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ORIGINAL RESEARCH

Identification of *fosA10*, a Novel Plasmid-Mediated Fosfomycin Resistance Gene of *Klebsiella pneumoniae* Origin, in *Escherichia coli*

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Purpose: Several subtypes of plasmid-mediated fosfomycin resistance gene *fosA* in *Enterobacteriaceae* have been reported worldwide and have caused concern. The present study characterized a novel member of *fosA* gene located on a plasmid from *Escherichia coli*.

Materials and Methods: A fosfomycin-resistant *E. coli* isolate PK9 was recovered from a chicken meat sample in 2018. The presence of *fosA* genes was detected by PCR and sequencing. Whole-genome sequencing (WGS), conjugation, and cloning were performed to identify the mechanism responsible for fosfomycin resistance. Oxford Nanopore MinION sequencing was carried out to characterize the plasmid carrying fosfomycin resistance gene and the genetic context of the novel *fosA* variant.

Results: A novel *fosA* gene with significant homology (>98%) with *fosA6* and *fosA5* genes was identified by WGS and was named *fosA10*. FosA10 shared 56.1% to 98.6% amino acid sequence identity with other reported plasmid-mediated FosA enzymes. Fosfomycin resistance and *fosA10* gene were successfully transferred to *E. coli* C600 by conjugation. Cloning confirmed that FosA10 could confer fosfomycin resistance (MIC > 128 μg/mL). The *fosA10* gene was localized on a 53kb IncFII (F35:A-:B-) plasmid. The Δ*lysR-fosA10-*Δ*hp* fragment (4328 bp), located between two copies of IS10R, showed 100% identity with the chromosomal sequences of 17 *Klebsiella pneumoniae* strains of ST664 and one of ST3821 in GenBank.

Conclusion: Our findings indicated that the *fosA10* gene of *E. coli* might be captured from the chromosome of *K. pneumoniae* by IS*10*, which further demonstrated that *K. pneumoniae* might act as a reservoir of *fosA*-like genes acquired by *E. coli*.

Keywords: fosfomycin, resistance, plasmid, animal products

Introduction

In recent years, the widespread occurrence of extended-spectrum β-lactamases (ESBLs)-producing and carbapenem-resistant *Enterobacteriaceae* (CRE) in human clinic has renewed interest in the use of old antimicrobial agents, such as colistin and fosfomycin in the treatment of infections caused by multidrug-resistant pathogens.[1–3] Fosfomycin exhibits broad-spectrum bactericidal activity against Gram-positive and Gram-negative bacteria, and is listed as one of the first-line options for the treatment of uncomplicated lower urinary tract infections caused by ESBL-producing *Escherichia coli*.[4,5]

Fosfomycin interferes with the biosynthesis of cell wall by inhibiting production of UDP-N-acetylglucosamine enolpyruvyl transferase (MurA).[6] Besides, fosfomycin enters

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the bacterial cell via two transporters, glycerol-3-phosphate transporter (GlpT) and hexose phosphate transporter (UhpT).[7,8] Several mechanisms underlying fosfomycin resistance have been identified in *E. coli*, including modification or overexpression of MurA, inactivation of transporters and their regulatory genes (such as *uhpA*, *cyaA*, and *ptsI*), and acquisition of fosfomycin-modifying enzymes.[9] A variety of fosfomycin-modifying enzymes, such as FosA, FosB, FosC, FosL, have been described.[10–12] FosA is the most frequently reported group of enzymes in *Enterobacteriaceae*.[12,13] As a glutathione S-transferase, FosA inactivates fosfomycin by catalyzing the addition of glutathione to fosfomycin.[13] Until now, nine subtypes of fosA genes have been identified.[10,12,14-17] *fosA3*, the most widespread *fosA* gene in *E. coli*, has been detected in human and animal isolates from more than 12 countries, especially in food-producing animal-associated isolates from China.[12,18-20] All *fosA* subtypes, except *fosA2* and *fosA7*, are identified in plasmids, and they are usually originated from the chromosomal gene of *Enterobacteriaceae* species.[14,16,17,21-23]

In this study, we reported a novel plasmid-encoded *fosA* variant, *fosA10*, in an ESBL-producing *E. coli* isolate, and characterized the genetic context of *fosA10* to identify its origin.

