Evolution and Comparative Genomics of F33:A−:B− Plasmids Carrying $\text{bla}_{\text{CTX-M-55}}$ or $\text{bla}_{\text{CTX-M-65}}$ in Escherichia coli and Klebsiella pneumoniae Isolated from Animals, Food Products, and Humans in China

Jing Wang, Zhen-Ling Zeng, Xin-Yi Huang, Zhen-Bao Ma, Ze-Wen Guo, Lu-Chao Lv, Ying-Bi Xia, Li Zeng, Qian-Hua Song, Jian-Hua Liu

*College of Veterinary Medicine, Key Laboratory of Zoonosis of Ministry of Agricultural and Rural Affairs, South China Agricultural University, Guangzhou, China

ABSTRACT To understand the underlying evolution process of F33:A−:B− plasmids among Enterobacteriaceae isolates of various origins in China, the complete sequences of 17 $\text{bla}_{\text{CTX-M}}$-harboring F33:A−:B− plasmids obtained from Escherichia coli and Klebsiella pneumoniae isolates from different sources (animals, animal-derived food, and human clinics) in China were determined. F33:A−:B− plasmids shared similar plasmid backbones comprising replication, leading, and conjugative transfer regions and differed by the numbers of repeats in $\text{yddA}$ and $\text{traD}$ and by the presence of group II intron, except that pHNAH9 lacked a large segment of the leading and transfer regions. The variable regions of F33:A−:B− plasmids were distinct and were inserted downstream of the addiction system $\text{pemI}/\text{pemK}$, identified as the integration hot spot among F33:A−:B− plasmids. The variable region contained resistance genes and mobile elements or contained segments from other types of plasmids, such as IncI1, IncN1, and IncX1. Three plasmids encoding CTX-M-65 were very similar to our previously described pHN7A8 plasmid. Four CTX-M-55-producing plasmids contained multidrug resistance regions related to that of F2:A−:B− plasmid pHK23a from Hong Kong. Five plasmids with IncN and/or IncX replication regions and IncI1-backbone fragments had variable regions related to those of pE80 and p42-2. The remaining five plasmids with IncN replicons and an IncI1 segment also possessed closely related variable regions. The diversity in variable regions was presumably associated with rearrangements, insertions, and/or deletions mediated by mobile elements, such as IS26 and IS1294.

IMPORTANCE Worldwide spread of antibiotic resistance genes among Enterobacteriaceae isolates is of great concern. F33:A−:B− plasmids are important vectors of resistance genes, such as $\text{bla}_{\text{CTX-M-55}}$, $\text{bla}_{\text{NDM-1}}$, $\text{fosA3}$, and $\text{rmtB}$, among E. coli isolates from various sources in China. We determined and compared the complete sequences of 17 F33:A−:B− plasmids from various sources. These plasmids appear to have evolved from the same ancestor by mobile element-mediated rearrangement, acquisition, and/or loss of resistance modules and similar IncN1, IncI1, and/or IncX1 plasmid backbone segments. Our findings highlight the evolutionary potential of F33:A−:B− plasmids as efficient vectors to capture and diffuse clinically relevant resistance genes.

KEYWORDS IncFII, antimicrobial resistance, expanded-spectrum $\beta$-lactamases, IncFII

Plasmids are crucial vehicles for worldwide spread of antibiotic resistance genes in Gram-negative bacteria. Several plasmid families, such as IncF, IncI1, IncI2, IncX, IncA/C, and IncHI2, play an important role in the global dissemination of expanded-
spectrum β-lactamase genes, AmpC β-lactamase genes, carbapenemase genes, plasmid-mediated quinolone resistance genes, and colistin resistance gene mcr-1 in *Enterobacteriaceae* isolates (1–3).

F33:A−B− plasmids are some of the most prevalent replicon sequence types (RSTs) among IncF multiresistance plasmids from *Escherichia coli* isolates of animal origin in China (4). Our previous studies confirmed that F33:A−B− plasmids were major vehicles for fosA3-blaCTX-M-65−rmtB transmission among food animals and pets in Guangdong Province, China (5, 6); similar F33:A−B− plasmids associated with rmtB-blaCTX-M-65 were disseminated in pets in southern China and in a pig farm and its environment in eastern China (7, 8). In addition, F33:A−B− or IncN-F33:A−B− plasmids were responsible for the dissemination of fosA3 and blaCTX-M-55/65 genes in *E. coli* from chickens in China and pigs, chickens, and dairy cows in northeast China (9–11). Furthermore, oqxAB colocalated with blaCTX-M-55 on F33:A−B− and IncN-F33:A−B− plasmids was identified in food-producing animals, chicken meat, and humans in China (12–15). Recently, F33:A−B− plasmids were also described as carriers of blanDM from porcine *E. coli* isolates in China (16). Interestingly, two plasmids, p397Kp and p477Kp, which are highly similar to our previously sequenced *E. coli* F33:A−B− pHN7A8 plasmid (blaCTX-M-65, fosA3, and rmtB) collected from a dog (17), were identified in clinical *Klebsiella pneumoniae* isolates from the Bolivian Chaco region (18). Taken together, previous studies suggest that F33:A−B− plasmids involved in the spread of blaCTX-M− blanDM, fosA3, rmtB, and oqxAB have been efficiently and widely disseminated in *E. coli* strains of various origins, particularly animals, in China (see Table S1 in the supplemental material).

Here, we aimed to determine and compare the complete sequences of 17 F33:A−B− plasmids harboring blaCTX-M obtained from *E. coli* and *K. pneumoniae* isolates from different sources (animals, animal-derived food products, and human clinics), providing new insights into the evolution of F33:A−B− plasmids among *Enterobacteriaceae* isolates of various origins in China.

