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First Report of a Clinical, Multidrug-Resistant *Enterobacteriaceae* Isolate Coharboring Fosfomycin Resistance Gene *fosA3* and Carbapenemase Gene bla_{KPC-2} on the Same Transposon, Tn*1721*

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In order to understand the genetic background and dissemination mechanism of carbapenem resistance and fosfomycin resistance in *Enterobacteriaceae* isolates, we studied a clinical *Escherichia coli* strain HS102707 isolate and an *Enterobacter aerogenes* strain HS112625 isolate, both of which were resistant to carbapenem and fosfomycin and positive for the bla_{KPC-2} and *fosA3* genes. In addition, a clinical *Klebsiella pneumoniae* strain HS092839 isolate which was resistant to carbapenem was also studied. A 70-kb plasmid was successfully transferred to recipient *E. coli* J53 by a conjugation test. PCR and Southern blot analysis showed that bla_{KPC-2} was located on this plasmid. The complete sequence of pHS102707 showed that this plasmid belongs to the P11 subfamily (IncP1) and has a replication gene, several plasmid-stable genes, an intact type IV secretion system gene cluster, and a composite transposon Tn*1721*-Tn*3* that harbored bla_{KPC-2}. Interestingly, a composite IS*26* transposon carrying *fosA3* was inserted in the Tn*1721-tnpA* gene in pHS102707 and pHS112625, leading to the disruption of Tn*1721-tnpA* and the deletion of Tn*1721-tnpR*. However, only IS*26* with a truncated Tn*21-tnpR* was inserted in pHS092839 at the same position. To our knowledge, this is the first report of *fosA3* and bla_{KPC-2} colocated in the same Tn*1721*-Tn*3*–like composite transposon on a novel IncP group plasmid.

The increasing incidences of carbapenem-resistant bacteria, which are frequently resistant to most antibiotics, have renewed interest in revisiting the clinical use of old antibiotics, such as fosfomycin, for treating infections (1–3). Fosfomycin remains active against most *Enterobacteriaceae* isolates, with low-level resistance mediated most often by mutations in chromosomal loci, including *glpT* (4), *murA* (3), and so on. Recently, two novel plasmid-encoded fosfomycin-inactivating enzymes, FosA3 and FosC2, were found among CTX-M-producing *Escherichia coli* isolates in Japan, China, and South Korea (5–7). In the majority of isolates expressing these enzymes, the corresponding genes are located on an IS*26*-flanked composite transposon inserted into the vicinity of bla_{CTX-M}.

The most prevalent class A carbapenemases in clinical *Enterobacteriaceae* isolates are the *Klebsiella pneumoniae* carbapenemase (KPC) enzymes, and the KPC-2 variant is the most common (8). In Europe and the United States, bla_{KPC-2} is frequently located within a Tn*3*-based transposon, Tn*4401* (8, 9). Similarly, Shen et al. (10) showed that the majority of carbapenem-resistant isolates, obtained from six eastern cities in China, carried bla_{KPC-2} within related but more complex chimeric elements. They comprise Tn*1721*, Tn*3*, Tn*4401*, and IS*Kpn8* fragments and a segment similar to the plasmid RA3. This complex composite transposon possesses Tn*1721*-derived termini that match the intact transposase gene and an internal bla_{KPC-2}-bearing 2-kb fragment showing significant sequence similarity to Tn*4401* (10). In this study, we report the coexistence of *fosA3* and bla_{KPC-2} on a single transposon found within a conjugative IncP plasmid, which was identified in two temporally and spatially related clinical isolates belonging to distinct *Enterobacteriaceae* species. To our knowledge, this is the first report of a transposon carrying the fosfomycin resistance gene *fosA3* and the carbapenemase gene bla_{KPC-2}.

MATERIALS AND METHODS

Bacterial strains and antibiotic susceptibility testing. Two clinical isolates, of *E. coli* strain HS102707 and *Enterobacter aerogenes* strain HS112625, were collected after they were found to not be sensitive to imipenem and fosfomycin in routine antibiotic susceptibility testing. Among 78 bla_{KPC}-positive clinical *K. pneumoniae* isolates for which we sequenced the genetic environment of bla_{KPC}, strain HS092839 has a bla_{KPC-2} environment similar to that of *E. coli* strain HS102707 or *E. aerogenes* strain HS112625; that is, a similar bla_{KPC-2}-carrying transposon was inserted into a similar 70-kb plasmid. Therefore, the *K. pneumoniae* HS092839 isolate was also included in this study.

