

Complete Sequence of a Novel IncR-F33:A–:B– Plasmid, pKP1034, Harboring *fosA3*, *bla*_{KPC-2}, *bla*_{CTX-M-65}, *bla*_{SHV-12}, and *rmtB* from an Epidemic *Klebsiella pneumoniae* Sequence Type 11 Strain in China

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A high fosfomycin resistance rate was observed in *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (KPC-KP) in our previous study, but little is known about its mechanisms. In this study, we explored the prevalence of plasmid-mediated fosfomycin resistance determinants among fosfomycin-resistant KPC-KP strains from a Chinese university hospital and determined the complete sequence of a novel *fosA3*-carrying plasmid isolated from an epidemic *K. pneumoniae* sequence type (ST) 11 strain. A total of 97 KPC-KP strains were studied, of which 57 (58.8%) were resistant to fosfomycin, including 44 (45.4%) harboring *fosA3* and 1 harboring *fosA*. All *fosA3*-positive strains belonged to the dominant ST11-pulse type (PT) A clone according to multilocus sequence typing and pulsed-field gel electrophoresis, suggesting clonal dissemination. The *fosA*-positive isolate belonged to ST11-PTE. The *fosA3*-carrying plasmid pKP1034 is 136,848 bp in length and is not self-transmissible. It is a multireplicon plasmid belonging to IncR-F33:A–:B–. Besides *fosA3*, a variety of other resistance determinants, including *bla*_{KPC-2}, *rmtB*, *bla*_{CTX-M-65}, and *bla*_{SHV-12}, are identified in pKP1034, which would allow for coselection of *fosA3* by most β -lactams and/or aminoglycosides and facilitate its dissemination despite limited use of fosfomycin in China. Detailed comparisons with related plasmids revealed that pKP1034 is highly mosaic and might have evolved from alarming recombination of the *bla*_{KPC-2}-carrying plasmid pKPC-LK30 from Taiwan and the epidemic *fosA3*-carrying plasmid pHN7A8 from mainland China.

Increasing prevalence of *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-KP) has presented an alarming clinical threat. Fosfomycin seems to retain *in vitro* activity against many carbapenem-resistant *Enterobacteriaceae* and has been recently reintroduced into the fight against these superbugs, including KPC-KP strains (1–4). A previous study showed that only 43.4% of KPC-KP strains retained susceptibility to fosfomycin in a Chinese university hospital (5). Recently, Jiang et al. reported a comparable fosfomycin susceptibility rate (39.2%) in KPC-KP collected from 12 hospitals in China, and fosfomycin resistance could be attributed to the plasmid-mediated *fosA3* gene in 94 (55.6% of fosfomycin-resistant KPC-KP) strains (6).

FosA3 inactivates fosfomycin by glutathione S-transferase activity and was characterized first in CTX-M-producing *Escherichia coli* in Japan (7). Thereafter, it has been detected in food-producing animals, pets, patients, and healthy individuals in Asian countries (8–11). In most cases, the *fosA3* gene is associated with *bla*_{CTX-M} genes and sometimes with *rmtB* as well, and it resides on IS26-composite transposons, facilitating its dissemination. More recently, *fosA3* has been characterized in two atypical *bla*_{KPC}-carrying plasmids (6, 12). Here we explored the prevalence of plasmid-mediated fosfomycin resistance determinants among KPC-KP strains from a Chinese university hospital and determined the complete sequence of a novel *fosA3*-carrying plasmid pKP1034 from an epidemic KPC-KP sequence type (ST) 11 strain. To the best of our knowledge, this is the first fully sequenced plasmid from the dominant KPC-KP ST11 strain cocarrying *fosA3*, *bla*_{KPC-2}, *rmtB*, *bla*_{CTX-M-65}, and *bla*_{SHV-12}.

MATERIALS AND METHODS

A total of 97 nonduplicate KPC-KP strains were collected from the first affiliated hospital, Zhejiang University School of Medicine, between January 2010 and February 2013. MICs of fosfomycin were determined by the agar dilution method on Mueller-Hinton agar supplemented with glucose-6-phosphate (G-6-P) (25 mg/liter) and interpreted according to the Clinical and Laboratory Standards Institute guidelines (13). Only fosfomycin-resistant strains were included for further investigation. PCR was conducted to confirm the presence of *bla*_{KPC} and plasmid-mediated fosfomycin determinants (*fosA*, *fosA3*, *fosB*, *fosC2*), as described previously (9, 14). Since *rmtB* was prevalent in KPC-KP in our hospital, it was screened with PCR in all fosfomycin-resistant strains as well (5). Pulsed-field gel electrophoresis (PFGE) was performed using the XbaI restriction enzyme and interpreted by the criteria proposed by Tenover et al. (15). Multilocus sequence typing (MLST) was carried out according to proto-

