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Plasmids of Diverse Inc Groups Disseminate the Fosfomycin Resistance Gene *fosA3* among *Escherichia coli* Isolates from Pigs, Chickens, and Dairy Cows in Northeast China

Xiu-Mei Wang,[a] Zhimin Dong,[a] Stefan Schwarz,[b] Yao Zhu,[a] Xin Hua,[a] Yanhe Zhang,[a] Siguo Liu,[a] Wan-Jiang Zhang[a]

State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China[a]; Institute of Microbiology and Epizootics, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany[b]

ABSTRACT Thirty-nine fosfomycin-resistant *Escherichia coli* isolates carrying *fosA3* were obtained from pigs, chickens, dairy cows, and staff in four northeastern provinces of China between June 2015 and April 2016. The *fosA3* gene was colocated with bla_{CTX-M} genes on conjugative plasmids of the incompatibility groups IncN ($n = 12$), IncN-F33:A$^-$:B$^-$($n = 2$), IncF33:A$^-$:B$^-$($n = 14$), IncF14:A$^-$:B$^-$($n = 2$), and IncI1/sequence type 136 (ST136) ($n = 9$). Four different genetic contexts of *fosA3* were detected among the 39 *E. coli* isolates. Three potential epidemic plasmids circulated among *E. coli* strains from this region.

KEYWORDS CTX-M, antibiotic resistance, resistance genes, horizontal dissemination

Due to the increasing prevalence of bacterial infections caused by multidrug-resistant (MDR) or extensively drug-resistant (XDR) Gram-negative pathogens (1, 2), one of the older antibiotics, fosfomycin, has been reintroduced into clinical use (3). While resistance to fosfomycin is mainly due to chromosomal mutations (4), two plasmid-borne fosfomycin resistance genes, *fosA3* and *fosC2*, were first identified in human *Escherichia coli* in Japan in 2010 (5). Thereafter, a relatively high prevalence of fosfomycin-resistant *E. coli* isolates carrying *fosA3* was found in food animals, pets, and wild rodents in China, although fosfomycin has not been approved for veterinary use in China (6–8).

So far, information on the distribution of fosfomycin-resistant *E. coli* isolates of animal origin in China has been limited to the southern and central parts of China. However, available information on the occurrence and epidemiological characteristics of fosfomycin-resistant *E. coli* in other regions of China is scarce. In this study, a total of 370 *E. coli* isolates were collected from diseased animals and humans (pigs, $n = 115$; chickens, $n = 95$; dairy cows, $n = 98$; dairy farm staff, $n = 1$) and healthy animals and humans (pigs, $n = 13$; chickens, $n = 19$; dairy cows, $n = 24$; dairy farm staff, $n = 5$) in northeast China between June 2015 and April 2016.

The MICs of fosfomycin for 370 isolates, determined as previously described (6), revealed that 39 isolates (10.5%) were resistant to fosfomycin (>512 mg/liter) (see Table S1 in the supplemental information). PCR assays for plasmid-mediated fosfomycin resistance genes *fosA3*, *fosA*, and *fosC2* (7) showed that all 39 isolates were positive only for the *fosA3* gene. Additional antimicrobial susceptibility testing by broth microdilution (9, 10) revealed that all 39 *fosA3*-positive isolates were also resistant to florfenicol, cefotaxime, gentamicin, and tetracycline but susceptible to colistin (see Table S1). PCR amplification and sequencing (11, 12) revealed that all of the *fosA3*-positive isolates also

Received 25 April 2017 **Returned for modification** 22 May 2017 **Accepted** 27 June 2017

Accepted manuscript posted online 3 July 2017

Citation Wang X-M, Dong Z, Schwarz S, Zhu Y, Hua X, Zhang Y, Liu S, Zhang W-J. 2017. Plasmids of diverse Inc groups disseminate the fosfomycin resistance gene *fosA3* among *Escherichia coli* isolates from pigs, chickens, and dairy cows in northeast China. Antimicrob Agents Chemother 61:e00859-17. https://doi.org/10.1128/AAC.00859-17.

Address correspondence to Siguo Liu, siguo_liu@hvri.ac.cn, or Wan-Jiang Zhang, wjzhang@hvri.ac.cn.

