occurred. The corresponding *bla* gene was carried by Tn*3* elements associated with plasmids of different incompatibility groups, which subsequently were responsible for the rapid spread of TEM variants. Moreover, Tn*3* was shown to efficiently transpose the bla_{TEM} ampicillin resistance gene marker (8, 22).

Similarly, Tn*4401* is a Tn*3*-like transposon that is associated with bla_{KPC} genes (13). This transposon was identified in isolates from different geographical origins, and of different sequence types (ST), in *Enterobacteriaceae* (1, 4, 11, 12, 13) and in *P. aeruginosa* (13, 18). In all cases, Tn*4401* was inserted at different loci and on plasmids varying in size and incompatibility group (1, 4, 13). We have demonstrated here that Tn*4401* is capable of transposition with 5-bp target site duplication and without target site specificity. This work confirms that KPC genes benefit from all the genetic tools available—active transposons, self-transferable plasmids, and efficient ST types—for their rapid spread to various bacterial species.

TABLE 1. Results of the three independent transposition experiments performed with the recombinant plasmid pTn*4401*

Expt	Frequency (%) of event/recipient cell[a]						
	Tc^R Cm^s	Tc^R Cm^r	Total for Tc^R	Recipient strain J53Az^r	Cointegration events	Transposition events	Total
1	9×10^1 (82)	2×10^1 (18)	1.1×10^2	392×10^5	5×10^{-7}	2.3×10^{-6}	2.8×10^{-6}
2	8×10^1 (80)	2×10^1 (20)	1.6×10^2	324×10^5	9.9×10^{-7}	3.9×10^{-6}	4.9×10^{-6}
3	1×10^2 (91)	1×10^1 (9)	1.1×10^2	350×10^5	4.9×10^{-7}	4.9×10^{-6}	5.4×10^{-6}
Avg ± SD					$6.6 \times 10^{-7} \pm 2.8 \times 10^{-7}$	$3.7 \times 10^{-6} \pm 1.3 \times 10^{-6}$	$4.4 \times 10^{-6} \pm 1.4 \times 10^{-6}$

[a] Tc^R, transconjugant; Tc^R Cm^s, transconjugants in *E. coli* J53 Az^r Tic^r Gen^r Az^r Cm^s; Tc^R Cm^r, transconjugants in *E. coli* J53 Az^r Tic^r Gen^r Az^r Cm^r.

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