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Characterization of a Novel IncHI2 Plasmid Carrying Tandem Copies of $bla_{CTX-M-2}$ in a *fosA6*-Harboring *Escherichia coli* Sequence Type 410 Strain

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The extended-spectrum β-lactamase gene $bla_{CTX-M-2}$ is mainly associated with IS*CR1* embedded in complex *sul1*-type integrons, but information on the genetic context of plasmids harboring the IS*CR1*-$bla_{CTX-M-2}$ module remains limited. In this study, a $bla_{CTX-M-2}$-harboring plasmid (pYD786-1) belonging to the sequence type 2 (ST2)-IncHI2 plasmid type and isolated from an *Escherichia coli* ST410 clinical strain was sequenced and analyzed. pYD786-1 belongs to the APEC-O1-R-type IncHI2 plasmids, which are widely distributed in human, poultry, and livestock strains. It contains a multidrug resistance mosaic region (MRR) consisting of a Tn*21*::In2 transposon backbone augmented by acquisition of duplicate IS*CR1*-$bla_{CTX-M-2}$ modules. Tn*2411*, a Tn*21*::In2 precursor, likely played a role in the generation of the MRR in pN13-01290_23, the putative progenitor plasmid of pYD786-1, found in a foodborne *Salmonella* strain. Tn*21*/Tn*2411*::In::IS*CR1*-$bla_{CTX-M-2}$ derivatives, including pYD786-1, have been identified in strains from Europe, South America, and the United States, suggesting potential global dissemination of the $bla_{CTX-M-2}$ modules mediated by this vehicle.

CTX-M-type β-lactamases are the most common extended-spectrum β-lactamases (ESBLs) worldwide and include five major subgroups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) (1). In the United States, CTX-M-15 of the CTX-M-1 group is the predominant ESBL in *Escherichia coli*, especially among the epidemic sequence type 131 (ST131) strains, followed by CTX-M-14 of the CTX-M-9 group (2–5). CTX-M-2 was identified in Argentina in 1989, first in different *Salmonella* serovars and then in diverse species of *Enterobacteriaceae* (1). Although it is not observed as frequently as CTX-M-1- and CTX-M-9-group enzymes, CTX-M-2 is endemic in South America (1, 6), caused a regional outbreak in Japan (7), circulates in animal and human strains in some European countries (8), and has been reported in North America (4, 5). In South America and Europe, $bla_{CTX-M-2}$ is associated mainly with complex *sul1*-type integrons containing IS*CR1* (9, 10), while in Japan most cases are associated with IS*Ecp1* (7, 11). Only a few studies have examined the incompatibility (Inc) types of the plasmids carrying $bla_{CTX-M-2}$ (7, 8, 11, 12). In a survey of human and poultry isolates from Europe, bla_{CTX-M} genes were found to be associated with IncHI2-type plasmids, and all $bla_{CTX-M-2}$-carrying plasmids belonged to the IncHI2 APEC-O1-R type (8, 13, 14). Besides bla_{CTX-M}, IncHI2 plasmids can recruit multiple resistance genes, including bla_{CMY-2} (accession no. KT347600.1) (15), bla_{VIM} (accession no. LN555650.1) (16), bla_{IMP}, *armA*, *qnrA*, *oqxAB*, *fosA3*, and *mcr-1* (12, 17–21). So far, no IncHI2 plasmid carrying $bla_{CTX-M-2}$ has been characterized fully. Here we report a novel IncHI2 plasmid carrying two copies of $bla_{CTX-M-2}$ in tandem.