TABLE 1 Characterization of *fosA3*-positive *E. coli* isolates and their *fosA3*-carrying plasmids

						fosA3-carrying plasmids			
Isolate[a]	Host	MLST	Phylogroup	Other cotransferred resistance genes[b]	Context of *fosA3*	Size	Replicon type	EcoRI RFLP[c]	Addiction system(s)
SY301B	Pig	ST761	B2	$bla_{CTX-M-65}$, *floR*, *cfr*, *tet*(A)	III	pECXH3 (~97 kb)	N (ST7)	A1	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SY301F	Pig	ST3315	B2	$bla_{CTX-M-55}$, *floR*, *cfr*, *rmtB*, *strA*, *strB*, *tet*(A)	I	~105 kb	N (ST7)	A2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SY303P	Pig	ST5714	B2	$bla_{CTX-M-65}$, *floR*, *rmtB*, *tet*(A)	III	~100 kb	N-F33:A$^-$:B$^-$	NT	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SY301L	Pig	ST5442	B2	$bla_{CTX-M-55}$, *floR*, *oqxA*, *oqxB*, bla_{TEM-1}, *tet*(A)	II	~97 kb	F33:A$^-$:B$^-$	C1	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SY303M	Pig	ST3944	B2	$bla_{CTX-M-14}$, *floR*, *cfr*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~97 kb	N	A1	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SY305M	Pig	ST3944	B2	$bla_{CTX-M-14}$, *floR*, *cfr*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~97 kb	N	A1	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SY286M	Cow	ST93	B2	$bla_{CTX-M-14}$, *rmtB*, *aadA2*, bla_{TEM-1}	IV	pECM13 (113,006 bp)	IncI1 (ST136)	E1	*pemKI*, *hok-mok-sok*
SY287M	Cow	ST98	B2	$bla_{CTX-M-14}$, *floR*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	pECM13-like	IncI1 (ST136)	E1	*pemKI*, *hok-mok-sok*
SY284B	Cow	ST4683	B1	$bla_{CTX-M-14}$, *floR*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	pECM13-like	IncI1 (ST136)	E1	*pemKI*, *hok-mok-sok*
CH293B	Chicken	ST10	B2	$bla_{CTX-M-55}$, *floR*, *strA*, *strB*, *tet*(A)	I	~97 kb	F33:A$^-$:B$^-$	C1	*pemKI*, *hok-mok-sok*, *srnB-srnC*
CH292B	Chicken	ST10	B2	$bla_{CTX-M-55}$, *floR*, *cfr*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11 (92,545 bp)	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
CH291M	Chicken	ST410	B2	$bla_{CTX-M-65}$, *floR*, *rmtB*	III	~100 kb	N-F33:A$^-$:B$^-$	C3	*pemKI*, *hok-mok-sok*, *srnB-srnC*
CH281F	Chicken	ST5889	A	$bla_{CTX-M-65}$, *floR*, *rmtB*	III	~100 kb	N (ST7)	A2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
CH282M	Chicken	ST1437	A	$bla_{CTX-M-65}$, *floR*, *rmtB*, *oqxA*, *oqxB*	III	~100 kb	N (ST7)	A2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
CH285F	Chicken	ST448	B2	$bla_{CTX-M-3}$, *floR*, *strA*, *strB*, *tet*(A)	I	~105 kb	F14:A$^-$:B$^-$	NT	*pemKI*, *hok-mok-sok*
DH286F	Chicken	ST2518	B2	$bla_{CTX-M-3}$, *rmtB*, *strA*, *strB*, *tet*(A)	I	pECF12 (77,822 bp)	F33:A$^-$:B$^-$	D1	*pemKI*
DH286M	Chicken	ST617	B2	$bla_{CTX-M-55}$, *floR*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	~120 kb	F33:A$^-$:B$^-$	NT	*pemKI*, *hok-mok-sok*, *srnB-srnC*