**RESULTS AND DISCUSSION**

**Strains and F33:A−B− resistance plasmids.** Complete nucleotide sequences were determined for 17 F33:A−B− transmissible plasmids obtained from 15 *E. coli* isolates from food-producing animals, food, and patients and 2 *K. pneumoniae* isolates from pork (Table 1). The plasmids ranged in size between 55,683 and 145,804 bp and contained 2 to 11 resistance genes (Table 1 and 2). Eleven *E. coli* isolates carrying F33:A−B− plasmids subjected to multilocus sequence typing (MLST), either previously (9, 14) or in this study, were identified as 11 different sequence types (STs), and two *K. pneumoniae* isolates belonged to ST35. They were distinct from previously described isolates carrying F33:A−B− resistance plasmids (see Table S1 in the supplemental material), further indicating the important role of F33:A−B− plasmids in the horizontal transfer of resistance genes between bacteria and in the adaptation of these plasmids to different hosts with genomic differences (Table 1).

**The backbone sequences of F33:A−B− plasmids are highly conserved.** All plasmids possessed the same overall backbone organization as F33:A−B− plasmid pHN7A8, corresponding to replication, leading, and transfer regions, with the exception of pHNAH9 (Fig. 1). The replication regions of all 17 plasmids (repA2/copB-repA1-repA4) were identical to those of many F33:A−B− plasmids. The leading region contained genes related to plasmid maintenance and stability, such as peml/pemK, stbA/stbB, and sok/hok/mok, and showed 99% identity to many F33:A−B− plasmids, but a large segment of the leading region was absent in pHNAH9, which might have been the result of a recombination event between ycgA and trbI. The integration hot spot among F33:A−B− plasmids was identified downstream of the addiction system peml/pemK, in which the variable region comprising backbone segments from other plasmid types and/or resistance modules was inserted and bounded at both ends by fragments of IS1, with the exception that pemK was truncated by IS1294 in pHNF460-1 (Fig. 2, 3, and 4). In addition, five plasmids in this study and plasmid pHN7A8 carried a putative group II
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**TABLE 1 General features of F33:A−B− plasmids analyzed in this study and of related reference plasmids for comparative analysis**

| Strain     | Species     | MLSTb | Plasmid     | GenBank accession no. | Location                   | Yr of isolation | Isolate origin     | Reference or source |
|------------|-------------|-------|-------------|------------------------|----------------------------|-----------------|-------------------|---------------------|
| HN7A8      | E. coli     | ND    | pHN7A8      | JN232517               | Guangdong Province, China  | 2008            | Dog               | 17                  |
| FPK460#    | E. coli     | 354   | pHNFP460-1  | KJ20575                | Guangdong Province, China  | 2010            | Pig               | 29                  |
| 04NH83     | E. coli     | ND    | pHN04NH83   | MG197488               | Guangdong Province, China  | 2009            | Duck              | 29                  |
| MOCh2      | E. coli     | 2732  | pHMC02      | MG197490               | Guangdong Province, China  | 2009            | Chicken           | 29                  |
| FPKD271#   | E. coli     | ND    | pHNFKD271   | MG197490               | Guangdong Province, China  | 2010            | Duck              | 29                  |
| FPKU92#    | E. coli     | ND    | pHNFKU92    | MG197491               | Guangdong Province, China  | 2013            | Duck              | This study          |
| GDKP4177#  | E. coli     | ND    | pHNMGDP4177 | MG197492               | Guangdong Province, China  | 2014            | Pig               | This study          |
| AHC9       | E. coli     | 48    | pHNAH9      | MG197493               | Anhui Province, China      | 2011            | Chicken           | 9                   |
| AHC17      | E. coli     | 4483  | pHNAH17     | MG197494               | Anhui Province, China      | 2011            | Chicken           | 9                   |
| AHC24      | E. coli     | 155   | pHNAH24     | MG197495               | Anhui Province, China      | 2011            | Chicken           | 9                   |
| AHC33      | E. coli     | 101   | pHNAH33     | MG197496               | Anhui Province, China      | 2011            | Chicken           | 9                   |
| HN02       | E. coli     | 4464  | pHNNHC02    | MG197497               | Henan Province, China      | 2009            | Chicken           | 9                   |
| HZMCC14*   | E. coli     | 1290  | pHNMCC14    | MG197498               | Guangdong Province, China  | 2011            | Chicken meat      | This study          |
| HZMPC32*   | E. coli     | New   | pHNMPC32    | MG197499               | Guangdong Province, China  | 2011            | Pork              | This study          |
| HZMPC51-2* | K. pneumoniae| 35    | pHNMPC51    | MG197500               | Guangdong Province, China  | 2011            | Pork              | This study          |
| HZMPC43-3* | K. pneumoniae| 35    | pHNMPC43    | MG197501               | Guangdong Province, China  | 2011            | Pork              | This study          |
| ZYTF32*    | E. coli     | 58    | pHNZY32     | MG197502               | Guangdong Province, China  | 2013            | Patient           | 14                  |
| ZYTM118*   | E. coli     | New   | pHNZY118    | MG197503               | Guangdong Province, China  | 2013            | Patient           | 14                  |
| 397Kp      | K. pneumoniae| 726   | p397Kp      | LNB97474               | Bolivia                    | 2013            | Patient           | 18                  |
| 477Kp      | K. pneumoniae| 726   | p477Kp      | LNB97475               | Bolivia                    | 2013            | Patient           | 18                  |
| HN02       | E. coli     | ND    | pHN02       | KJ20575                | Guangdong Province, China  | 2010            | Pig               | 16                  |
| HN04       | E. coli     | ND    | pHN04       | KJ20575                | Guangdong Province, China  | 2010            | Pig               | 16                  |
| HZMCC14*   | E. coli     | 1290  | pHNMCC14    | MG197498               | Guangdong Province, China  | 2011            | Chicken meat      | This study          |
| HZMPC32*   | E. coli     | New   | pHNMPC32    | MG197499               | Guangdong Province, China  | 2011            | Pork              | This study          |
| HZMPC51-2* | K. pneumoniae| 35    | pHNMPC51    | MG197500               | Guangdong Province, China  | 2011            | Pork              | This study          |
| HZMPC43-3* | K. pneumoniae| 35    | pHNMPC43    | MG197501               | Guangdong Province, China  | 2011            | Pork              | This study          |
| ZYTF32*    | E. coli     | 58    | pHNZY32     | MG197502               | Guangdong Province, China  | 2013            | Patient           | 14                  |
| ZYTM118*   | E. coli     | New   | pHNZY118    | MG197503               | Guangdong Province, China  | 2013            | Patient           | 14                  |
| 397Kp      | K. pneumoniae| 726   | p397Kp      | LNB97474               | Bolivia                    | 2013            | Patient           | 18                  |
| 477Kp      | K. pneumoniae| 726   | p477Kp      | LNB97475               | Bolivia                    | 2013            | Patient           | 18                  |