Materials and Methods

Bacterial Isolation

E. coli isolate PK9 was recovered from a local broiler meat outlet in Faisalabad, Pakistan, in March 2018 as previously described.[24]

Antimicrobial Susceptibility Testing and Detection of *fos* Genes

The MICs of 13 antimicrobial agents, including ampicillin, cefotaxime, ceftazidime, cefoxitin, florfenicol, streptomycin, doxycycline, ciprofloxacin, imipenem, colistin, amikacin, gentamycin, and tigecycline, were determined by either agar dilution or broth microdilution method (colistin and tigecycline) according to the Clinical Laboratory Standards Institute (CLSI) guideline.[25] Besides, the agar dilution method using Mueller-Hinton agar supplemented with 25 μg/mL of glucose-6-phosphate (G6P) was applied to determine the fosfomycin MICs as recommended.[25] *E. coli* ATCC 25922 was used as a quality control standard. The results were interpreted according to the breakpoints of the CLSI.[25]

The presence of known *fos* genes (*fosA1* to *fosA7* and *fosC*) in PK9 was investigated by PCR amplification as previously described.[14,15,22,23,26,27]

Whole-Genome Sequencing

Whole genomic DNA of the *fosA10*-positive *E. coli* isolate was extracted using HiPure Bacterial DNA Kit (Magen, Guangzhou, China). Whole-genome sequencing (WGS) was performed by Novogene (Beijing Novogene Bioinformatics Co., Ltd., Beijing, China) using Illumina HiSeq 2500 technology (Illumina, San Diego, CA, USA). De novo assembly was performed using SOAPdenovo (version 2.04). To obtain the complete sequence of the *fosA10*-carrying plasmid, we then sequenced *E. coli* PK9 on Oxford Nanopore MinION. The assemblies of long Nanopore reads and the short Illumina reads were combined via Unicycler (version 0.4.8). The resulting contigs were uploaded into the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/services/). The resistance genes, plasmid type, and multilocus sequence type (MLST) of PK9 were analyzed by ResFinder 3.2, PlasmidFinder, and MLST, respectively.

Conjugation Experiment

Conjugation experiments were performed using isolate PK9 and *E. coli* strain C600 (high-level resistance to streptomycin) as donor and recipient strains, respectively.[28] Transconjugants were selected on MacConkey agar plates containing fosfomycin (64 μg/mL) and streptomycin (3000 μg/mL) for counter-selection.

Cloning of *fosA10* Gene

The *fosA10* gene from *E. coli* PK9 was cloned into the pMD19-T vector using primers listed as followed: PK9-F: 5′-TCATTAGGGGATTCATCAGT-3′ and PK9-R: 5′-AGATAGTGGAGCGGAGACC-3′. Then, it was transferred into *E. coli* DH5α (Takara, Shiga, Japan) by heat shock. Transformants were selected on LB agar plates containing 100 μg/mL ampicillin. Recombinant clones were analyzed by PCR followed by Sanger sequencing.

Phylogenic Analyses and Homology Alignment

The amino acid sequence of FosA10 was predicted based on the nucleotide sequence of fosA10 gene, and its amino acid sequence was homologously aligned with the amino acid sequence of fosfomycin enzymes in other *Enterobacteriaceae* obtained from GenBank by

T-Coffee.[29,30] The phylogenic tree was constructed by distance method using Neighbor-Joining algorithm of MEGA 5. Branch lengths were drawn to scale and were proportional to the number of amino acid substitutions with 500 bootstrap replications.