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TABLE 1 MICs of the studied strains

Strain	MIC (mg/liter) of[a]:							
	AK	IMP	CAZ	ETP	CTX	MEM	TZP	FOS
E. coli J53	2	0.25	0.25	0.06	0.064	0.015	4	1
K. pneumoniae HS092839 (Kp-39)	>256	>32	>256	>32	>256	32	>256	4
E. coli J53 HS092839 transconjugant	2	4	8	1.5	4	1	>256	0.38
Enterobacter aerogenes HS112625 (Ea-25)	8	8	32	24	32	8	>256	1024
E. coli J53 HS112625 transconjugant	2	4	8	1	6	0.5	>256	48
E. coli HS102707 (Ec-07)	8	6	6	1	4	0.5	>256	>1,024
E. coli J53 HS102707 transconjugant	1.5	4	6	1	4	0.25	>256	>1,024

[a] AK, amikacin; IMP, imipenem; CAZ, ceftazidime; ETP, ertapenem; CTX, cefotaxime; MEM, meropenem; TZP, piperacillin-tazobactam; FOS, fosfomycin.

The MICs of amikacin, ceftazidime, cefotaxime, imipenem, ertapenem, meropenem, piperacillin-tazobactam, and fosfomycin were determined by the Etest technique (AB Biodisk, Sweden) for the three isolates mentioned above, their transconjugants, and recipient *E. coli* J53. The results of the susceptibility tests were interpreted by Clinical and Laboratory Standards Institute (CLSI) criteria for *E. coli* and even for species other than *E. coli* (11).

MLST. The sequence types (STs) of *E. coli* strain HS102707 were were determined by analyzing housekeeping genes in two schemes: (i) eight genes used in the Pasteur project, including *dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA*, and (ii) seven genes used in the Achtman project, including *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*. The ST of *K. pneumoniae* strain HS092839 was determined with seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*). The results were compared with information in the multilocus sequence typing (MLST) databases (available at http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html, http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html, and http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Bacterial conjugation. Conjugation was performed using donor and recipient *E. coli* J53 cells mixed at a ratio of 1:1 in broth culture as described previously (12). Transconjugants were selected on MacConkey agar containing ampicillin (100 mg/liter) and sodium azide (100 mg/liter). Putative transconjugant colonies were selected and identified by the Vitek system and further confirmed by a bla_{KPC-2} PCR assay.

Plasmid analysis. Plasmids were extracted from 100-ml overnight cultures with the Qiagen plasmid midi kit (Qiagen, Germany) and examined by agarose gel electrophoresis. Plasmid sizes were estimated by comparison with *E. coli* V517 plasmid bands (54.2, 7.3, 5.6, 5.2, 3.9, 3.1, 2.7, and 2.1 kb) (13). The primers targeting bla_{KPC} genes were described previously (14).

Sequencing of pHS102707. One microgram of pHS102707 was used to generate a fragment library using the Ion Plus fragment library kit (Life Technologies). DNA sequencing and sequence assembly were performed using the Ion Torrent platform (Life Technologies). Gaps were closed by sequencing amplicons generated by primer-walking PCR performed on linking clones. Open reading frames were identified and annotated by searching against the NCBI nonredundant protein database.

Southern hybridization. Purified plasmids were electrophoresed in a 1.0% agarose gel, transferred to a positively charged nylon membrane (Roche Applied Science), and probed with a PCR-amplified bla_{KPC-2} probe according to the protocol specified by the DIG-High Prime DNA labeling and detection kit (Roche Applied Science).

Analysis of the genetic environment of bla_{KPC-2}. As preliminary screening suggested that pHS102707, pHS112625, and pHS092839 shared a bla_{KPC-2} genetic context similar to that of pKP048 (10), a PCR mapping approach was used to examine the relevant regions of the former plasmids. A series of primers with a common annealing temperature of ~60°C (see Table S1 and Fig. S1 in the supplemental material) was designed to allow for the generation of overlapping PCR fragments spanning the region of interest. When a standard primer pair failed to yield a product, alternative outer primers from the same PCR mapping set were used to span the region of variation. All relevant amplicons obtained were sequenced.