*Plasmids with names in bold typeface were sequenced in this study. Abbreviations: NA, not available; ND, not determined. Strains isolated from Guangzhou, Foshan, or Shenzhen are indicated by an asterisk, pound sign, or triangle.

*The HZMPC32 isolate was identified as a new MLST with alleles adk457, furC65, gyrB5, icd16, msh11, purA8, and recA6. The ZYTM118 isolate was previously identified as a new MLST with alleles adk64, furC23, gyrB58, icd91, msh307, purA7, and recA2 (13).

intron inserted downstream of the ycaA gene, as identified in pHNEC55, p397Kp, and p477Kp but not pECB11, pSLK172-2, p42-2, and pE80 (Table 2). Furthermore, different numbers of 6-bp tandem repeats (GCTACT) in ydaA were present in F33:A−B− plasmids (Table 2). The transfer regions of plasmids were highly similar to those of plasmid pHN7A8 and other F33:A−B− plasmids and differed by the numbers of CAACAGCCG tandem repeats in the traD gene (Table 2). However, possibly due to multiple recombination events, pHNAH9 lacked large parts of the tra-trb region, which might account for its conjugation failure (9). Plasmids related to pHN7A8 producing CTX-M-65. Plasmids pHNNHC02 (E. coli, chicken) and pHNMPC32 (E. coli, pork) showed high gene synteny with pHN7A8 (E. coli, dog), p397Kp (K. pneumoniae, human), and p477Kp (K. pneumoniae, human) (Fig. 1 and 2A). These plasmids differed by only three to eight nucleotide changes, by various numbers of 6-bp repeats in ydaA and/or 9-bp repeats in traD (Table 2), and by the absence of a 2,095-bp segment (fosA3-orf1-IS26) on pHNMPC32 and p477Kp. The generation of a circular molecule by recombination between the two copies of IS26 in the same orientation could lead to the insertion or loss of the fosA3 segment.

Plasmid pHNHA33 (E. coli, chicken) was highly related to pHN7A8 but with a different arrangement of multidrug resistance region (MMR) (Fig. 1 and 2). The segments corresponding to the typical transposition unit (ISEcp1-blaCTX-M-65-IS903-iroN) inserted in Tn1722 were identical in two plasmids, with a single nucleotide change in ISEcp1. The rearrangement of this segment in pHNHA33 may have arisen from IS26-mediated homologous recombination or transposition. In pHN7A8, the fosA3 resistance module was followed by an incomplete Tn2 sequence containing β-lactam resistance gene blaTEM-1B, interrupted by IS1294 at a resolvase gene (tnpR). Unlike a typical mobile element, IS1294 lacks terminal inverted repeats (TIRs), fails to generate direct repeats (DRs) of the target site, and exhibits a target site insertion with preferred tetranucleotide sequence GTTC or CTTG (19). In pHNHA33, the fosA3 module was truncated by
IS1294, followed by a partial Tn2 (ΔtnpR-bla<sub>TEM-1b</sub>), leading to the replacement of 525 bp downstream of fosA3, IS26, and 221-bp ΔTn2 sequences compared with pHN7A8. The insertion of an extra copy of IS1294 at the GTTC target site within the fosA3 module followed by homologous recombination between IS1294 elements may have led to the deletion of the 1,566-bp region plus one IS1294 element (Fig. 2B). On the other hand, the pHNCE55 (E. coli, pig) MRR possessed a structure related to those of pHN7A8 and pHNHNC02, but likely recombination events between IS<sub>26</sub> elements resulted in the acquisition of a 9,637-bp region harboring bla<sub>NDM-1</sub> and loss of the bla<sub>CTX-M-65</sub> module (Fig. 2A and B).