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MATERIALS AND METHODS

A total of 760 ESBL-producing *E. coli* strains collected between 2009 and 2014 were screened for fosfomycin resistance by plating on Mueller-Hinton agar plates containing 128 μg/ml of fosfomycin and 25 μg/ml of glucose-6-phosphate. A total of 10 strains stably resistant to fosfomycin were identified as a result. *E. coli* transformants with fosfomycin resistance could be obtained for 2 of these 10 strains by electroporation. One of the 2 strains harbored *fosA3* (22), and the other harbored *fosA6* (23). *E. coli* YD786 is an extraintestinal pathogenic *E. coli* (ExPEC) ST410 strain isolated from the urine of a female patient admitted to a hospital in Pennsylvania in 2012. It was sequenced and assembled as previously described (23), and it carries four plasmids, including the $bla_{CTX-M-2}$-bearing plasmid pYD786-1 (accession no. KU254578.1) and the *fosA6*-carrying plasmid pYD786-2 (accession no. KU254579.1). Replicon sequence typing (RST) and plasmid double-locus sequence typing (pDLST) were conducted *in silico* based on a previously described scheme (12, 24; https://cge.cbs.dtu.dk/services/PlasmidFinder/; https://cge.cbs.dtu.dk/services/pMLST/). Nucleotide BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to find homologues of IncHI2 plasmid replication regions. Easyfig 2.0 was used to compare pYD786-1 to related plasmids. A phylogenetic tree was constructed by ClustalW alignment (http://www.genome.jp/tools/clustalw/).

Accession number(s). The nucleotide sequence of pYD786-1 has been deposited in the GenBank database under accession number KU254578.1.

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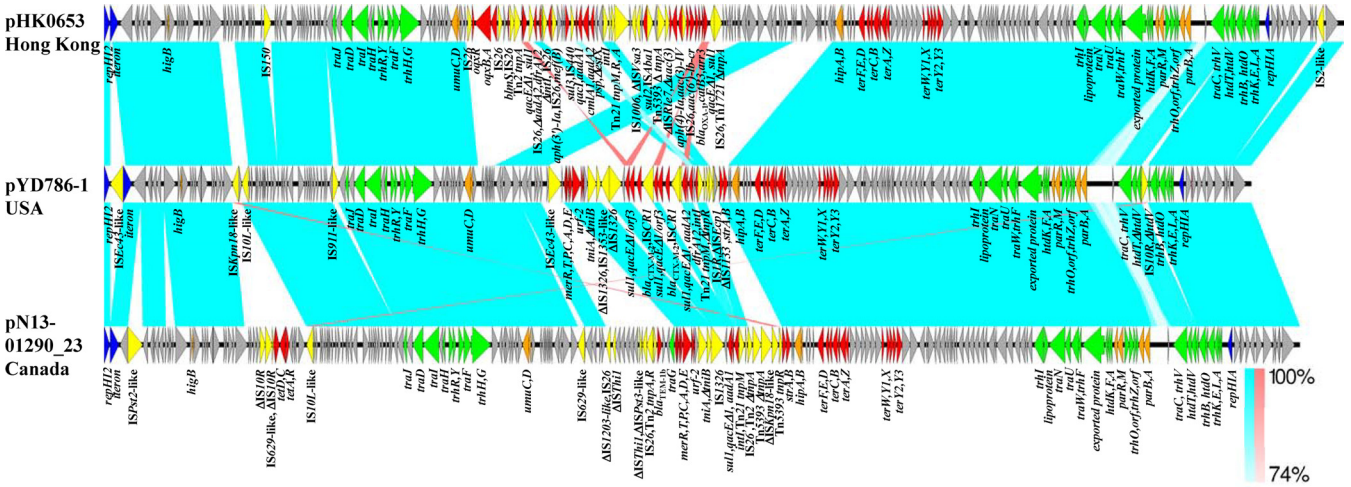

FIG 1 Comparison of plasmid pYD786-1 and the related IncHI2 plasmids pHK0653 (accession no. KT334335.1) and pN13-01290_23 (accession no. CP012931.1) (rearranged and inversely displayed for comparison of structures). Genes with different functions are color coded as follows: blue, plasmid replication genes; orange, plasmid stability, partitioning, and maintenance genes; green, conjugal transfer genes; red, resistance genes; yellow, mobile genetic elements; and light gray, genes with other functions. Cyan shading indicates shared backbone regions and antimicrobial resistance regions with high degrees of homology (74% to 100% identity of nucleotide sequences).

