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Double Copies of *bla*$_{KPC-3}$::Tn*4401a* on an IncX3 Plasmid in *Klebsiella pneumoniae* Successful Clone ST512 from Italy

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A carbapenem-resistant sequence type 512 (ST512) *Klebsiella pneumoniae* carbapenemase 3 (KPC-3)-producing *K. pneumoniae* strain showing a novel variant plasmid content was isolated in Palermo, Italy, in 2014. ST512 is a worldwide successful clone associated with the spread of *bla*$_{KPC}$ genes located on the IncFIIk pKpQIL plasmid. In our ST512 strain, the *bla*$_{KPC-3}$ gene was unusually located on an IncX3 plasmid, whose complete sequence was determined. Two copies of *bla*$_{KPC-3}$::Tn*4401a* caused by intramolecular transposition events were detected in the plasmid.

Extensively drug-resistant (XDR) and pandrug-resistant *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* strains of the hyperepidemic clonal complex 258 (CC258) are detected worldwide as hospital-acquired pathogens and are frequently responsible for outbreaks. In particular, sequence type 258 (ST258), ST512, ST11, and ST340 are the most frequently detected variants of KPC-producing *K. pneumoniae* isolates (1, 2).

During May and June 2011, a countrywide Italian survey focusing on the diffusion of carbapenem-nonsusceptible *K. pneumoniae* isolates showed that the most frequent lineages belonged to CC258 (ST258 or ST512) (3). The epidemiology of KPC-producing *K. pneumoniae* in Palermo, Italy, also confirmed the emergence of ST258 beginning in 2008 (4). More recently, a 6-month surveillance performed in Sicily suggested that a major epidemiological change is likely ongoing in this geographic area, with ST258 still being prevalent but circulating along with several additional STs, including ST307 and ST273 (4). In particular, only one isolate of ST512 (i.e., *K. pneumoniae* strain 45) was identified on a total of 94 KPC-producing *K. pneumoniae* strains (4). This unique isolate of ST512 was further investigated and described in this study.

As shown in Table 1, the ST512 *K. pneumoniae* 45 strain showed an XDR phenotype (4). It was screened by PCR for the following plasmid-mediated quinolone resistance and β-lactamase genes: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *qepA*, *oqxAB*, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{OXA}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{LAP}$, *bla*$_{CTX-M}$, and *bla*$_{CMY}$ (5–8). Positive amplicons underwent Sanger DNA sequencing to identify the variant genes. *K. pneumoniae* 45 was positive for the *bla*$_{KPC-3}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *aac(6')-Ib-cr*, *bla*$_{SHV-11}$, and *oqxAB* genes. The implementation of the PCR-based replicon typing (PBRT) kit (Diatheva) indicated that plasmids carried by strain 45 were not typeable. Therefore, the *bla*$_{KPC-3}$-carrying plasmid was transformed in *Escherichia coli* DH5α competent cells (Invitrogen) and selected on Luria-Bertani agar plates (Sigma) containing ampicillin (50 μg/ml). The transformants were then screened by PCR for the presence of *bla*$_{KPC}$ genes. Plasmid DNA of one representative *bla*$_{KPC-3}$-positive transformant (named p45) was purified using an Invitrogen plasmid midi kit (Invitrogen) and fully sequenced. A shotgun library was obtained, and sequencing was performed with the 454-GS Junior platform, according to the standard sequencing procedure (Roche Diagnostics). Plasmid coverage was >80×. The reads were aligned and assembled using the Newbler assembler software version 2.0.01.14 (Roche Diagnostics). We found that plasmid p45 was split into three contigs, and the complete sequence was reconstructed by a PCR-based gap closure method. Open reading frames (ORFs) were predicted and annotated using the Artemis software (Wellcome Trust Sanger Institute, United Kingdom). Each predicted protein was compared against the protein database using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene sequences were compared and aligned with GenBank data using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The IncX3 plasmids pKpS90 and pIncX-SHV (GenBank accession no. JX461340 and JN247852, respectively) were used as references for annotation and comparative analyses.