Results and Discussion

Identification of a Novel Plasmid-Mediated Fosfomycin Resistance Gene *fosA10*

E. coli PK9 was found to be resistant to ampicillin, cefotaxime, ceftazidime, cefoxitin, streptomycin, gentamycin, and ciprofloxacin, and intermediate to doxycycline (Table 1). Notably, it was highly resistant to fosfomycin with an MIC > 128 μg/mL (Table 1). However, no *fosC2* or *fosA* gene (*fosA1–A7*) was detected from PK9 by PCR amplification. Conjugation experiment showed that the fosfomycin resistance of PK9 was transferred to the recipient *E. coli* C600 (Table 1), indicating the presence of plasmid-mediated fosfomycin resistance gene.

WGS data showed that *E. coli* PK9 belonged to ST38. In a 1918 bp contig, a 420 bp open reading frame (ORF) encoding FosA-like protein was identified. This novel *fosA* gene was then named *fosA10* as the next available number according to published data and NCBI.[17] *fosA10* had 69.7%, 67.8%, 73.4%, 73.1%, 98.3%, 99.3%, 58.2%, 64.0%, 93.9%, and 53.6% nucleotide identity with *fosA1* to *fosA9* and *fosC2*, respectively. Besides, the amino acid sequence of FosA10 enzyme encoded by *fosA10* shared 70.0%, 68.6%, 79.0%, 78.3%, 98.6%, 97.8%, 62.9%, 65.0%, 97.8%, and 56.1% identity with FosA1 to FosA9 and FosC2, respectively (Figures 1 and S1).

To determine whether *fosA10* could confer resistance to fosfomycin, we constructed a recombinant plasmid pMD19-T + fosA10. The MIC of fosfomycin for *E. coli* DH5α transformed with pMD19-T + fosA10 was >128μg/mL, more than 64-fold higher than that of *E. coli* DH5α carrying pMD19-T alone (Table 1).

Genetic Context and Origin of the *fosA10* Gene

Hybrid assembly of the MinION long reads and HiSeq short reads revealed that PK9 had a circular 5,423,354 bp chromosome and three plasmids (Table 2). One plasmid, designated pHNPK9-FOS, carries *fosA10*. pHNPK9-FOS was a 53,736 bp plasmid containing 69 predicted ORFs, and belonged to IncFII (F35:A-:B-). It possessed a typical IncF-type backbone, encoding genes for replication, transfer, maintenance, and stability functions (Figure 2). A variable region of 4328 bp consisting of *fosA10* and two copies of IS*10* element was inserted into a hypothetical gene of the plasmid backbone. The insertion was surrounded by a 9-bp direct repeat sequences (DRs) (TACCTGGTG) suggesting mobilization of this *fosA10* gene by composite transposon formed by IS10 (Figure 3). In addition to *fosA10* gene, the region surrounded by IS*10* includes a truncated transcriptional regulator gene *lysR* (190 bp) and a 1060 bp sequence (containing a truncated hypothetical gene) located upstream and downstream of

Table 1 Antimicrobial Susceptibility of *fosA10*-Carrying *Escherichia coli* Strain PK9 and Transconjugant or Transformant

Strain[a]	PK9	Transconjugant (*E. coli* C600 +pHNPK9-FOS)	*E. coli* C600	*E. coli* DH5α (pMD19-T +fosA10)	*E. coli* DH5α (pMD19-T)
Fosfomycin	>128	>128	2	>128	2
Ampicillin	>128	4	4	>128	>128
Cefotaxime	>128	0.03	0.125	0.125	0.06
Ceftazidime	>128	0.5	0.06	0.5	0.5
Cefoxitin	64	2	4	2	2
Streptomycin	256	>256	>256	2	2
Gentamicin	16	0.5	0.5	0.25	0.25
Florfenicol	4	2	2	2	2
Doxycycline	8	0.25	0.5	0.25	0.25
Ciprofloxacin	64	0.004	0.008	0.002	0.002
Colistin	0.25	0.125	0.125	0.125	0.125
Amikacin	4	0.5	0.5	0.5	0.5
Imipenem	0.125	0.5	0.125	0.25	0.125
Tigecycline	0.25	0.25	0.25	0.25	0.25