RESULTS AND DISCUSSION

Comparative analysis of the IncHI2 plasmid backbone. pYD786-1 is a 226.6-kb circular plasmid that possesses an IncHI2 plasmid backbone composed of replication (*repHIA*, *repHI2*, and iterons), partitioning (*parAB* and *parMR*), conjugation (*tra*, *trh*, and *htd* genes), and tellurium resistance (*terABCDEFWXYZ*) gene regions, complete or partial toxin-antitoxin systems (*hipA/hipB* and *higB*), and a *umuCD* operon involved in SOS mutagenesis (Fig. 1). Twenty-four fully sequenced IncHI2 plasmids were available in GenBank as of 5 April 2016, and they could be divided into three subtypes based on phylogenetic analysis of the replication regions, including *repHIA* (876 bp), *repHI2* (1,089 bp), and its iterons (1,425 to 1,930 bp long) (see Fig. S1 in the supplemental material). Of these 24 plasmids, 15 plasmids clustered with the R478 type and 8 plasmids, including pYD786-1, to the APEC-O1-R type, whereas 1 plasmid, from *Salmonella enterica* strain CFSAN00205, was distinct. All of the R478-type plasmids were assigned to ST1 by pDLST, including two ST1-like plasmids (pRH-R178 and p34977-263.138kb; *smr0199* is absent) (see Fig. S1). In contrast, in the APEC-O1-R group, 5 plasmids, including pYD786-1, belonged to ST2 or were closest to ST2 (one nucleotide difference in *smr0018*), and 3 plasmids from China belonged to ST3. The R478 plasmid was the first IncHI2 plasmid identified, from a clinical strain of *Serratia marcescens* in 1969 (13), while pAPEC-O1-R was recovered from an avian ExPEC strain in 2006 (14); both were recovered in the United States. Four of the APEC-O1-R-type (ST2-IncHI2) plasmids were from North America, and the other four were from China, including plasmids from *Salmonella* spp., *E. coli*, and *Shigella* spp. from human, poultry, and swine (see Fig. S1). ST3-IncHI2 plasmids are reportedly widely disseminated in Chinese poultry and livestock farms, and they carry *fosA3*, $bla_{CTX-M-9}$-group genes ($bla_{CTX-M-14/65/27}$), and/or *oqxAB* (17, 19). In previous studies, ST1-IncHI2 plasmids were associated with $bla_{CTX-M-9}$ in Europe, while ST2- or APEC-O1-R-type IncHI2 plasmids linked to $bla_{CTX-M-2}$ were found in *Salmonella* spp. from Belgium (poultry) and French Guiana (human feces), suggesting their distribution in Europe and South America (8, 12). pYD786-1 is the only fully sequenced $bla_{CTX-M-2}$-bearing ST2-IncHI2 plasmid whose backbone is closely related to those of the other 7 APEC-O1-R-type plasmids (see Fig. S1). It has a replication region identical to those of pN13-01290_23, pHK0653, 1205p1, pEC5207, and pAPEC-O1-R, except that pHK0653, 1205p1, and pEC5207 have an 81-bp fragment deletion in the iterons and pAPEC-O1-R has a nucleotide substitution in *repHIA*. Comparison of three sequences (pYD786-1, pN13-01290_23, and pHK0653) revealed conservation of the backbone, including the tellurium resistance gene cluster, and substantial differences in the multidrug resistance mosaic region (MRR), reflecting the common origin of an APEC-O1-R-type IncHI2 plasmid and the contributions of various subsequent lateral gene transfer events (Fig. 1).