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TABLE 1 Characterization of 57 fosfomycin-resistant KPC-KP strains

Strain	PT ^a	ST ^b	wzi allele	No.	Resistance determinant
<i>fos</i> negative ^c (n = 12)	A1	11	wzi209	1	<i>rmtB-bla</i> _{KPC}
			wzi209	1	<i>bla</i> _{KPC}
			wzi209	3	<i>rmtB-bla</i> _{KPC}
	A2	11	wzi209	1	<i>rmtB-bla</i> _{KPC}
	B	11	wzi108	4	<i>rmtB-bla</i> _{KPC}
C	11	wzi108	1	<i>bla</i> _{KPC}	
D	494	wzi141	1	<i>bla</i> _{KPC}	
<i>fos</i> positive (n = 45)	A1	11	wzi209	34	<i>fosA3-rmtB-bla</i> _{KPC}
	A2	11	wzi209	2	<i>fosA3-rmtB-bla</i> _{KPC}
	A3	11	wzi64	1	<i>fosA3-rmtB-bla</i> _{KPC}
	A4	11	wzi209	3	<i>fosA3-rmtB-bla</i> _{KPC}
				3	<i>fosA3-bla</i> _{KPC}
	A5	11	wzi209	1	<i>fosA3-rmtB-bla</i> _{KPC}
	E	11	UT ^d	1	<i>fosA-bla</i> _{KPC}

^a PT, pulse type.

^b ST, sequence type.

^c Strains negative for all plasmid-mediated fosfomycin resistance determinants screened in this study.

^d UT, untypeable.

cols on the MLST website for *K. pneumoniae* (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>), and *wzi* sequencing was conducted to predict the capsular (K) types of these *K. pneumoniae* isolates (16).

Conjugation experiments were carried out by broth mating for a representative *fosA3*-positive strain KP1034 (ST11-PTA1) with *E. coli* J53Azi as the recipient. Transconjugants were selected on lysogenic agar plates supplemented with sodium azide (150 mg/liter), fosfomycin (100 mg/liter), and G-6-P (25 mg/liter). The transformation experiment was conducted using total plasmids extracted from KP1034 and *E. coli* DH5 α competent cells (TaKaRa Bio, Japan) as the recipient, according to the user manual. Transformants were then selected on LB agar containing fosfomycin (100 mg/liter) and G-6-P (25 mg/liter) after overnight incubation. Antimicrobial susceptibility (to imipenem, meropenem, ertapenem, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, gentamicin, amikacin, ciprofloxacin, colistin, tigecycline, and fosfomycin) was determined for KP1034 and its *E. coli* DH5 α transformant KP1034-TF by Etest, except for fosfomycin, for which the MICs were determined as described above. The aforementioned resistance determinants were detected in KP1034-TF by PCR as well. Nuclease S1-PFGE was performed for both KP1034 and KP1034-TF to make sure that only one plasmid was transferred to KP1034-TF and to determine the approximate size of the plasmid.

Finally, *fosA3*-carrying plasmid pKP1034 was extracted from KP1034-TF using the Qiagen plasmid maxi kit (Qiagen, Valencia, CA). The plasmid was sequenced by a whole-genome shotgun approach, as previously described (17). In brief, pKP1034 DNA was randomly sheared using a Hydroshear DNA shearing device (Gene Machines, USA), and 2.0- to 4.0-kb fragments were cloned into a pMD19-T vector (TaKaRa Bio, Japan) and then transformed into *E. coli* DH5 α . End sequencing was conducted using an ABI 3730xl sequencer (Applied Biosystems, USA). The average coverage was approximately 7 \times across the plasmid. Sequence data were assembled to 25 contigs, including 3 bigger ones, with the Phred/Phrap/Consed software suite (University of Washington, Seattle, WA, USA). Six pairs of primers were designed according to ends of the 3 bigger contigs, and gaps were filled with PCR and Sanger sequencing. The plasmid sequence was initially annotated with RAST (<http://rast.nmpdr.org>) and then curated manually using the BLASTn and BLASTp algorithms (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Easyfig 2.0 was used to map whole plasmids and to conduct limited comparisons between closely related plasmids. The multiresistance region (MRR) on pKP1034 was mapped with reference to the MRRs of pHN7A8, pKPC-LK30, pKP0

48, and pHS102707 (GenBank accession no. JN232517, KC405622, FJ62 8167, and KF701335, respectively).