JL12G	Cow	ST559	B2	$bla_{CTX-M-55}$, *floR*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11-like	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
JL15P	Cow	ST209	B2	$bla_{CTX-M-55}$, *floR*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11-like	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
JL17P	Cow	ST359	A	$bla_{CTX-M-65}$, *tet*(A)	III	~100 kb	N (ST7)	NT	*pemKI*, *hok-mok-sok*, *srnB-srnC*
JT14G	Cow	ST5689	B1	$bla_{CTX-M-14}$, *floR*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~95 kb	N	A3	*pemKI*, *hok-mok-sok*, *srnB-srnC*
JT14P	Cow	ST5714	B1	$bla_{CTX-M-14}$, *floR*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~95 kb	N	A4	*pemKI*, *hok-mok-sok*, *srnB-srnC*
HL12L	Cow	ST195	D	$bla_{CTX-M-55}$, *floR*, *strA*, *strB*, *tet*(A)	I	~97 kb	F33:A$^-$:B$^-$	C1	*pemKI*, *hok-mok-sok*, *srnB-srnC*
HL36B	Cow	ST4680	B1	$bla_{CTX-M-55}$, *strA*, *strB*, *tet*(A)	I	~100 kb	N	A3	*pemKI*
HL40C	Farmer	ST4680	B1	$bla_{CTX-M-14}$, *floR*, *aadA2*, bla_{TEM-1}	IV	~105 kb	IncI1 (ST136)	E2	None
HB37B	Chicken	ST1725	B1	$bla_{CTX-M-55}$, *floR*, *rmtB*, *strA*, *strB*, *tet*(A)	I	pECF12-like	F33:A$^-$:B$^-$	D1	*pemKI*
HB35D	Chicken	ST48	B2	$bla_{CTX-M-123}$	III	~90 kb	F14:A$^-$:B$^-$	NT	*pemKI*, *hok-mok-sok*, *srnB-srnC*
HB38B	Cow	ST2055	B1	$bla_{CTX-M-14}$, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~140 kb	IncI1 (ST136)	E3	*pemKI*
HB38L	Cow	ST5693	B1	$bla_{CTX-M-14}$, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~120 kb	IncI1 (ST136)	E4	None
HB18F	Cow	ST1081	B1	$bla_{CTX-M-14}$, *floR*, *rmtB*, *aadA2*, bla_{TEM-1}	IV	~140 kb	IncI1 (ST136)	E3	*pemKI*
HB13B	Chicken	ST167	B2	$bla_{CTX-M-55}$, *rmtB*, *strA*, *strB*, *tet*(A)	I	pECF12-like	F33:A$^-$:B$^-$	D2	*pemKI*
HB13M	Cow	ST4463	B2	$bla_{CTX-M-65}$, *rmtB*, *tet*(A)	III	~120 kb	N	NT	None
HB312G	Cow	ST685	B2	$bla_{CTX-M-65}$, *floR*, *rmtB*, *strA*, *strB*, *tet*(A)	III	~120 kb	IncI1 (ST136)	E4	None
SH312M	Pig	ST1488	B2	$bla_{CTX-M-55}$, *floR*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11-like	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SH312N	Pig	ST1313	B2	$bla_{CTX-M-65}$, *floR*, *oqxA*, *oqxB*	III	~120 kb	N	B	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SH21F	Pig	ST209	B2	$bla_{CTX-M-55}$, *floR*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11-like	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SH21G	Pig	ST354	D	$bla_{CTX-M-55}$, *floR*, *cfr*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11-like	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SH21M	Pig	ST648	D	$bla_{CTX-M-55}$, *floR*, bla_{TEM-1}, *strA*, *strB*, *tet*(A)	II	pECB11-like	F33:A$^-$:B$^-$	C2	*pemKI*, *hok-mok-sok*, *srnB-srnC*
SH33L	Cow	ST3743	B1	$bla_{CTX-M-65}$, *tet*(A)	III	~100 kb	IncI1 (ST136)	E5	None