As shown in Fig. 1, our results indicated that p45 is 63,203 bp in size and shows the typical IncX3 scaffold, including the replicase gene, *tax*, and *pilX* gene clusters (9). The *bla*$_{KPC-3}$ gene is located in the Tn*3*-like element Tn*4401a* (10). However, we observed that two copies of Tn*4401a* were present at a distance of 8,658 bp within the IncX3 plasmid scaffold of p45.

One copy of Tn*4401a* was integrated within an IS*3000*-a1 element, which was interrupted by the *bla*$_{KPC-3}$::Tn*4401a* insertion. The duplication of a 5-bp sequence was identified at the site of integration, immediately flanking the inverted repeat right (IRR) and inverted repeat left (IRL) of Tn*4401a*. The *bla*$_{SHV-11}$ gene was identified 4 kb from the *bla*$_{KPC-3}$::Tn*4401a* integration site, followed by an IS*26* element. The same structure carrying IS*26*-*bla*$_{SHV-11}$-IS*3000* was previously detected in plasmid pIncX-SHV

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TABLE 1 Antimicrobial susceptibility profiles of the original KPC-producing *K. pneumoniae* isolates and transformants[a]

Antibiotic(s)	MIC (μg/ml) (category) for[c] *K. pneumoniae* 45 (ST512 IncX3, two bla_{KPC} genes)	*K. pneumoniae* SG1[b] (ST512 pKpQIL, one bla_{KPC} gene)	*K. pneumoniae* 44[b] (ST258 pKpQIL, one bla_{KPC} gene)	*E. coli* DH5α 45-1T (IncX3, two bla_{KPC} genes)	*E. coli* DH5α 48-1T (pKpQIL, one bla_{KPC} gene)
Piperacillin-tazobactam	≥128 (R)	≥128 (R)	≥128 (R)	≥128 (R)	32 (S)
Ticarcillin-clavulanate	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)
Cefoxitin	≥128 (NA)	≥128 (NA)	≥128 (NA)	16 (NA)	16 (NA)
Cefpodoxime	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)
Ceftriaxone	≥256 (R)	≥256 (R)	≥256 (R)	32 (R)	4 (R)
Cefotaxime	≥128 (R)	≥128 (R)	≥128 (R)	8 (R)	4 (R)
Cefotaxime-clavulanate	≥128 (NA)	≥128 (NA)	≥128 (NA)	4 (NA)	0.25 (NA)
Ceftazidime	≥256 (R)	≥256 (R)	≥256 (R)	64 (R)	16 (R)
Ceftazidime-clavulanate	≥256 (NA)	≥256 (NA)	≥256 (NA)	32 (NA)	4 (NA)
Cefepime	≥32 (R)	≥32 (R)	≥32 (R)	8 (R)	2 (S)
Aztreonam	≥32 (R)	≥32 (R)	≥32 (R)	≥32 (R)	≥32 (R)
Imipenem	≥32 (R)	≥32 (R)	≥32 (R)	2 (S)	≥0.25 (S)
Meropenem	≥16 (R)	≥16 (R)	≥16 (R)	1 (S)	≤0.5 (S)
Ertapenem	≥8 (R)	≥8 (R)	≥8 (R)	1 (I)	≤0.125 (S)
Doripenem	≥4 (R)	≥4 (R)	≥4 (R)	0.5 (S)	0.25 (S)
Gentamicin	1 (S)	≤0.5 (S)	2 (S)	≤0.5 (S)	≤0.5 (S)
Tobramycin	≥16 (R)	≥16 (R)	≥16 (R)	≤0.5 (S)	≤0.5 (S)
Amikacin	16 (I)	16 (I)	32 (R)	≤2 (S)	≤2 (S)
Ciprofloxacin	≥4 (R)	≥4 (R)	≥4 (R)	≤0.125 (S)	≤0.125 (S)
Levofloxacin	≥16 (R)	≥16 (R)	≥16 (R)	≤0.5 (S)	≤0.5 (S)
Trimethoprim-sulfamethoxazole	2 (S)	2 (S)	2 (S)	≤0.25 (S)	≤0.25 (S)
Colistin	4 (R)	≤0.25 (S)	≥8 (R)	≤0.125 (S)	≤0.125 (S)
Polymyxin B	1 (NA)	0.5 (NA)	≥8 (R)	≤0.125 (S)	≤0.125 (S)
Doxycycline	8 (NA)	8 (NA)	4 (NA)	≤1 (NA)	≤1 (NA)
Minocycline	16 (NA)	8 (NA)	4 (NA)	≤1 (NA)	≤1 (NA)
Tigecycline	0.5 (S)	0.5 (S)	0.5 (S)	≤0.125 (S)	≤0.125 (S)