Genetic analysis of the MRR of pYD786-1. The MRR of pYD786-1 is composed of a Tn*21*-derived transposon carrying a mercuric resistance gene operon, followed by an IS*Ecp1* transposition unit and a Tn*5393*::IS*1133* element characterized by the presence of the *strA-strB* streptomycin resistance genes (Fig. 1 and 2). Insertion of IS*1R* truncates *tnpR* of Tn*21* and IS*Ecp1* at the 5′ end, while IS*Ecp1* disrupts IS*1133* at the 5′ end. The Tn*21* derivative apparently underwent the following insertion events, as reported previously (25): (i) a Tn*402*-derived class 1 integron inserted into *urf-2* of Tn*21* and generated two direct repeats (DRs), and (ii) the insertion of IS*1326* disrupted *tniB*, followed by the transposition of IS*1353*, which interrupted IS*1326*, thus creating a nested complex structure almost identical to that of In2-bearing Tn*21* (Fig. 2A). However, In2 contained only one gene cassette, *aadA1*, while in pYD786-1, *dfrA12-gcuF-aadA2* gene cassettes were incorporated into the integron, presumably by replacing *aadA1* with IntI1-mediated recombination or by homologous recombination with similar integrons, thus generating In27 (Fig. 2A) (26–28). In27 in pYD786-1 contains a strong P_c promoter (PcS) (−35 sequence, TTGACA; −10 sequence, TAAACT) (Fig. 2B) identical to that of In4 in Tn*1696* (accession no. U12338.1), in