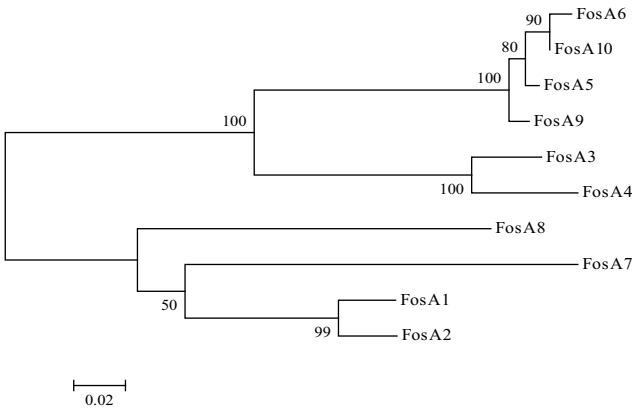

Figure 1 Phylogenetic tree obtained for the FosA proteins. The protein GenBank accession numbers are FosA^{TN}(FosA1), AAA98399; FosA2, ACC85616; FosA3, AB522970; FosA4, AB908992; FosA5, AJE60855; FosA6, NG051497; FosA7, KKE03230; FosA8, CP013990; FosA9, PRJEB32329; and FosA10, MT074415.

fosA10, respectively. BLAST homology analysis demonstrated that the sequence of the 1670 bp region surrounded by IS*10* had 100% nucleotide identity with chromosome sequences of 18 *K. pneumoniae* strains from Austria, USA, UK, Morocco, and Japan (Figure 3 and Table S1), suggesting its mobilization from the chromosome of one *K. pneumoniae* strain to plasmid. All the 18 *K. pneumoniae* strains belong to ST644 except one that belongs to ST3821 (Table S1). In addition, a WGS contig (GenBank accession number MUJB01000052) of an *E. coli* strain AUH_IMP168 collected in 2013 from Lebanon also contained *fosA10* and two incomplete IS*10* elements caused by short-read DNA sequencing. The fragment containing *fosA10* surrounded by IS*10* in *E. coli* strain AUH_IMP168 was also 100% identical to the chromosome sequences of 18 *K. pneumoniae* strains