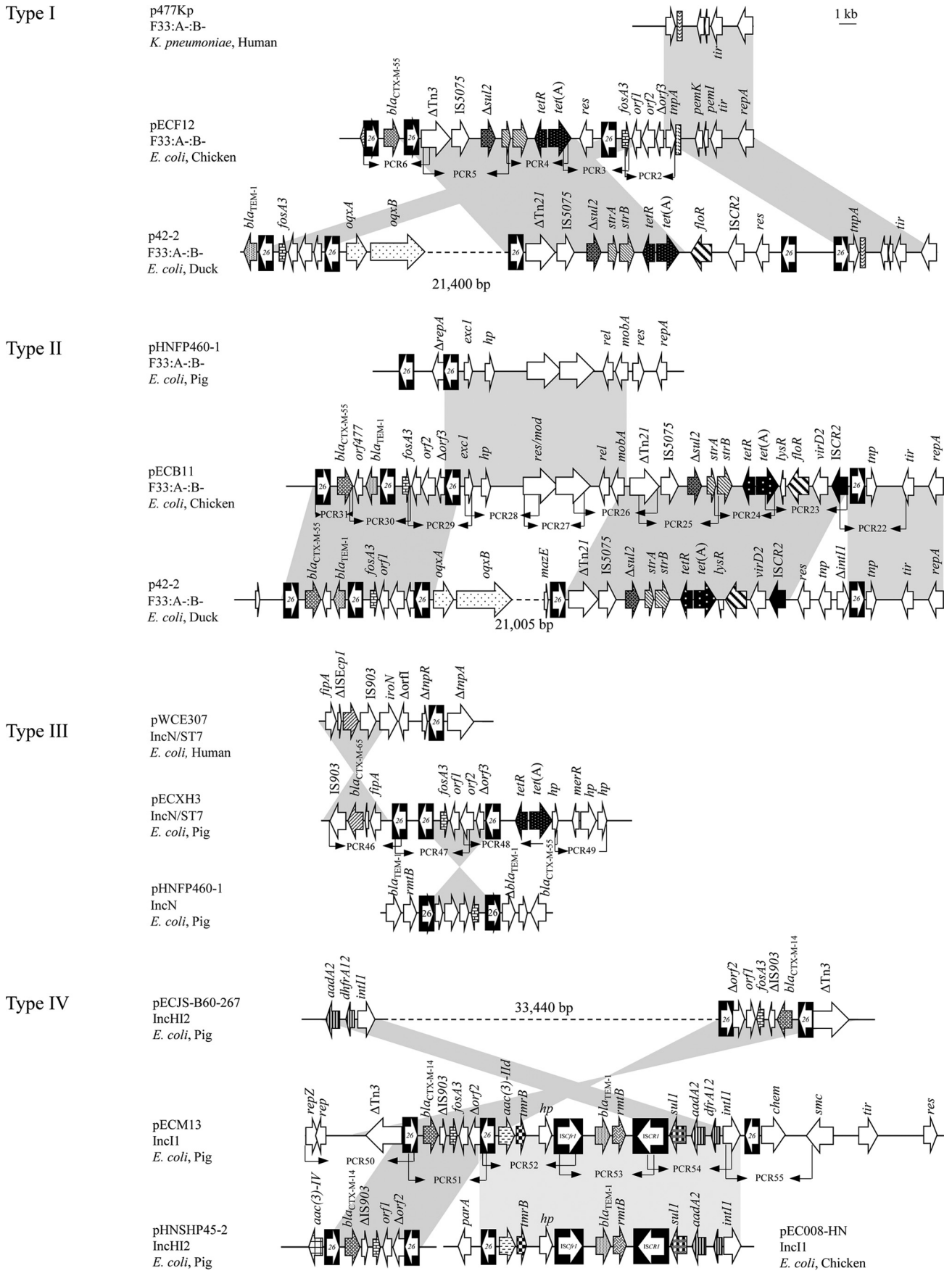
[a]The first two capital letters in each strain name represent the city in which the isolate was obtained: SY, Shenyang (Liaoning Province); CH, Changchun (Jilin Province); DL, Dehui (Jilin Province); JL, Jilin (Jilin Province); JT, Jiutai (Jilin Province); HL, Hailaer (Inner Mongolia autonomous region); HB, Harbin (Heilongjiang Province); SH, Suihua (Heilongjiang Province). Isolates from healthy animals are underlined.