Nucleotide sequence accession number. The annotated sequence of pKP1034 has been deposited in GenBank under accession number KP893385.

RESULTS AND DISCUSSION

Of the 97 KPC-KP strains, 57 (58.8%) were resistant to fosfomycin, including 44 (45.4%) harboring *fosA3* and 1 (1.0%) harboring *fosA*. The fosfomycin resistance rate in KPC-KP was comparable to that (60.8%) recently reported by Jiang et al., although a higher breakpoint for fosfomycin resistance (256 mg/liter) was adopted in our study (6). Consistent with the previous study, the high-level incidence of fosfomycin resistance in KPC-KP in our study was mainly due to acquisition of *fosA3* (77.2%). Molecular typing identified two STs (ST11 and ST494) based on MLST and five pulse types (PTA through PTE) based on PFGE (Table 1; see also Fig. S1 in the supplemental material). However, PTA comprised five subtypes, PTA1 to PTA5, and PTA1 was the major subtype. Note that all minor subtypes but PTA2 were found only in strains collected in 2013 (data not shown), suggesting increasing diversity of the genetic backgrounds of these fosfomycin-resistant KPC-KP strains over time. All *fosA3*-positive strains belonged to the dominant clone ST11-PTA, indicating clonal dissemination. Eleven of the 12 *fosA*- and *fosA3*-negative strains also fell into the ST11 clone. Six of them were grouped into PTA, 4 into PTB, and 1 into PTC, with the remaining one belonging to ST494-PTD. The *fosA*-positive strain belonged to ST11-PTE. The results of *wzi* sequencing showed high-level consistency with that of PFGE (Table 1). A total of 4 *wzi* alleles (*wzi209*, *wzi64*, *wzi108*, and *wzi141*) were found, and the main allele *wzi209* was first reported and showed relatively low-level identity to alleles reported by Brisse et al. (16). Thus, the K type of the dominant clone could not be predicated according to the reference database, and classical K typing should be performed in the future (16). However, *wzi* sequencing did serve as a reliable typing method and seemed to demonstrate a discriminatory power lower than that of PFGE but higher than that of MLST. According to *wzi* sequencing, all *fosA3*-harboring isolates clustered in the novel *wzi209* allele except one, which had