Nucleotide sequence accession numbers. The complete sequences of the plasmid pHS102707, a 15,464-bp region of pHS112625, and a 15,499-bp region of pHS092839 were deposited in GenBank under the accession numbers KF701335, KF724506, and KJ210592, respectively. The sequences of the relaxase genes of pHS092839 and pHS112625 were deposited under accession numbers KJ210594 and KJ210593, respectively.

RESULTS AND DISCUSSION

Fosfomycin resistance is uncommon among *Enterobacteriaceae* clinical isolates. As part of a broader study focused on carbapenem-resistant *Enterobacteriaceae* isolates obtained at Huashan Hospital, Shanghai, from August 2006 to December 2011, two isolates, from *E. coli* strain HS102707 (Ec-07) and *E. aerogenes* strain HS112625 (Ea-25), which exhibited resistance to fosfomycin and intermediate resistance to imipenem, were chosen for further characterization. A third multidrug-resistant, fosfomycin-sensitive *Enterobacteriaceae* isolate from this same collection, *K. pneumoniae* strain HS092839 (Kp-39), carrying a similar plasmid and Tn*1721* transposon structure, was also analyzed for comparison. The MICs of selected antimicrobial agents for Ec-07, Ea-25, and Kp-39 were determined (Table 1), which confirms that all three were resistant or intermediately resistant to imipenem. The MICs of fosfomycin for the Kp-39, Ec-07, and Ea-25 transconjugants were 0.38 mg/liter, 48 mg/liter, and >1,024 mg/liter, respectively. All three isolates tested positive for bla_{KPC-2} by PCR, and Ec-07 and Ea-25 were also positive for *fosA3*.

The *E. coli* (Ec-07) and *K. pneumoniae* (Kp-39) isolates were also characterized by MLST and found to belong to ST46 and ST11, respectively, according to corresponding species-specific MLST schemes of Pasteur. Consistent with previous reports, ST11 has been shown to be a predominant *K. pneumoniae* sequence type identified among clinical isolates from China (15, 16). In contrast, *E. coli* ST46 (or ST5 in Achtman MLST schemes) has not been reported to be a dominant clone in China or elsewhere (17–19).