[b]Resistance genes were transferred by conjugation and determined by PCR.

[c]RFLP patterns were assigned to the same major RFLP profiles (A–E) when they differed in ≤3 bands. The numbers (e.g., A1, A2 etc.) indicate minor variations within the RFLP patterns. NT, not typeable.

harbored bla_{CTX-M} genes, and 15 (38.5%) of them produced CTX-M-55 (Table 1). In addition, 29 (74.4%), 22 (56.4%), 20 (51.3%), and 20 (51.3%) isolates carried *floR*, *tet*(A), bla_{TEM-1}, and *rmtB* genes, respectively (Table 1). Pulsed-field gel electrophoresis (PFGE) analysis of the original 39 *fosA3*-positive *E. coli* isolates revealed great genetic diversity of the isolates, with 29 major XbaI patterns (see Table S2 in the supplemental material). The results of multilocus sequence typing (MLST) and phylogenetic analysis also revealed a great diversity. Thirty-seven different sequence types (STs) were distributed among 39 *fosA3*-positive *E. coli* isolates (Table 1). Most of these strains belonged to the phylogenetic grouping B2 (60.0%), followed by B1 (25.6%), A1 (7.7%), and D (7.7%).

Conjugation by filter mating was conducted as previously described (13) to determine the transferability of *fosA3*-carrying plasmids. Plasmids of the transconjugants were analyzed by S1 nuclease PFGE and Southern blot hybridization using a *fosA3*-specific probe. Each transconjugant carried a single *fosA3*-positive plasmid, which ranged in size between 70 and 140 kb and was assigned to IncFII (n = 18), IncN (n = 12), or IncI1 (n = 9) replicon type (14–16) (Table 1). Restriction fragment length polymorphism (RFLP) analysis was successfully performed on 34 *fosA3*-carrying plasmids, but only five EcoRI RFLP profiles were detected, suggesting the presence of


Type I
p477Kp
F33:A-:B-
K. pneumoniae, Human
1 kb
pECF12
F33:A-:B-
E. coli, Chicken
p42-2
F33:A-:B-
E. coli, Duck
21,400 bp
Type II
pHNFP460-1
F33:A-:B-
E. coli, Pig
pECB11
F33:A-:B-
E. coli, Chicken
p42-2
F33:A-:B-
E. coli, Duck
21,005 bp
Type III
pWCE307
IncN/ST7
E. coli, Human
pECXH3
IncN/ST7
E. coli, Pig
pHNFP460-1
IncN
E. coli, Pig
Type IV
pECJS-B60-267
IncHI2
E. coli, Pig
33,440 bp
pECM13
IncI1
E. coli, Pig
pHNSHP45-2
IncHI2
E. coli, Pig
pEC008-HN
IncI1
E. coli, Chicken

several epidemic plasmids that may be responsible for the dissemination of *fosA3* in northeast China.

Based on several parameters (i.e., size, EcoRI RFLP profile, and replicon type), four representative *fosA3*-positive plasmids, namely, pECF12 (F33:A$^-$:B$^-$), pECB11 (F33:A$^-$:B$^-$), pECM13 (IncI1), and pECXH3 (IncN), were subjected to complete plasmid sequencing using the next-generation Illumina MiSeq system. The draft sequences of the plasmids were assembled by the GS *de novo* Assembler (version 2.8), and gap closure of the plasmids was done by PCR and Sanger sequencing. Except for pECXH3, from which only a fragment of 16,293 bp carrying the *fosA3* gene was obtained, the remaining three plasmids were successfully sequenced and assembled. A PCR mapping approach served to determine the genetic context of the *fosA3* gene in the remaining *fosA3*-positive plasmids. Based on the obtained sequences harboring *fosA3* in this study, a series of primers (Fig. 1; see also Table S3 in the supplemental material) were designed to amplify several overlapping PCR fragments containing *fosA3*. Subsequently, all relevant PCR amplicons were cloned into pEASY-T1 and sequenced by primer walking (Invitrogen, Beijing, China). Four genetic environments of *fosA3* were identified among the 39 plasmids and designated types I through IV (Fig. 1).

The type I genetic environment is associated with the F33:A$^-$:B$^-$ plasmid pECF12, which is 77,822 bp in length, has a mean G+C content of 53.1%, and contains 52 open reading frames (ORFs) with known functions (see Table S4 in the supplemental material). The pECF12 backbone of 59,497 bp showed high homology with F33:A$^-$:B$^-$ plasmids, such as p477kp from human *Klebsiella pneumoniae* (17) and p42-2 (GenBank accession number KT990220, duck *E. coli*, China). The multidrug resistance region (MDRR) of 18,325 bp in pECF12 is bracketed on the left-hand side by the IS*26* element and on the right-hand side by the ΔIS*1* element. It contains three resistance genes, $bla_{CTX\text{-}M\text{-}55}$, *tet*(A), and *fosA3*. The segment of 3,270 bp (IS*26*-*fosA3*-*orf1*-*orf2*-Δ*orf3*) represents one of the most common genetic environments of *fosA3* which was detected among eight *fosA3*-positive *E. coli* isolates from different cities and hosts in the present study (Table 1).

Plasmid pECB11, which harbors the type II structure of *fosA3* environments, has a size of 92,545 bp, of which 61,191 bp represents the IncFII typical backbone segment encoding genes for plasmid replication, maintenance, conjugative transfer, and stability functions (see Table S5 in the supplemental material). The 31,354-bp MDRR of pECB11 has a mosaic structure, scattering four IS*26* elements flanking two different fragments that harbor several resistance genes. The first segment of 7,724 bp corresponds to the IS*26*-formed composite transposon carrying the $bla_{CTX\text{-}M\text{-}55}$, $bla_{TEM\text{-}1}$, and *fosA3* genes, which has been seen in several plasmids (e.g., p42-2). The second segment of 13,378 bp, containing the resistance genes *floR*, *tet*(A), *strA*, *strB*, and Δ*sul2*, shared high nucleotide sequence identity with that found in p42-2 except for a 1,966-bp insertion between the 5′ end of the IS*CR2* and upstream of the IS*26* element (Fig. 1).