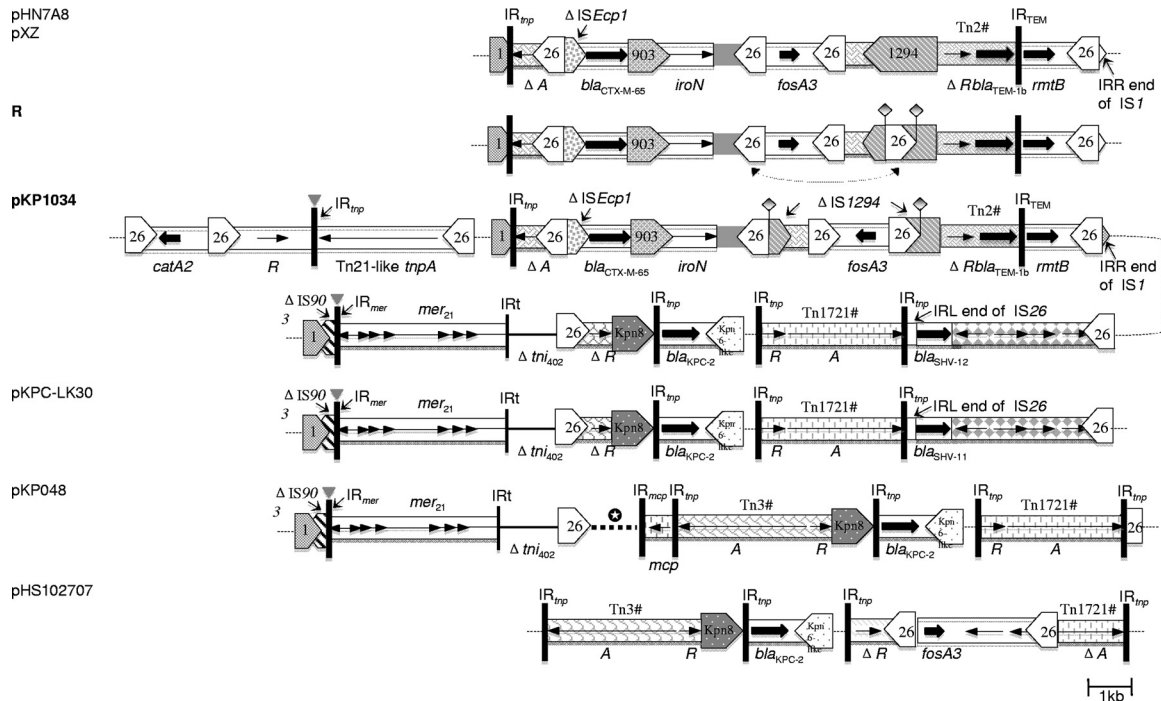


FIG 2 The MRR of plasmid pKP1034, indicated with bold type, compared with related MRRs of *fosA3*-positive plasmids pHN7A8 (JN232517.1) and pXZ (JF927996.1), *bla*_{KPC-2}-positive plasmids pKPC-LK30 (KC405622.1) and pKP048 (FJ628167.2), and plasmid pHS102707 (KF701335.1) cohaboring *fosA3* and *bla*_{KPC-2}. Various transposons and other modules have different shading and are generally labeled once for each plasmid. A pound sign (#) indicates that a transposon is incomplete. IS elements are pointed boxes labeled with the number/name. Tall bars represent the 38-bp inverted repeat (IR) of transposons, as indicated. Positions/orientations of selected resistance and other genes are indicated by arrows, generally labeled once for each plasmid. A, *tnpA*; R, *tnpR*. Gray triangles indicate the positions of IS4321 elements that have been removed for ease of comparison. Dashed lines represent plasmid backbone. The star inside a black circle indicates more MRR components of the plasmid pKP048 between the surrounding elements, including resistance genes *mph2*, *armA*, *sul1*, *bla*_{DHA-1}, and *qnrB4*. The structure named R above pKP1034 is hypothetical and is intended to facilitate comparison between related MRRs. Paired squares represent flanking sequences that are reverse complementary to one another. Dashed arrowed lines indicate where homologous recombination (HR) happens to invert the region between two IS26 elements in opposite orientations.

passing an abundance of scaffold genes and another multidrug resistance region. The IncR region containing genes responsible for replication (*repB*), partition (*parAB*), and stability (*umuCD*) is identified on this module, which is conserved among most sequenced IncR plasmids, such as pEFER, pKP1780, pKPN101-IT, pK245, pKPS30, and pKPS77 (GenBank accession no. CU928144, JX424614, JX283456, DQ449578, KF793937, and KF954150, respectively). However, *vagCD* (toxin-antitoxin system) and *resD* (multimer resolution) genes commonly located upstream of *repB* on IncR plasmids are missing in pKP1034, probably due to IS1-mediated deletion. The downstream region contains additional genes for plasmid stability, including *stbAB*, *ardAB* (antirestriction protein), *parB*-like gene, and *psiAB*, which together with the IncR region exhibits the highest identity (>99%) to that of plasmid pKPHS2 (GenBank accession no. CP003224), a *bla*_{KPC}-carrying plasmid identified in *K. pneumoniae* from Shanghai. Located next to the pKPHS2-like region was a region closely related to those of two *bla*_{NDM-1}-carrying plasmids pKOX-NDM1 and pRJF866 (GenBank accession no. JQ314407 and KF732966, respectively), found in *Klebsiella oxytoca* and *K. pneumoniae*, respectively. This region consists of *psiAB*, *ardA*, *umuCD* (involved in SOS mutagenesis), and *parAB*. Although lack of a functional transfer system has likely prevented extensive spread of pKP1034 across various *K. pneumoniae* lineages, the abundance of genes for plasmid maintenance and stability has likely ensured its stable

inheritance in strains harboring it, leading to clonal dissemination of *fosA3*-carrying KPC-KP in our hospital.