CTX-M-55-producing plasmids pHMPC43, pHMPC51, pHNGD4P177, and pHMNC02 MRRs: homologous recombination in IS26. Plasmids pHNGD4P177 (pig) and pHMNC02 (chicken) were identical except for the numbers of repeats in yddA and traD (Table 2) (Fig. 1 and 2C). The pHNGD4P177 and pHMNC02 MRRs were similar to the MRR in F2A-:B—plasmid pHK23a (GenBank accession number JQ432559) recovered from a slaughtered pig in Hong Kong (Fig. 2C) (20). A 5,847-bp region found in pHNGD4P177 and pHMNC02, containing three IS26 elements flanking two different segments associated with parts of a 5'-conserved segment (5'-CS) and 3'-CS and the fosA3 resistance module, was identical to that of pHK23a with opposite orientation. This observation may be associated with homologous recombination between IS26 elements located in inverse orientations. In addition, a structure comprising a truncated bla<sub>TEM-1b</sub> and bla<sub>CTX-M-55</sub> within its typical (ΔSEcp1-bla<sub>CTX-M-55</sub>-orf477) transposition unit with a 127-bp spacer was found downstream. A similar structure was found in pHK23a with two nucleotide changes giving bla<sub>CTX-M-55</sub> rather than bla<sub>CTX-M-55</sub>.

Plasmids pHMPC51 and pHMPC43 from ST35 K. pneumoniae isolates from pork showed high similarity but differed by only three nucleotide changes and repeats in yddA (Table 2) (Fig. 1 and 2C). The pHMPC43/pHMPC51 MRRs lacked the fosA3 module and one IS26 element compared with MRRs in pHNGD4P177/pHMNC02, which could be explained by IS26-mediated homologous recombination.
Variable regions of IncN1-F33:A–B– plasmids pHNAH9, pHNAH17, pHNF460-1, pHNFD271, and pHN04NHB3 are closely related. The pHNDG4P177-like plasmid MRR may have acquired an approximately 25.8-kb segment to generate the variable region of pHNAH17, as the genetic structure upstream and downstream of this ~25.8-kb segment showed 100% nucleotide identity with that found in pHNDG4P177 MRR (Fig. 3).

As a multireplicon plasmid, pHNAH17 harbored an approximately 3-kb segment (ΔrepA-iteron I-CUP6-resP) corresponding to IncN1 plasmid replication region (Fig. 3). In pHNAH17, IncN replication initiation gene repA was truncated by IS26 at the 5′ end, and 31 tandem repeats of 37 bp were observed within an iteron region which played an important role in determining plasmid replication and copy number control (21, 22). A 9,962-bp segment was located upstream of an IncN replication region and contained 10 putative open reading frames (ORFs). The segment shows 99% identity to a fragment of IncN plasmid pABWA45_3 (CP022157) together with the downstream IncN replication region, suggesting that pABWA45_3-like plasmids may have been the sources (Fig. 3).

An approximately 9.4-kb fragment was located downstream of an IncN region and displayed 99% identity to that of the archetypal IncN1 plasmid R64 (AP005147) back-
bone carrying several ORFs such as the vagCD addiction system (Fig. 3) (23). pHNAH17 may have acquired this segment from an IncI1 plasmid by the following two main events: (i) insertion of IS\textsubscript{1294} at target site 5'-GAAC into \textit{ydjA} and (ii) transposition of IS\textsubscript{1294} together with the adjacent IncI1 segment by rolling-circle replication through ori\textsubscript{IS} to an alternative ter\textsubscript{IS} look-alike sequence (19) (Fig. 3). Although we failed to identify ter\textsubscript{IS} look-alike sequence GTTC in the 5'-end of an IncI1 fragment in pHNAH17, it is possible that a longer IncI1 segment ends in GTTC and is mobilized by IS\textsubscript{1294} but is truncated by IS\textsubscript{26} downstream of \textit{arsR2}.

The variable region of pHNAH9 differed from that of pHNAH17 by (i) an ~25.8-kb segment in the opposite orientation that was truncated at IS\textsubscript{26} downstream of Δ\textit{Tn2} by insertion of IS\textsubscript{1294} and (ii) deletions involving the structure corresponding to IS\textsubscript{26}-5'-CS-3'-CS (Fig. 3). It suggested that the ~25.8-kb fragment was inserted into a pHNGD4P177-like MRR through IS\textsubscript{26} homologous recombination and IS\textsubscript{1294} transposition to generate pHNAH17 and that a similar insertion with the opposite orientation had occurred during pHNAH9 evolution together with IS\textsubscript{26}-mediated deletions.