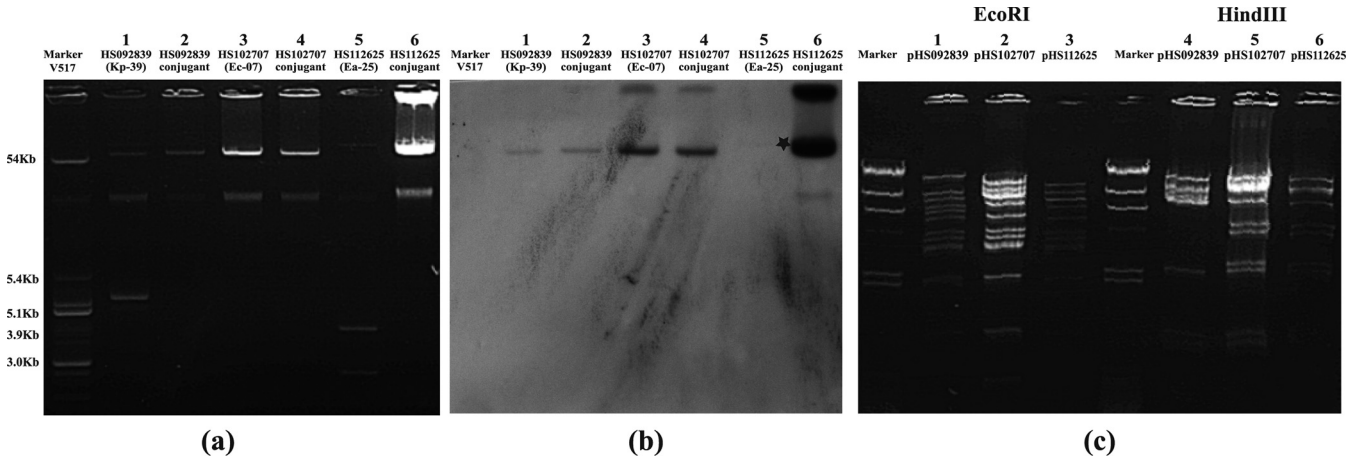

FIG 1 Electrophoretic profiles of plasmids (a), hybridization with a bla_{KPC-2}-specific probe (b), and plasmids digested with EcoRI and HindIII (c). (a and b) In lanes 1, 3, and 5, plasmids were extracted from parental isolate *K. pneumoniae* HS092839, *E. coli* HS102707, and *E. aerogenes* HS112625, respectively; in lanes 2, 4, and 6, plasmids were extracted from *E. coli* J53 HS092839 transconjugant, *E. coli* J53 HS102707 transconjugant, and *E. coli* J53 HS112625 transconjugant, respectively. The asterisk in panel b indicates a faint bla_{KPC-2}-positive band. (c) In lanes 1, 2, and 3, plasmids derived from isolates *K. pneumoniae* HS092839, *E. coli* HS102707, and *E. aerogenes* HS112625 were digested with EcoRI; in lanes 4, 5, and 6, plasmids derived from isolates *K. pneumoniae* HS092839, *E. coli* HS102707, and *E. aerogenes* HS112625 were digested with HindIII.

***fosA3* and bla_{KPC-2} are carried on the same conjugative plasmid.** In order to further explore the genetic basis of resistance, we performed conjugation experiments with each of the three primary isolates serving as a donor and the azide-resistant *E. coli* J53 as the recipient. Transconjugants were selected on medium containing ampicillin and azide. Next, electrophoretic plasmid profiles of the parental isolates were compared with those of matching transconjugants. Based on these data, it was evident that the parent Ea-25 and Kp-39 isolates carried multiple plasmids. By contrast, Ec-07 appeared to carry only a single plasmid. The three transconjugants derived from each of the primary clinical isolates appeared to each harbor a single plasmid of approximately 70 kb in size (Fig. 1a). As expected, plasmid bands of the transconjugants matched corresponding bands of each of the parent isolates. bla_{KPC-2}-directed PCR and Southern blot analysis of the plasmid DNA extracted from both parent donor isolates and the transconjugants showed that bla_{KPC-2} was located on these ~70-kb plasmids (Fig. 1b). Electrophoresis of EcoRI- and HindIII-digested plasmids purified from the three transconjugants showed that the restriction profiles of Ec-07-derived pHS102707, Kp-39-derived pHS092839, and Ea-25-derived pHS112625 were similar, but some differences were still seen among these three plasmids (Fig. 1c). The different MICs for fosfomycin between the Ec-07 and Ea-25 transconjugants may be due to the differences between plasmid pHS102707 and pHS112625.

Complete sequence of the *E. coli*-derived pHS102707 plasmid. In order to further define the genetic context of bla_{KPC-2} and *fosA3* in *E. coli* strain HS102707, we determined the entire sequence of the plasmid pHS102707, which was 69,453 bp in length with a G+C content of 48.9%. Using the approach by Norman et al. (20), we determined that pHS102707 carries genes involved in replication, stability, propagation, and adaptation (Fig. 2). It has one replication gene, *trfA*, in a replication module, and several plasmid stability genes, including *parAB*, *higA*, and *higB*, etc. bla_{KPC-2} was located within a complex chimeric element derived from Tn*1721*, Tn*3*, and other mobile elements. This complex element was inserted in the stability module of pHS102707. Critically, consistent with its ready mobilization by conjugation, pHS102707 encodes a full complement of conjugation machinery, including a type IV secretion system (T4SS), a relaxase, and a cognate origin of transfer (*oriT*) sequence. The pHS102707 T4SS is encoded by the *trbA-trbP* and *traA-traM* gene clusters.

