First Report of a Clinical, Multidrug-Resistant Enterobacteriaceae Isolate Coharboring Fosfomycin Resistance Gene fosA3 and Carbapenemase Gene blaKPC-2 on the Same Transposon, Tn1721

Gang Li,a,b Ying Zhang,b Dexi Bi,c Pinghua Shen,b Fuqi Ai,b Hong Liu,b Yueru Tian,b Yiming Ma,b Bei Wang,b Kumar Rajakumar,d,e Hong-Yu Ou,c Xiaofei Jiangb

Department of Laboratory Medicine, Jinshan Hospital, Shanghai Medical College, Fudan University, Shanghai, China; Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, China; State Key Laboratory for Microbial Metabolism and School of Life Sciences & Biotechnology, Shanghai Jiaotong University, Shanghai, China; Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, United Kingdom; Department of Clinical Microbiology, University Hospitals of Leicester NHS Trust, Leicester, United Kingdom

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 blaKPC-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 blaKPC-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 Tn1721-Tn3 that harbored blaKPC-2. Interestingly, a composite IS26 transposon carrying fosA3 was inserted in the Tn1721-tnpA gene in pHS102707 and pHS112625, leading to the disruption of Tn1721-tnpA and the deletion of Tn1721-tnpR. However, only IS26 with a truncated Tn21-tnpR was inserted in pHS092839 at the same position. To our knowledge, this is the first report of fosA3 and blaKPC-2 colocalized in the same Tn1721-Tn3–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 a IS26-flanked composite transposon inserted into the vicinity of blactx-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, blaKPC-2 is frequently located within a Tn3-based transposon, Tn4401 (8, 9). Similarly, Shen et al. (10) showed that the majority of carbapenem-resistant isolates, obtained from six eastern cities in China, carried blaKPC-2 within related but more complex chimeric elements. They comprise Tn1721, Tn3, Tn4401, and ISKpn8 fragments and a segment similar to the plasmid RA3. This complex composite transposon possesses Tn1721-derived termini that match the intact transposase gene and an internal blaKPC-2-bearing 2-kb fragment showing significant sequence similarity to Tn4401 (10). In this study, we report the coexistence of fosA3 and blaKPC-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 blaKPC-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 blaKPC-positive clinical K. pneumoniae isolates for which we sequenced the genetic environment of blaKPC, strain HS092839 has a blaKPC-2 environment similar to that of E. coli strain HS102707 or E. aerogenes strain HS112625; that is, a similar blaKPC-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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The MICS of amikacin, ceftazidime, cefotaxime, imipenem, ertapenem, and piperacillin-tazobactam are determined by the Etest technique (AB Biodisk, Sweden) for the three isolates (1). Putative transconjugant colonies were selected and identified by the approach 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 pH11022, and a 15,499-bp region of pHS092839 were deposited in GenBank under the accession numbers KP701335, KP724596, and KJ210592, respectively. The sequences of the relaxase genes of pHS092839 and pH11022 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 Tn1721 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 intermediate resistant to imipenem. The MICs of fosfomycin for the Kp-39, Ec-07, and Ea-25 were 0.25 mg/liter, 0.25 mg/liter, and 0.06 mg/liter, respectively. All three isolates tested positive for bla\(^\text{KPC-2}\) 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 ST15 in Achtman MLST schemes) has not been reported to be a dominant clone in China or elsewhere (17–19).
foscA3 and blaKPC-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. Successfully transconjugated recipient strains were confirmed by resistance to ampicillin and fosfomycin, and by conjugation experiments with each of the three primary isolates serving as a donor and the azide-resistant E. coli J53 serving 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 strain 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 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. blaKPC-2-directed PCR and Southern blot analysis of the plasmid DNA extracted from both parent isolates and the transconjugants showed that blaKPC-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 plasmids pHS102707 and pHS112625.

Complete sequence of the E. coli-derived pHS102707 plasmid. In order to further define the genetic context of blaKPC-2 and foscA3 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. blaKPC-2 was located within a complex chimeric element derived from Tn1721, Tn3, 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 (MOBp, MOBp, MOBp, MOBp, MOBp, and MOBp) and 31 subfamilies (23–25). MOBp 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., MOBp, 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 are grouped as the P11 relaxase subfamily (Fig. 2b). Relaxase protein sequence comparison of pHS102707 revealed 30.5% identity with RP4 (IncP1), the prototype plasmid for the P11 subfamily. The relaxase most similar to that of pHS102707, pXFA501, exhibited 47% identity; no identity was found with any other non-P11 subfamily relaxases by NCBI blast.

The blaKPC-2 and foscA3 genes are borne on a novel mosaic transposon. The foscA3 gene in pHS102707 is embedded in a variant of the recently described blakPC-2-bearing transposon found on pKP048 (Fig. 3). blakPC-2 is located in a Tn1721-Tn3 chimeric element flanked by two 38-bp inverted repeat sequences, IRR and IRL2. In pHS102707 and pHS092839, Tn1721-Tn3 was inserted close to the gene klcA, but in pKP048, it was located next to a truncated IS26-tnpA. pKP048 belongs to the family (IncF), while
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 \( \text{bla}\text{KPC-2} \)-flanking regions among pHS102707, pHS092839, and pKP048 on pHS102707 showed a high degree of synteny. However, several insertion and/or deletion events had taken place within the Tn1721-like region. Tn1721 was intact in pKP048, carrying intact termini and transposase genes. In pHS092839, IS26 and a truncated Tn21-tnpR had been inserted into Tn1721, resulting in the truncated Tn1721-tnpA and deletion of Tn1721-tnpR. Remarkably, in pHS102707, a DNA fragment newly found in this study, IS26-orf3-orf2-orf1-fosA3-IS26-Tn21-tnpR, had been inserted into Tn1721-tnpA. The IS26 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 IS26-associated context on different plasmids from \( E.\ coli \) and \( K.\ pneumoniae \) (5, 26) and has frequently been linked physically to one of several \( \text{bla}\text{CTX-M} \) variants and, occasionally, to the aminoglycoside resistance-encoding \( \text{rmtB} \) gene. Our findings, combined with those of the present study, suggest that the IS26 composite transposon is highly mobile, appearing in the plasmid harboring \( \text{bla}\text{CTX-M} \) as well as the transposon with \( \text{bla}\text{KPC-2} \). Additionally, it is interesting to note that the two IS26 insertions in pHS102707 and pHS092839 occurred in the exactly the same position as in pKP048. pHS102707 has an IS26-mediated composite transposon in comparison to pHS112625, as well as the transposon with \( \text{bla}\text{KPC-2} \). Additionally, it is interesting to note that the two IS26 insertions in pHS102707 and pHS092839 occurred in the exactly the same position as in pKP048. pHS102707 has an IS26-mediated composite transposon in comparison to pHS092839, highlighted here by the insertion of fosA3. Since IS26 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 IS26-flanked composite transposon which has mobilized fosA3 onto a \( \text{bla}\text{KPC-2} \)-bearing Tn1721-Tn3-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
a single mobilizable element that itself resides on a larger conjugative plasmid.

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FIG 3 Comparative analysis of the blaKPC-2 and fosA3 bearing mosaic Tn1721-Tn3-derived transposons present on pHS102707. The genes are depicted as arrows according to the direction of transcription. blaKPC-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.