The variable region of pHNFP460-1 was related to pHNAH9 (Fig. 3). The arrangement of the ~25.8-kb fragment was identical to that of pHNAH9 and differed by the absence of a 37-bp repeat within the iteron region (Table 2). However, IS\textsubscript{1294} was inserted in \textit{pemI}/\textit{pemK}, leading to the replacement of 53 bp of the 3' end of \textit{pemK} and the genetic structure (Δ\textit{IS1}-Δ\textit{Tn2}-IS\textsubscript{26}). Furthermore, an approximately 8.2-kb segment consisting of three IS\textsubscript{26} elements flanking two different parts, located between the IncN fragment and the \textit{fosA3} module, was present in pHNFP460-1. The first part corresponded to an additional IncN replication region, which encompassed a truncated (81-bp-shorter) \textit{repA} gene, iteron region II with five tandem 37-bp repeats, \textit{gshB} encoding glutathione synthetase, and 114 bp of the \textit{oriIS} end of IS\textsubscript{1294}. The second part consisted of an incomplete \textit{Tn2} and \textit{rrmB} gene, pHNFP460-1 had an 80-bp longer \textit{Tn2} without IS\textsubscript{1294} insertion than pHN7A8 and a deletion of 335 bp downstream of \textit{rrmB}. This ~8.2-kb segment was identical to a fragment of pABWA45_3 with the exception of an IS\textsubscript{075} insertion, further suggesting that pABWA45_3-like plasmids may be among the sources of IncN1-F33:A:-8-- plasmids (Fig. 3).
FIG 4  Comparison of the variable regions of (A) pHNZY32, pHNZY118, and pHNAH24; (B) pHNMCC14; (C) pHNFKU92; (D) pE80; (E) pECB11; (F) p42-2; and (G) pSLK172-2. The extents and directions of antibiotic resistance in antibiotic resistance genes (thick arrows) and in other
The variable regions of pHNFD271 and pHN04NHB3 were highly similar, with five nucleotide changes (Fig. 3). As observed in IncN1-F33:A—B—plasmids, these plasmids harbored a fragment of pABWA45_3-like plasmids without the second IncN replication region and one IS26 element. IS26-mediated deletion or insertion could readily explain the observed absence or presence. In addition, an approximately 18-kb segment comprising two parts associated with IS1294 and IS26 was present in pHNFD271 and pHN04NHB3. The first of these (~2.9 kb) coded four putative ORFs and was found in multiple plasmids, particularly in F—A13:B—plasmids such as pKPN528-3 (CP020856) and pK245 (QQ449578), suggesting that the F—A13:B—plasmid may be one source. The second part (~15.1 kb) was similar (six single nucleotide polymorphisms [SNPs]) to that of Inc11 pC271 plasmids (LN735561) and exhibited 99% identity with 58% coverage to those of pHNAH9, pHNAH17, and pHNFP460-1. A sequence of approximately 50 bp downstream of the first segment in other F—A13:B—plasmids was highly homologous to the Inc11 fragment, which might have been a site of recombination between these two parts. Therefore, this ~18-kb hybrid segment might have resulted from recombination between an IncN1 fragment and an IncFIA-like fragment. Similarly to pHNAH9 and pHNFP460-1, IS1294—a captured hybrid Inc11 segment together with an IncN fragment—was inserted into the fosA3 module located in the opposite orientation at the GTTC target site, resulting in the deletion of a 1,390-bp fragment within the fosA3 module.

The variable regions of pHNAH9, pHNAH17, pHNFP460-1, pHNFD271, and pHN04NHB3 may have been generated from pHN04D177-like MRR by similar insertions plus deletions or insertions of the appropriate regions.

**Variable regions of pHNZY32, pHNZY118, pHNAH24, pHNMC014, and pHNFKU92 are closely related to those of pECB11, pE80, p42-2, and pSLK172-2.** The variable region of pHNZY32 (E. coli, patient) was the largest and consisted of six regions bounded by IS26 or IS1294 (Fig. 4A). The first segment corresponded to an approximately 2.8-kb structure (AIS1-ΔTn2-IS26-5’CS-3’CS), as observed in other plasmids in this study (e.g., pHNMP43 and pHNMC02 with the same 3’CS/IS26 boundary).

The second segment (~15 kb) contained multiple resistance genes that included *floR* (florfenicol resistance), *tetA/tetR* (tetracycline resistance), *strA/ΔstrB* (streptomycin resistance) associated with Tn5393, and *sul2* (sulfonamide resistance); *isc2* mobile elements; and incomplete Tn5051-like transposon TnAO22 (EU696790) interrupted by IS5075 at 38-bp IR... A similar fragment was previously detected in IncN/C plasmids lacking IS5075-TnAO22 regions such as p112298-tetA (KY986974, Citrobacter freundii) and pAR060302 (FJ621588, E. coli).

The third region was similar to that of pHNFD271 with an opposite orientation and contained an IncN1 pABWA45_3-like plasmid segment and the ~18-kb hybrid Inc11 segment. However, IS5075 was inserted into a hypothetical protein in pHNZY32 and appeared to target a specific sequence similar to the 38-bp TIR of Tn21 (Fig. 4A; see also Fig. S1 in the supplemental material). IS5075 has been described to target a specific position in the 38-bp TIRs of Tn21/Tn501 family transposons (24). Similar IS5075 insertion was observed downstream of iveron II, further suggesting that IS5075 displays insertion site specificity not limited to 38-bp TIR (Fig. 4A; see also Fig. S1).