Approximately 50% of *Gammaproteobacteria* plasmids are potentially transmissible (conjugative and mobilizable) (23). Relaxase is the only component common to all transmissible plasmids (24, 25). Based upon putative protein sequence similarities, a total of 741 sequences of relaxase encoded by 673 plasmids have been taken from 1,730 plasmid sequences deposited in GenBank (25). All the plasmids with relaxase can be classified into one of six families (MOB_P, MOB_F, MOB_V, MOB_Q, MOB_H, and MOB_C) and 31 subfamilies (23–25). MOB_P was the most represented (273 plasmids) by a degenerate primer MOB typing (DPMT) method (26). Within each family, subfamilies corresponding to phylogenetic clades contain more members (e.g., MOB_{F12}, which harbors relaxases encoded by IncF plasmids, and MOBP11, which groups relaxases encoded by IncP plasmids) (23). Phylogenetic analysis shows that the three relaxases encoded by pHS102707, pHS092839, and pHS112625 were grouped as the P11 relaxase subfamily (Fig. 2b). Relaxase protein sequence comparison of pHS102707 revealed 30.5% identity with RP4 (Inc P1α), the prototype plasmid for the P11 subfamily. The relaxase most similar to that of pHS102707, pXFAS01, exhibited 47% identity; no identity was found with any other non-P11 subfamily relaxases by NCBI blast.

The bla_{KPC-2} and *fosA3* genes are borne on a novel mosaic transposon. The *fosA3* gene in pHS102707 is embedded in a variant of the recently described bla_{KPC-2}-bearing transposon found on pKP048 (Fig. 3). bla_{KPC-2} is located in a Tn*1721*-Tn*3* chimeric element flanked by two 38-bp inverted repeat sequences, IRR and IRL2. In pHS102707 and pHS092839, Tn*1721*-Tn*3* was inserted close to the gene *klcA*, but in pKP048, it was located next to a truncated IS*26*-*tnpA*. pKP048 belongs to the F family (IncF), while