Besides *fosA3*, a variety of other resistance determinants, including *bla*_{KPC-2}, *rmtB*, *bla*_{CTX-M-65}, *bla*_{SHV-12}, *bla*_{TEM-1}, and *catA2*, are present in pKP1034, which allows coselection of *fosA3* by most β-lactams and/or aminoglycosides frequently used in clinic and, therefore, has further facilitated stable inheritance and wide dissemination of *fosA3* in our hospital despite limited use of fosfomycin. The mosaic, modular MRR in pKP1034 could be divided into three segments of distinct phylogenetic origins (Fig. 2). The first one is located upstream of the pHN7A8-derived module and carries *catA2* flanked by two inversely oriented IS26 elements at each end. In detail, this segment contains a small composite transposon carrying *catA2* bracketed by directly oriented copies of IS26, which has been commonly found on several fully sequenced plasmids, such as pENT-8a4, pEC-IMPQ, pEC-IMP, pVC1447, and pK245 (GenBank accession no. CP008899.1, EU855788.1, EU855787.1, KM083064.1, and DQ449578.1, respectively). However, compared with its counterparts in the above plasmids, 11 nucleotides immediately downstream of the first IS26 are missing in pKP1034, indicating that the small IS26-flanked composite transposon in pKP1034 might have originated from an independent capture event. The downstream structure encodes a resolvase, a Tn21-like transposase, and an inversely oriented IS26, which constitutes a bigger composite transposon together with

the small one upstream. *catA2* could be horizontally transferred via either the small composite transposon or the bigger one. The second segment is almost identical to the MRR in pHN7A8 and contains *fosA3*, *bla_{CTX-M-65}*, *bla_{TEM-1}*, and *rmtB*. However, the IS1294 adjacent to *fosA3* in pKP1034 was interrupted by IS26, and homologous recombination (HR) occurred afterward between the newly inserted IS26 and the one located upstream of *fosA3*, inverting the intervening structure. The third segment carries *bla_{KPC-2}*, *bla_{SHV-12}*, and a mercury resistance operon, which is highly related to the MRR in pKPC-LK30. However, *bla_{SHV-12}* instead of *bla_{SHV-11}* is identified in pKP1034. *bla_{SHV-12}* is carried by an IS26-flanked composite transposon, as previously reported (24, 25). Nonetheless, the IS26 adjacent to Tn1721 was truncated, probably by Tn1721, at the same location as its counterpart in pKPC-LK30. The immediate genetic surroundings of *bla_{KPC-2}* originated from the typical Tn3-Tn4401 integration structure reported in China (6), and, like pKPC-LK30 and pKPHS2, Tn3 was truncated at the Tn3 *tnpR* as well, probably due to IS26-mediated deletion. So far, only two plasmids, pFOS18 and pHS102707, co-harboring *bla_{KPC-2}* and *fosA3* have been fully sequenced, and they were identified from *K. pneumoniae* and *E. coli*, respectively (6, 12). In both cases, *fosA3* was acquired by two atypical plasmids carrying only *bla_{KPC-2}* via IS26-composite transposons embedding it, which is different from pKP1034 in our study.

A total of 11 copies of IS26 are dispersed through pKP1034, mainly in the MRR. Nevertheless, no target duplication repeats flanking IS26 are observed. This indicates that HR might play a key role in the generation of pKP1034. Detailed comparisons suggest that pKP1034 may have evolved from several recombination events of the two closely related plasmids pHN7A8 and pKPC-LK30. First, in plasmid pHN7A8, the composite transposon carrying *catA2* and an extra copy of IS26 in the same orientation as the IS26 at the boundary of *catA2* were inserted and truncated genes *traI* and *traB*, respectively. Second, HR between the two directly oriented IS26 elements occurred, deleting a circular molecule containing the intervening structure plus one copy of IS26. Afterward, the excised circular molecule was inserted next to the IS26 downstream of *bla_{SHV-11}* in plasmid pKPC-LK30 via another HR event between IS26 elements. Last, insertion of an extra IS1 element between the *repB* gene and the *resD* gene in pKPC-LK30 followed by HR between the directly oriented IS1 elements led to generation of a pKP1034-like plasmid. The third and fourth recombination events could have occurred in either order.

In conclusion, we report a high fosfomycin resistance rate in KPC-KP strains in a Chinese university hospital, mainly driven by clonal spread of *fosA3*-carrying KPC-KP ST11 strains. pKP1034, a novel *fosA3*-carrying plasmid from an representative KPC-KP strain, is a multireplicon plasmid belonging to IncR-F33:A–:B–. Besides *fosA3*, it carries a variety of other resistance determinants, including *bla_{KPC-2}*, *rmtB*, *bla_{CTX-M-65}*, *bla_{SHV-12}*, *bla_{TEM-1}*, and *catA2*. It is highly mosaic and could evolve from an alarming recombination of the *bla_{KPC}*-carrying plasmid pKPC-LK30 from Taiwan and the epidemic *fosA3*-bearing plasmid pHN7A8 reported in mainland China.

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We declare no conflicts of interest.

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