The fourth segment (4.9 kb) contained the replication region and putative plasmid addiction system (*ddp3-bis-repX-pir-stbD/E*) and was identical to plasmids such as pC25 (KP722020) with the same IS26/IS1294 boundary. In addition, this segment showed 98% identity to IncX1 plasmid pOLAS2, although *repX* encoding a putative replication
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| Plasmid       | Resistance genes          | Replicon type | No. of 6-bp repeats in ydDA | No. of 9-bp repeats in traD | No. of 37-bp repeats in iteron I (IncN) | Group II intron | Addiction systems |
|---------------|---------------------------|---------------|------------------------------|-----------------------------|----------------------------------------|----------------|-------------------|
| pHN7A8        | bla(CTX-M-65) | blαTEM-1b, fosA3, mrtB | F33:A–B | 8 | 9 | + | pemKl, hok-sok, smBC |
| pHNP460-1     | bla(CTX-M-65) | blαTEM-1b, fosA3, mrtB | N1-F33:A–B | 7 | 11 | 30 | + | pemKl, hok-sok, vagCD, smBC |
| pHN04NB8      | bla(CTX-M-65) | blαTEM-1b, fosA3, mrtB | N1-F33:A–B | 8 | 11 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| pHNMC02       | bla(CTX-M-65) | ΔblαTEM-1b, fosA3 | F33:A–B | 7 | 11 | + | pemKl, hok-sok, smBC |
| pHNFK271      | bla(CTX-M-65) | blαTEM-1b, fosA3, mrtB | N1-F33:A–B | 7 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| pHNFKU92      |bla(CTX-M-65) | ΔblαTEM-1b, fosA3, mrtB | X1-F33:A–B | 7 | 11 | + | pemKl, hok-sok, vagCD, smBC |
| pHNGD4P177    | blαTEM-1b, fosA3 | F33:A–B | 8 | 13 | + | pemKl, hok-sok, smBC |
| pHNA9         | blαTEM-1b, fosA3 | N1-F33:A–B | 7 | 11 | 31 | + | pemKl, hok-sok, smBC |
| pHNAH17*      | blαTEM-1b, fosA3 | N1-F33:A–B | 7 | 11 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| pHNAH24       | blαTEM-1b, fosA3, mrtB | N1-X1-F33:A–B | 7 | 11 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| pHNNAH33      | blαTEM-1b, fosA3, mrtB | F33:A–B | 8 | 11 | + | pemKl, hok-sok, smBC |
| pHNHNC02*     | blαTEM-1b, fosA3, mrtB | F33:A–B | 8 | 11 | + | pemKl, hok-sok, smBC |
| pHNMC14       | blαTEM-1b, fosA3, mrtB | F33:A–B | 8 | 11 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| pHNMP332      | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 8 | + | pemKl, hok-sok, smBC |
| pHNMP531      | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 12 | + | pemKl, hok-sok, smBC |
| pHNMP43       | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 12 | + | pemKl, hok-sok, smBC |
| pHNZY32       | blαTEM-1b, fosA3, mrtB | N1-X1-F33:A–B | 7 | 11 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| pHNZY118      | blαTEM-1b, fosA3, mrtB | N1-X1-F33:A–B | 7 | 11 | 31 | + | pemKl, hok-sok, vagCD, smBC |
| p397Kp        | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 8 | + | pemKl, hok-sok, smBC |
| p477Kp        | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 8 | + | pemKl, hok-sok, smBC |
| pHNEG55       | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 8 | + | pemKl, hok-sok, smBC |
| pECE11        | blαTEM-1b, fosA3, mrtB | F33:A–B | 7 | 8 | + | pemKl, hok-sok, smBC |
| p42-2         | blαTEM-1b, fosA3, mrtB | X1-F33:A–B | 6 | 6 | + | pemKl, ΔvagCD, smBC |
| pE80c         | blαTEM-1b, fosA3, mrtB | X1-F33:A–B | 8 | 8 | 28 | + | pemKl, ΔvagCD, smBC |
| pSLK17-2-2    | blαTEM-1b, fosA3, mrtB | X1-F33:A–B | 6 | 4 | + | pemKl, ΔvagCD, smBC |

*pHNAH17 was previously detected to carry mrtB by PCR [9], but plasmid sequencing and further PCR in this study demonstrated that mrtB was not present.

*pHNHNC02 was formerly detected to harbor vagCD by PCR [9], but plasmid sequencing and further PCR in this study confirmed that vagCD was not present.

*One nucleotide was absent in one 37-bp repeat in iteron I in pE80, which might have represented a sequencing problem.
protein belonging to Rep_3 type family was absent in pOLA52 (25). It appeared that IS1294 was inserted into an IncX1-like segment and mobilized together with the adjacent IncX1-like segment, which was truncated by IS26.

The fifth segment (~7.9 kb) was downstream of IS50 and contained aph(3′)-Ila (aminoglycoside), Δble (bleomycin resistance), and oqxAB (quinolone/olaquindox resistance). oqxAB was flanked by two IS26 elements in the same orientation and constituted composite transposon Tn6010, which was first identified in pOLA52 (26).

The last segment comprising the fosA3 module, ΔblaTEM-1B, the typical blaCTX-M-55 transposition unit, was identical to segments in the other plasmids in this study such as pHNMC02 and pHNAH17 with the same IS26/IS1 boundary.

Compared with that of pHNZY32, the variable regions of other similar plasmids differed by one nucleotide change (in pHNHAH24) or three nucleotide changes (in pHNZY118, obtained from the same hospital as pHNZY32); deletions of 14 bp of the 5′ end of IS5075 with ΔTnAO22 downstream and one IS5075 element (in pHNMCC14); or deletions involving the first and second IncN1 plasmid segments and an approximately 12.8-kb hybrid IncI1 segment (in pHNFKU92) (Fig. 4). IS26-mediated deletion may have been responsible for the creation of the similar structures present in these closely related plasmids. In addition, the latter two plasmids harbored an incomplete Tn5393 consisting of 81-bp TIRs, and the entire strA/strB genes, which were identical to those of plasmids pE80, pECB11, p42-2, and pSLK172-2 but 157 bp longer than that of pHNZY32 (Fig. 4).

The variable region of pHNZY32 was related to that of pE80, except for a rearrangement, insertions of mobile elements, and deletions involving ΔTn2 and rmtB (Fig. 4A and D). The fosA3 module consisted of two parts interrupted by IS26, and the first part was in the opposite location compared with pHNZY32. Insertion of an extra copy of IS26 in orf1, followed by homologous recombination between it and the upstream IS26, may explain the generation of the pE80 configuration (Fig. 4D). The pECB11 variable region similarly differed from pHNZY32 by (i) deletions involving structure (IS26-5′CS-3′CS) as well as a 2,157-bp segment comprising three putative ORFs and 527 bp of the terIS end of ISCR2; (ii) the presence of an IncN region associated with partial IS26, located in the opposite orientation without IS5075 insertion; and (iii) the absence of an ~47.4-kb fragment, including an IncN replication region, IncI1 segment, IncX1 segment, and oqxAB resistance region and the second IncN1 segment (Fig. 4E).