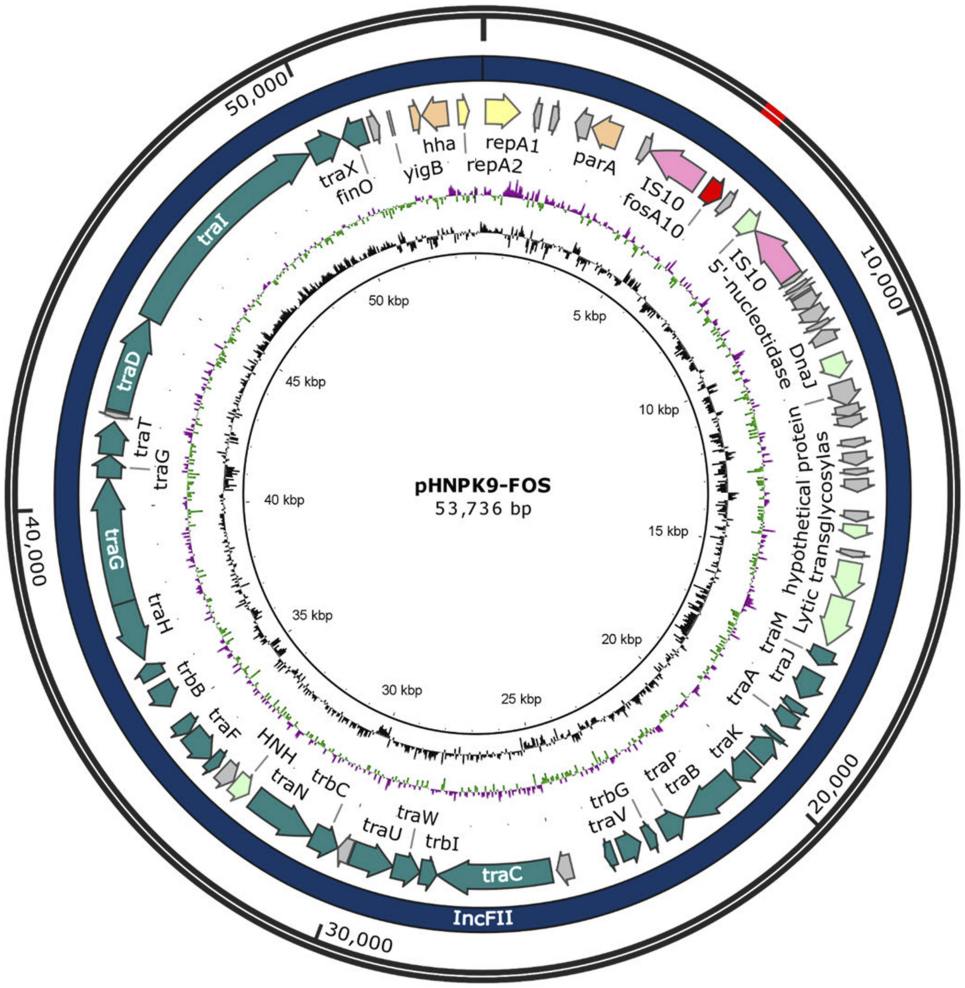

Figure 2 Map of pHNPK9-FOS. The map was carried out by BLAST tools, Sequin (version 15.50), BLAST Ring Image Generator (BRIG, version 0.95), Serial Cloner (version 2.6.1), and SnapGene (version 4.1.9). The outermost two rings indicate the size and plasmid type of pHNPK9-FOS, respectively. The next ring shows features extracted from the genome GenBank file of pHNPK9-FOS. The next two rings show GC content and GC skew. Each is plotted as the deviation from the average for the entire sequence.

Table 2 Resistance Genes and Plasmids Carried by *Escherichia coli* Strain PK9

Chromosome or Plasmid Name	Replicon of Plasmid	Size	Resistance Gene	Chromosomal Point Mutations
Chromosome	–	5,423,354 bp	*mdf(A), aac(6')-Ib-cr, tet(A), dfrA1, aac(3)-IIa, aac(6')-Ib-cr, aadA1, aph(3")-Ib, aph(3")-Ib, aph(6)-Id,* bla_{CMY-16}, $bla_{CTX-M-15}$, bla_{OXA-1}	GyrA: S83L, D87N ParC: S80I, E84G
pHNPK9-FOS	IncFII (F35:A-:B-)	53,736 bp	*fosA10*	-
pHNPK9-2	IncFII (F1:A-:B23)	135,056 bp	None	-
pHNPK9-3	Unknown	97,203 bp	None	-

mentioned above (Figure 3). However, the size of the spacer region between the 3′ end of *fosA10* and IS*10* was longer than that in pHNPK9-FOS (1663 bp vs 1060 bp), indicating occurrence of a different mobilization event of *fosA10* from *K. pneumoniae* chromosome.