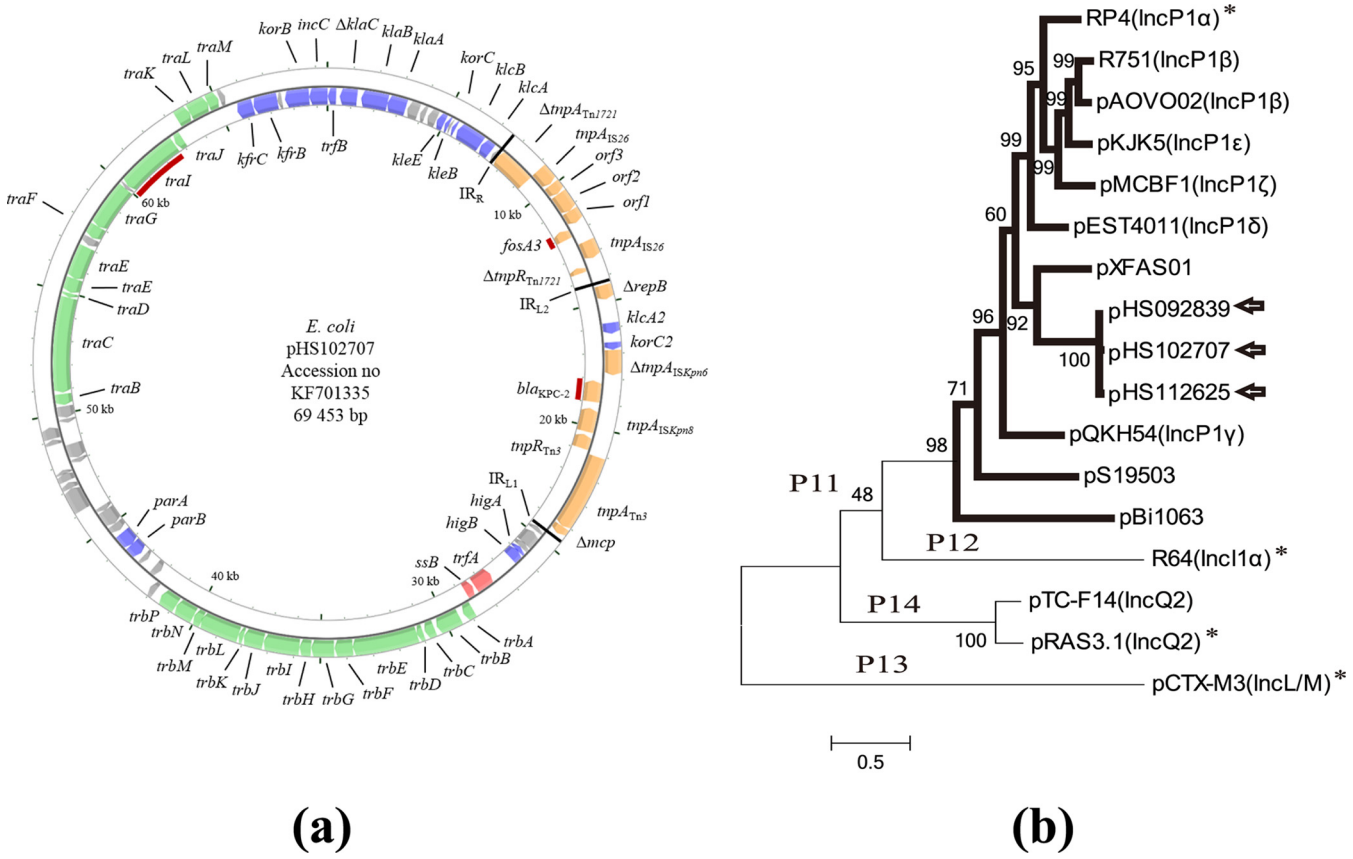

FIG 2 (a) Schematic map of pHS102707, an IncP plasmid found in the carbapenem-resistant *E. coli* isolate investigated in this study. Genes shown in red, blue, green, and orange are involved in replication, stability, propagation, and adaptation, respectively. Genes encoding unknown functions or that are not directly related to the above-mentioned roles are indicated in gray and shown unlabeled. Red bars highlight bla_{KPC-2}, *fosA3*, and the relaxase gene (*traI*). Tn*1721*-specific inverted repeats that define the boundaries of the associated mosaic transposon are labeled IR_{L1} and IR_R; a third matching internal repeat sequence is labeled IR_{L2}. (b) Inferred phylogenetic relationships of the plasmid-coding relaxase homologs. Seventeen protein sequences were aligned and the tree was generated with MEGA5 (21, 22) using the maximum-likelihood method. The relaxase sequences obtained in this study are indicated by blank arrows, while others were taken from Alvarado et al. (25). The sequences marked by an asterisk denote the ones taken from the prototype plasmid of each subfamily.

pHS102707, pHS092839, and pHS112625 belong to the P11 subfamily (IncP1). This suggests that a block mobilization of the complex transposon may happen among IncP1 plasmids. Interestingly, EcoRI and HindIII digestion profiles (Fig. 1c) show that the three P11 subfamily plasmids are not identical, and some differences exist.

The bla_{KPC-2}-flanking regions among pHS102707, pHS092839, and pKP048 on pHS102707 showed a high degree of synteny. The similar regions contained Tn*3*, bla_{KPC-2}, and the IS*Kpn6*-like transposase gene. However, several insertion and/or deletion events had taken place within the Tn*1721*-like region. Tn*1721* was intact in pKP048, carrying intact termini and transposase genes. In pHS092839, IS*26* and a truncated Tn*21-tnpR* had been inserted into Tn*1721*, resulting in the truncated Tn*1721-tnp*A and deletion of Tn*1721-tnpR*. Remarkably, in pHS102707, a DNA fragment newly found in this study, IS*26-orf3-orf2-orf1-fosA3*-IS*26*-Tn*21-tnpR*, had been inserted into Tn*1721-tnpA*. The IS*26* composite transposon in pHS102707 is the same as that found in *E. coli* isolated from livestock (GenBank accession no. JQ432559) (26).

The plasmid-borne *fosA3* fosfomycin resistance gene was first reported in *E. coli* isolates collected between 2002 and 2007 in Japan (7) and has since been reported in China and South Korea. Where characterized, *fosA3* has been found in an IS*26*-associated context on different plasmids from *E. coli* and *K. pneumoniae* (5, 26) and has frequently been linked physically to one of several bla_{CTX-M} variants and, occasionally, to the aminoglycoside resistance-encoding *rmtB* gene. Our findings, combined with those of the present study, suggest that the IS*26* composite transposon is highly mobile, appearing in the plasmid harboring bla_{CTX-M}, as well as the transposon with bla_{KPC-2}. Additionally, it is interesting to note that the two IS*26* insertions in pHS102707 and pHS092839 occurred in the exactly the same position as in pKP048. pHS102707 has an IS*26*-mediated composite transposon in comparison to pHS092839, highlighted here by the insertion of *fosA3*. Since IS*26* is often present in the vicinity of a long list of resistance genes, this insertion sequence is likely to contribute to the accelerated emergence of other elements carrying *fosA3* alongside various repertoires of preexisting resistance determinants.