Previously described F33:A—B—plasmid p42-2 from a duck and plasmid pSLK172-2 from a patient possessed pHNFKU92-related variable regions (Fig. 4F and G). p42-2 differed from pHNFKU92 by a deletion of 697 bp at the 5′ end of IS1294 upstream of ΔIS50 and by the presence of a 4,496-bp-longer hybrid IncI1 segment, which was truncated by IS26 at vagC/D. The insertion site of IS26 was the same as that of pE80, suggesting the presence of a similar IS26 insertion in vagC/D followed by homologous recombination with another IS26 upstream of TnAO22, leading to deletions of IncN1 and part of hybrid IncI1 segments in p42-2 (Fig. 4E). Similarly to pE80, pSLK172-2 harbored an identical hybrid IncI1 segment present in p42-2. The structure of pSLK172-2 further differed from pHNFKU92 as follows: (i) the opposite locations of 5′-CS and 3′-CS segments; (ii) an IS21 family element ISSso4-like insertion in IS1294; and (iii) acquisition of an IncN1 replication region located between Tn6010 and the fosA3 module.

Conclusion. Comparisons of plasmids in this study to previously described F33: A—B—plasmids revealed similar backbones and similar or distinct variable regions in E. coli isolates or K. pneumoniae of various origins from different geographic areas (mainly China). The distinct architectures of variable regions may have resulted from a series of molecular module rearrangements, acquisitions, or loss mediated by mobile elements such as IS26 and IS1294 via transposition or homologous recombination. We failed to observe DRs flanking IS26 as well as specific target site duplication patterns, suggesting that IS26-mediated insertion, deletion, or reorganization may have occurred by homologous recombination rather than transposition (27). Common mobile ele-
ments present in F33:A--B-- plasmids, especially IS26 and IS1294, not only are able to capture and mobilize antibiotic resistance genes but also are capable of acquiring fragments from other types of plasmids carrying genes involved in plasmid replication or stability or unknown functions through the process of horizontal transfer. In a similar way, other plasmids can also capture fragments of F33:A--B-- plasmids through a recombination event such as that previously observed between IncR and pHN7A8-like plasmid (28). Given that F33:A--B-- plasmids have become efficient vehicles for the dissemination of resistance genes and display the potential to capture more resistance genes or fragments from other type of plasmids through the process of evolution, their rapid spread and efficient persistence among Enterobacteriaceae could pose a serious threat to clinical therapy and public health.

MATERIALS AND METHODS

Bacterial isolates and plasmids. A total of 17 strains carrying F33:A--B--/IncN-F33:A--B-- plasmids were included in this study (Table 1). Isolates AHC9, AHC17, AHC24, AHC33, and HNC02 were found in chicken samples; their transconjugants/transformants carrying F33:A--B-- or IncN-F33:A--B-- plasmids were previously obtained (9). ZYTF32 and ZYTM118 were obtained from a female patient and a male patient, respectively, at the same hospital in Guangzhou, Guangdong Province, and transformants carrying IncN1-F33:A--B-- plasmids were previously described (14). Additionally, four CTX-M-55-producing E. coli strains were recovered from food-producing animals in 2009 to 2010 (29), and six CTX-M-55/65-producing E. coli plasmid carrying fluoroquinolone and retail meat in Guangdong Province, China, from 2011 to 2014. For each of the 17 isolates, transformants in E. coli DH5α were obtained previously or in this study by heat-shock transformation or selected by electroporation using 2 mg/liter cefotaxime, and a single transformant of each strain that had been demonstrated to carry blaCTX-M-55/65 and a single F33:A--B-- plasmid by PCR, sequencing, and pulsed-field gel electrophoresis with S1 nuclease was selected for further study (30, 31). MLST was performed according to previously described protocols (https://pubmlst.org/mplsmlstseqdef) (32, 33).

Sequencing and annotation. Seventeen plasmids were extracted from transformants using a Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany). pHN7A60-1 was sequenced by the use of the Roche 454 GS-FLX platform, and contigs were assembled with 454 GS de novo assembler Newbler version 2.8. pHN7A32 was sequenced using PacBio single-molecule real-time sequencing (RSII platform) (Pacific Biosciences, Menlo Park, CA). Raw sequence data were introduced into the nonhybrid Hierarchical Genome Assembly Process (HGAP version 3). The remaining 15 plasmids were sequenced using Illumina MiSeq technology (Illumina, San Diego, CA). Sequence reads were assembled into contigs by the use of SOAPdenovo version 2.04.

Initial analysis and annotation of contigs were performed using the RAST server (34), Isfinder (https://www.is.biotoul.fr), ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), RAC (http://rac.aihi .nq.edu.au/rac/), BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and Gene Construction kit 4.0 (Textco BioSoftware, Inc., Raleigh, NC). Gaps between contigs were closed by PCR and Sanger sequencing. The replicon types of these plasmids were analyzed using the Plasmid MLST Database (http://pubmlst.org/ plasmid/).

Accession number(s). The nucleotide sequences of all 17 plasmids obtained from this study were deposited in GenBank under the accession numbers listed in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00137-18.

FIG S1, TIF file, 0.04 MB.

TABLE S1, PDF file, 0.4 MB.

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REFERENCES

1. Carattoli A. 2013. Plasmids and the spread of resistance. Int J Med Microbiol 303:298–304. https://doi.org/10.1016/j.ijmm.2013.02.001.

2. Dobiasova H, Dolejska M. 2016. Prevalence and diversity of IncX plasmids carrying fluoroquinolone and β-lactam resistance genes in Escherichia coli originating from diverse sources and geographical areas. J Antimicrob Chemother 71:2118–2124. https://doi.org/10.1093/jac/dkw144.