[a] R, resistant; I, intermediate; S, susceptible; NA, not applicable.
[b] *K. pneumoniae* strains ST512 and ST258-44 were previously described in Capone et al. (18) and Bonura et al. (4).
[c] According to the EUCAST 2015 criteria. MICs were obtained by implementing ESB1F and GNX2F plates (Trek Diagnostics).

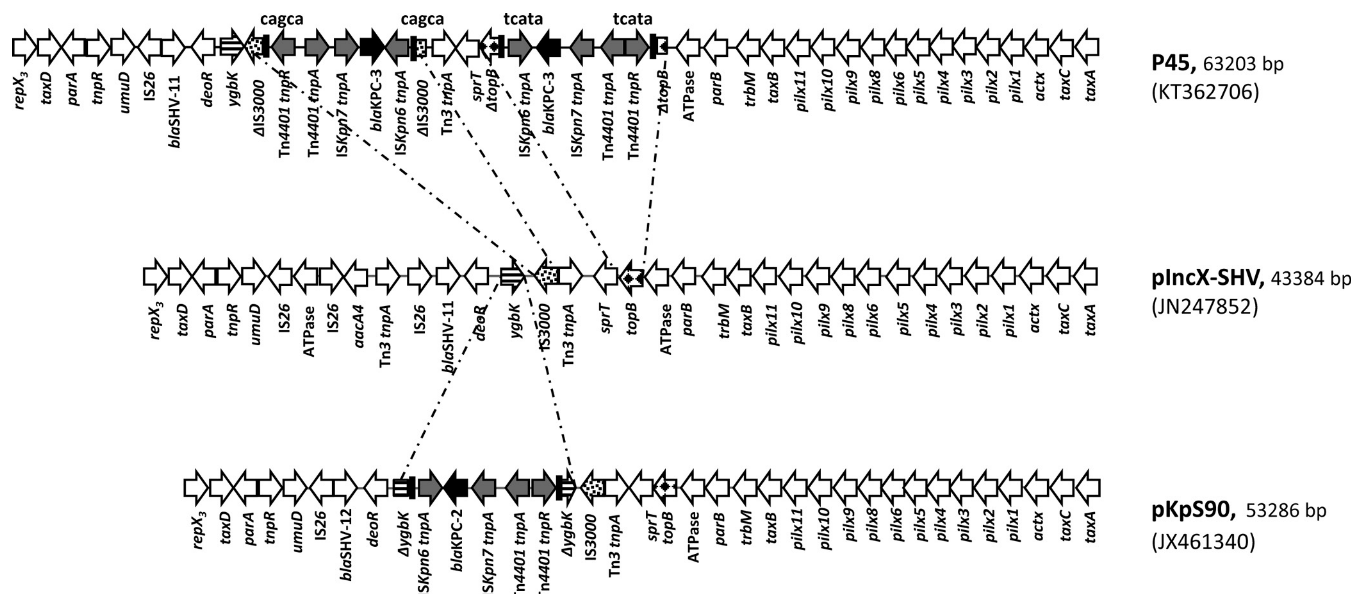

FIG 1 Major structural features of plasmids p45, pKpS90, and pIncX-SHV. Predicted open reading frames (ORFs) on plasmids are represented by white arrows. The ORFs of p45 were identified in this study; the ORFs of pKpS90 and pIncX-SHV were deduced from GenBank accession numbers JX461340 and JN247852, respectively. The transposase genes of the Tn*44011a* transposons flanked by IRR and IRL (thick black lines) are indicated by gray arrows; the bla_{KPC-3} genes inside Tn*4401a* are indicated by black arrows. The DNA sequences of duplicated target sites are indicated above the IRR and IRL. The *ygbK*, IS*3000*, and *topB* genes targeted by Tn*4401a* transposition events that occurred in the different plasmids are indicated by arrows filled with stripes, dots, and black squares, respectively.

(11; GenBank accession no. JN247852). In a similar plasmid (i.e., pKpS90), bla_{KPC-2}::Tn*4401a* was integrated in the same region as p45 but within a different site, causing the interruption of the *ygbK* gene (12). The progenitor of p45 was therefore a pIncX-SHV-like plasmid carrying the bla_{SHV-11} gene (11).

The second copy of Tn*4401a* in p45 was integrated in *topB* (a gene constantly present in all IncX3 plasmids) and in opposite orientation compared to that of the first transposon (Fig. 1). The target site duplication was also identified in this site, adjacent to the inverted repeats of the transposon. It is plausible that the acquisition of the second Tn*4401a* in p45 occurred by intramolecular transposition. In the literature, the simultaneous presence of two bla_{KPC} copies located in *trans* on different plasmids in the same bacterial host was previously reported in several collections (13–16). However, only two examples of in *cis* bla_{KPC} genes were previously described and differed from the arrangement described for p45. Two