A 16,293-bp *fosA3*-carrying segment containing 17 ORFs was identified in the IncN/ST9 plasmid pECXH3 (see Table S6 in the supplemental material). This segment contains the type III structure of *fosA3* genetic environments. In pECXH3, the *fosA3* gene was located in an IS*26*-formed composite transposon (IS*26*-*fosA3*-*orf1*-*orf2*-Δ*orf3*-IS*26*), which has been seen in plasmids of many replicon types found among isolates of diverse animal and human origins, such as pHNFP460-1 (KJ020575, pig *E. coli*, China, 2016), pEC012 (KT282968, chicken *E. coli*, China, 2016), and pSLK172-2 (CP017633, human *E. coli*, China, 2017) (Fig. 1).

Plasmid pECM13 has a size of 113,006 bp and an average G+C content of 50.6% and

FIG 1 Schematic presentation and comparison of the genetic environment of *fosA3* in four plasmids with other related plasmids. The arrows indicate the positions and directions of transcription of the genes. Gray shading indicates >99% nucleotide sequence identity. PCR2-PCR6, PCR22-PCR31, PCR46-PCR49, and PCR50-PCR55 used to investigate the type I, II, III, and IV structures of the *fosA3* environment are indicated by black arrowheads. Δ indicates a truncated gene. A 1-kb distance scale is displayed in the upper right corner. Diagrams were drawn from sequences deposited in the GenBank database under accession numbers LN897475 (p477kp), KT990220 (p42-2), KJ020575 (pHNFP460-1), HM440049 (pWCE307), KX254341 (pECJS-B60-267), KU341381 (pHNSHP45-2), and KP010147 (pEC008-HN).

comprises 58 putative ORFs (see Table S7 in the supplemental material). This plasmid was assigned to IncI1/ST136 and harbored a novel *fosA3* genetic environment, designated type IV. This genetic environment was detected in plasmids of two different replicon types among 11 *fosA3*-carrying *E. coli* isolates in this study, including the single isolate from a dairy farm worker. Like other resistance plasmids, pECM13 was divided into two parts, including the 90,949-bp plasmid backbone segment and a 22,057-bp MDRR. The pECM13 backbone is highly similar to that of other IncI1 plasmids, such as pEC15I_1 (KU932029, human *E. coli*, Finland, 2016) and pFAM22871_2 (KU355874, dairy cow *E. coli*, Switzerland, 2016). The MDRR of pECM13 also had a mosaic structure and contained five intact mobile elements (three IS*26* elements, IS*Cfr1*, and IS*CR1*) and nine resistance genes. Based on sequence comparisons, two transferable units existed in the MDRR of pECM13. The first transferable unit corresponded to the IS*26*-formed *fosA3*-carrying composite transposon, which has been found so far in the *mcr-1*-harboring plasmid pHNSHP45-2 from a pig *E. coli* isolate in China (18). The segment IS*26-tmrB*-IS*Cfr1-sul2-aadA2-dfrA12-intI1* was the second transferable unit, which has also been seen in another multidrug-resistant plasmid pEC088-HN (GenBank accession number KP010147) from a chicken *E. coli* isolate in China. Within this segment, the tunicamycin resistance gene, *tmrB*, coding for a protein of 180 amino acids (aa) was detected downstream of the *aac*(*3*)-*IId* gene.

Based on the known three complete sequences, a total of 73 overlapping PCRs were designed (see Table S3) to amplify 20, 25, and 27 partly overlapping regions covering the whole sequences of pECF12, pECB11, and pECM13, respectively. All replicons were cloned and sequenced. The results revealed that two, six, and two *E. coli* strains carried virtually the same pECF12-like, pECB11-like, and pECM13-like plasmids, respectively, except for a difference in a few nucleotides compared to the corresponding completely sequenced plasmids. These findings further demonstrate that three epidemic plasmids are maintained in northeast China and appear to be highly efficient vectors in the spreading of *fosA3* and several other resistance genes (e.g., $bla_{CTX\text{-}M}$ variants, *floR*, and *rmtB*).

Accession number(s). The complete sequences of three plasmids and a partial sequence from pECXH3 have been submitted to the NCBI database with accession numbers KY865321 (pECB11), KY865322 (pECF12), KY865323 (pECM13), and KY865324 (pECXH3).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00859-17.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (grant 2016YFD0501304).

We declare no conflicts of interest.

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