In conclusion, we report from this study an IS*26*-flanked composite transposon which has mobilized *fosA3* onto a bla_{KPC-2}-bearing Tn*1721*-Tn*3*-derived mosaic transposon in an ST11-type *K. pneumoniae* isolate. This brings together genes coding for resistance to two classes of last-line antimicrobial agents on

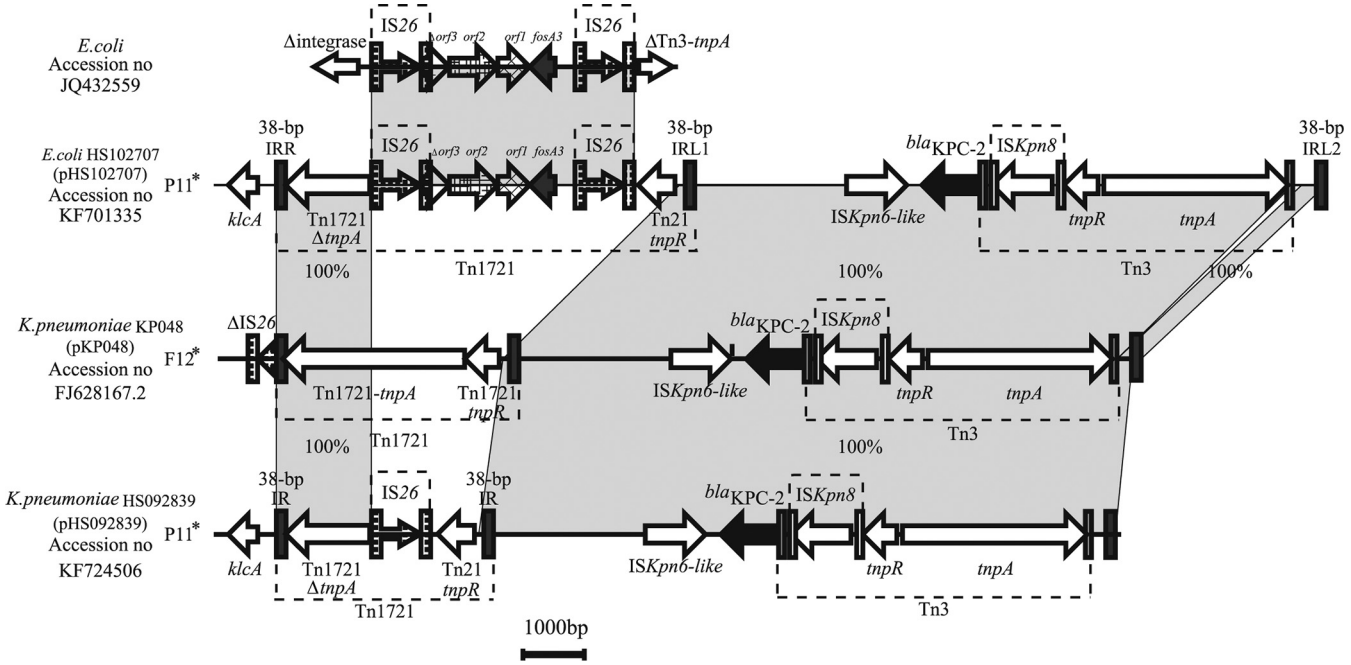

FIG 3 Comparative analysis of the bla_{KPC-2} and *fosA3* bearing mosaic Tn*1721*-Tn*3*-derived transposons present on pHS102707. The genes are depicted as arrows according to the direction of transcription. bla_{KPC-2} and *fosA3* are shown in black and dark gray, respectively. The inverted repeats are indicated by the variable, vertical gray bars. Regions with similar sequences are indicated in gray between the different plasmids. The asterisk indicates that the plasmid type was determined by the degenerate primer MOB typing (DPMT) method (25).

a single mobilizable element that itself resides on a larger conjugative plasmid.

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We declare no conflicts of interest.

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