bla_{KPC-3}::Tn*4401a* copies were identified in MNCRE44, an extraintestinal pathogenic *E. coli* strain belonging to the ST131 *H*30R subclade and found in the United States (13). Its plasmid (pMNCRE44_5) was a 116-kb hybrid of IncX3 and IncFIA(HI1) plasmids, and the two transposon copies were located as one copy for each of the two fused plasmid portions (13). The duplication of the similar transposon bla_{KPC-2}::Tn*4401b* was described for the IncN plasmid S9 from *K. pneumoniae* in the United States (17).

The MICs for several antibiotics for KPC-producing *K. pneumoniae* strains of ST512 and ST258 carrying bla_{KPC-3} on pKpQIL (18), those of KPC-producing *K. pneumoniae* 45 (ST512 and with IncX3) and those of their corresponding *E. coli* DH5α transformants, were determined implementing the microdilution ESB1F and GNX2F plates (Trek Diagnostics) (Table 1). As result, we noted that the *E. coli* DH5α transformant carrying the IncX3 plasmid with two copies of bla_{KPC-3}::Tn*4401a* showed significantly increased MICs for carbapenems, cephalosporins, and β-lactam–β-lactamase inhibitor combinations compared to those of the transformant carrying the classical pKpQIL plasmid. This phenomenon was not observed for the original *K. pneumoniae* strains of different STs and possessing different bla_{KPC} genetic backgrounds. The difference in MICs may be due not only to the double copy of the bla_{KPC} genes and their levels of gene expression (14) but also to different copy numbers of IncX3 and pKpQIL.

In conclusion, our study describes the change in the typical plasmid content of ST512 *K. pneumoniae*: the worldwide-described pKpQIL plasmid carrying bla_{KPC} (19; GenBank accession no. GU595196) was replaced by an IncX3 plasmid carrying two copies of bla_{KPC-3}::Tn*4401a*. The change in plasmid type in *K. pneumoniae* strain 45 might represent an important evolution of the ST512 lineage.

Nucleotide sequence accession number. The GenBank file for *K. pneumoniae* strain ST512 plasmid p45-IncX3 was compiled using Sequin (http://www.ncbi.nlm.nih.gov/Sequin/) and deposited at the NCBI GenBank under accession no. KT362706.

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