3. Jeannot K, Bolard A, Plésiat P. 2017. Resistance to polymyxins in Gram-negative organisms. Int J Antimicrob Agents 49:526–535. https://doi.org/10.1016/j.ijantimicag.2016.11.029.

4. Yang QE, Sun J, Li L, Deng H, Liu BT, Fang LX, Liao XP, Liu YH. 2015. IncF plasmid diversity in multi-drug resistant Escherichia coli strains from animals in China. Front Microbiol 6:964. https://doi.org/10.3389/fmicb.2015.00964.
5. Hou J, Yang X, Zeng Z, Lv L, Yang T, Lin D, Liu JH. 2013. Detection of the plasmid-encoded fosfomycin resistance gene fosA3 in Escherichia coli of food-animal origin. J Antimicrob Chemother 68:766–770. https://doi.org/10.1093/jac/dks465.

6. Hou J, Huang X, Deng Y, He L, Yang T, Zeng Z, Chen Z, Liu JH. 2012. Dissemination of the fosfomycin resistance gene fosA3 with CTX-M β-lactamase genes and mtbB carried on IncFI plasmids among Escherichia coli isolates from pets in China. Antimicrob Agents Chemother 56:2135–2138. https://doi.org/10.1128/AAC.05104-11.

7. Deng Y, He L, Chen S, Zheng H, Zeng Z, Liu Y, Sun Y, Ma J, Chen Z, Liu JH. 2011. F33:A- and F2A-:B - plasmids mediate dissemination of mtbB-blaCTX-M genes in Enterobacteriaceae isolates from pets in China. Antimicrob Agents Chemother 55:4926–4929. https://doi.org/10.1128/AAC.0133-11.

8. Yao Q, Zeng Z, Hou J, Deng Y, He L, Tian W, Zheng H, Chen Z, Liu JH. 2011. Dissemination of the mtbB gene on IncF and IncI plasmids among Enterobacteriaceae in a pig farm and its environment. J Antimicrob Chemother 66:2475–2479. https://doi.org/10.1093/jac/dkr328.

9. Yang X, Liu W, Liu Y, Wang J, Lv L, Chen X, He D, Yang T, Hou J, Tan Y, Xing L, Zeng Z, Liu JH. 2014. F33:A-:B- and IncI1-ST3, and IncI1-ST7 plasmids drive the dissemination of fosA3 and blaCTX-M-14 in Escherichia coli from chickens in China. Front Microbiol 5:5888. https://doi.org/10.3389/fmicb.2014.00668.

10. Wang XM, Dong Z, Schwarz S, Zhu Y, Hua X, Zang H, Liu Y, Sun Y, Cheng ST. 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 61:00859-17. https://doi.org/10.1128/AAC.00859-17.

11. Jiang W, Men S, Kong L, Ma S, Yang Y, Wang Y, Yuan Q, Cheng G, Zou W, Wang H. 2017. Prevalence of plasmid-mediated fosfomycin resistance gene fosA3 among CTX-M-producing Escherichia coli isolates from chickens in China. Foodborne Pathog Dis 14:210–218. https://doi.org/10.1089/fpd.2016.2230.

12. Liu BT, Yang QE, Li L, Sun J, Xiao XP, Fang LX, Yang SS, Deng H, Liu YH. 2013. Dissemination and characterization of plasmids carrying copies of blablaCTX-M genes in Escherichia coli isolates from food-producing animals. PLoS One 8:e73947. https://doi.org/10.1371/journal.pone.0073947.

13. Cheng Q, Zhang L, Wang H, Pan T, Li Y, Liu H, Shi G, Chen X, Liu JH. 2016. Complete sequence of the large IncF plasmid pKP1034, harboring fosA3, blaKPC-2, blaCTX-M-65, blaSHV-12, and mrtC from an epidemic Klebsiella pneumoniae sequence type 11 strain in China. Antimicrob Agents Chemother 60:3400–3405. https://doi.org/10.1128/AAC.00803-16.

14. Xiao M, He L, Gao B, Wang L, Liu JH. 2014. Increasing prevalence of extended-spectrum cephalosporin-resistant Enterobacteriaceae in food animals and the diversity of CTX-M genotypes during 2003–2012. Vet Microbiol 169:63–70. https://doi.org/10.1016/j.vetmic.2014.07.008.

15. Liu BT, Yang QE, Li L, Sun J, Xiao XP, Fang LX, Yang SS, Deng H, Liu YH. 2013. Nucleotide sequence of a large plasmid, pCU1. J Mol Biol 246:595–608. https://doi.org/10.1016/j.jmb.2004.08.008.

16. Liu BT, Yang QE, Li L, Sun J, Xiao XP, Fang LX, Yang SS, Deng H, Liu YH. 2013. Nucleotide sequence of a large plasmid, pCU1. J Mol Biol 246:595–608. https://doi.org/10.1016/j.jmb.2004.08.008.

17. Liu BT, Yang QE, Li L, Sun J, Xiao XP, Fang LX, Yang SS, Deng H, Liu YH. 2013. Nucleotide sequence of a large plasmid, pCU1. J Mol Biol 246:595–608. https://doi.org/10.1016/j.jmb.2004.08.008.

18. Liu BT, Yang QE, Li L, Sun J, Xiao XP, Fang LX, Yang SS, Deng H, Liu YH. 2013. Nucleotide sequence of a large plasmid, pCU1. J Mol Biol 246:595–608. https://doi.org/10.1016/j.jmb.2004.08.008.