The other two plasmid-located *fosA* genes, *fosA5* and *fosA6*, also originated from *K. pneumoniae* chromosome,[14,23] and have high similarity of nucleic acid sequence with *fosA10*, with difference in only four or seven nucleotides. Like *fosA10, lysR* genes truncated by IS*10* segment were identified upstream of *fosA5* (in plasmid pHKU1, GenBank accession number KC960485) and *fosA6* (in plasmid pYD786-2, KU254579.1),[14,23,31] and the insertion sites of IS*10* upstream of *fosA5* and *fosA10* were the same (Figure 3). In addition, IS*10* was found to be inserted downstream of *fosA5*. However, the fragments located downstream of *fosA5, fosA6*, and *fosA10* were varied. Although *fosA5, fosA6*, and *fosA10* have significant homology and were all derived from the *K. pneumoniae* chromosome, these mobilization events of $fosA^{kp}$ from the *K. pneumoniae* chromosome to plasmids seemed to occur separately. Nevertheless, the findings of several events of IS*10* insertion in *lysR* genes of *K. pneumoniae* in recent years implied that *lysR* gene was a hotspot for IS*10* insertion. Further capture of chromosomal *fosA* genes from *K. pneumoniae* by plasmids is possible.

In summary, we report the emergence of a novel plasmid-mediated *fosA* gene, *fosA10*, conferring high-level resistance to fosfomycin. *fosA10* gene was probably horizontally transferred from *K. pneumoniae* chromosome to *E. coli* plasmid by IS*10*. The increasing discovery of chromosomal *fosA* genes on plasmids is alarming and demonstrated that *Klebsiella spp*. might be an important reservoir of *fosA* genes for *E. coli*. Considering the apparent horizontal transferability of plasmids, further dissemination of these *fosA* genes among *E. coli* is possible and will constitute a serious threat to antimicrobial therapy. Therefore, we should pay more attention to the emergence and spread of plasmid-mediated *fosA* genes. Further studies on the occurrence and characterization of *fosA10*-carrying plasmids in *Enterobacteriaceae* from various sources (humans, animals, and the environment) will offer more

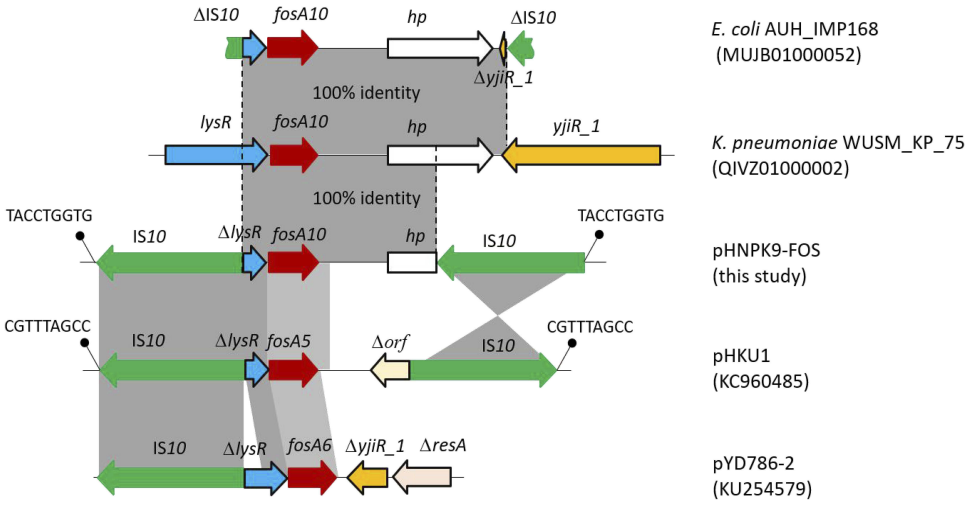

Figure 3 Genetic structure surrounding the new *fosA10* gene, *fosA5*, and *fosA6* gene. Genes and ISs are indicated by bold arrows. DR sequences are represented by diagonal bars. 100% and 98%~97% sequence identities were denoted by dark gray shading and light gray shading, respectively.

insights into the potential role of *fosA10* in the dissemination of fosfomycin resistance.

Accession Number

The complete nucleotide sequence of plasmid pHNK9-FOS has been deposited in GenBank under accession no. MT074415.

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Disclosure

The authors report no conflicts of interest in this work.

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