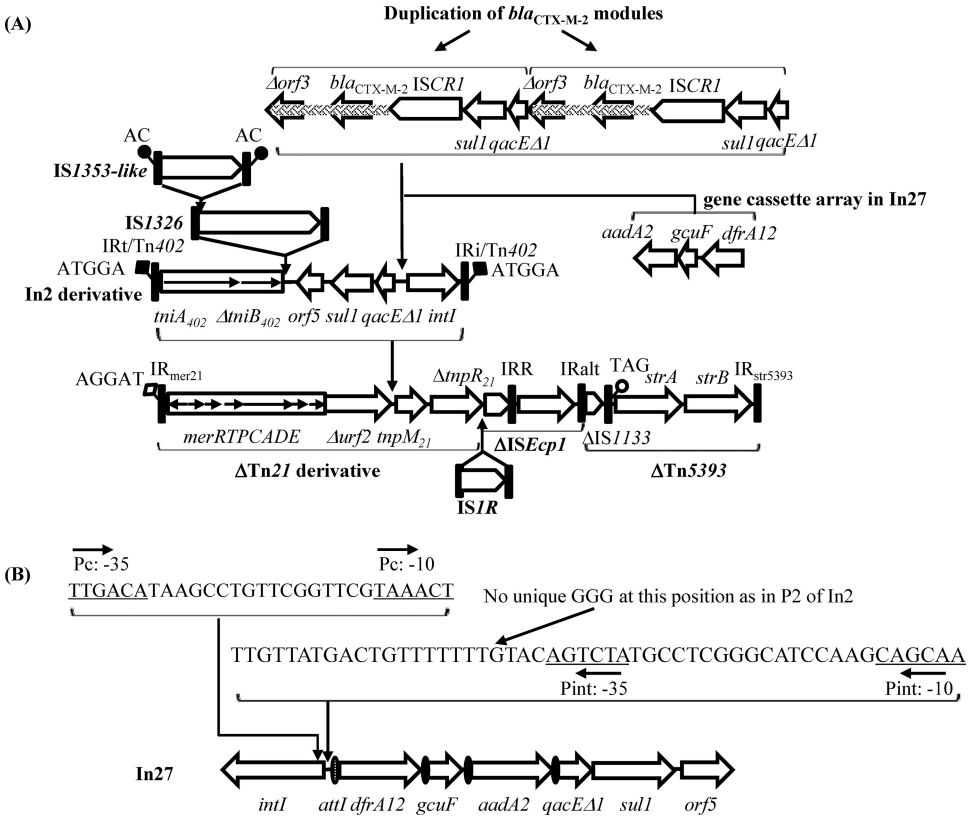

FIG 2 Schematic map of multidrug resistance mosaic region (MRR) in pYD786-1. (A) Evolution of the Tn*21*-like module in pYD786-1. The integration locations of the integron, IS*1326*, the IS*1353*-like element, and the gene cassette array are indicated by vertical arrows. Duplication of the $bla_{CTX-M-2}$ module is also indicated. DNA fragments shaded with basketwork showed 99% identity to those in the chromosome of *Kluyvera ascorbata* (accession no. AJ272538.2). The predicted open reading frames (ORFs) and insertion sequences are indicated by bold arrows and annotated above or below the maps, with arrowheads showing the direction of transcription. Putative direct repeat (DR) sequences are given to indicate the boundaries. Paired filled/unfilled squares or circles represent DRs of transposition units. The features shown are not necessarily drawn to scale. (B) Features of In27 and its promoter region in pYD786-1. *attI* and 59-be of the gene cassette are indicated by a filled oval. The promoters Pc (for the *dfrA12* gene) and Pint (for the *intI1* gene) are underlined. The position of unique GGG nucleotides in P2 of In2 is indicated. The position of and direction of transcription from the promoters are indicated by vertical and horizontal arrows, respectively.

contrast to the weak P_c promoter (PcW) (−35 sequence, TGGACA; −10 sequence, TAAGCT) in In2, as demonstrated experimentally by Jové et al. (29). Uniquely, In2 has extra GGG residues in the P2 region which are absent in the other integrons mentioned above, thus creating a functional P2 promoter for gene cassettes (Fig. 2B) (29), while In4 has a 19-bp duplication located in the *attI1* region. At least the following 3 Pc promoters are associated with In27, indicating possible recombination events: PcH1 (−35 sequence, TGGACA; −10 sequence, TAAACT) (accession no. EU780013.1), from Uruguay (6); PcS (accession no. EF592571.1), from French Guiana (8); and PcW (accession no. EF219134.3) in pJIE137, from Australia. However, the evolutionary process of the Pc/P2 promoters still remains obscure. In27 in pYD786-1 is inserted in an unusual location, as is In2 in Tn*21*, and the targeted positions of IS*1326* and IS*1353* are also identical to those for In2 in Tn*21*, indicating that the Tn*21*-like::In27 region in pYD786-1 is a descendant of In2-bearing Tn*21* and shares a common evolutionary lineage with In0, In2, and In5 (25, 30). In addition, pYD786-1 captured an IS*CR1*-$bla_{CTX-M-2}$ module characterized by partially duplicated 3′ conserved segments (3′-CS), as described previously (6, 31). Uniquely, however, the IS*CR1*-$bla_{CTX-M-2}$-3′-CS module was duplicated, generating two copies of $bla_{CTX-M-2}$ in tandem and thus forming an unusual complex class 1 integron (Fig. 2A).

Genetic context of IS*CR1*-$bla_{CTX-M-2}$-bearing integrons. The IS*CR1*-$bla_{CTX-M-2}$ module has been reported worldwide for various complex class 1 integrons, such as In35, In0, In2, In4, In27, In37, In131, In132, and many other types (1, 32). Most of these were identified in strains from Argentina or its adjacent countries in South America, whereas some were from Europe (6, 32). In Argentina, which is the major cradle of $bla_{CTX-M-2}$, the most common vehicle of $bla_{CTX-M-2}$ in *Enterobacteriaceae* is an In35::IS*CR1*::$bla_{CTX-M-2}$ element carrying the gene cassette array *aac*(6′)-*Ib*-bla_{OXA-2}-*orfD*. For instance, InV117 (PcS) in pAS1 was recovered from an endemic *Vibrio cholerae* strain, InS21 (PcS) in pS21 from an outbreak *Salmonella enterica* strain, In116 (PcIn116) (−35 sequence, TTGACA; −10 sequence, TGAACT) in pM16 from *Morganella morganii*, and In35 in pMAR12 from *Proteus mirabilis* (33).

Only a few reports have described the genetic contexts of IS*CR1*-$bla_{CTX-M-2}$-bearing integrons. One such combination is Tn*1696*-like::In35::IS*CR1*::$bla_{CTX-M-2}$. Tn*1696* is a close relative of Tn*21* that is always associated with In4. InV117 is located downstream of a Tn*1696*-like element. InV117 has >99.8% identity with InS21, In116, and In35, indicating their common origin and possible association with a Tn*1696*-like element (33). A fully sequenced multidrug resistance IncA/C_2 plasmid (accession no. CP007636.1), harbored by *V. cholerae* outbreak strain 2012EL-

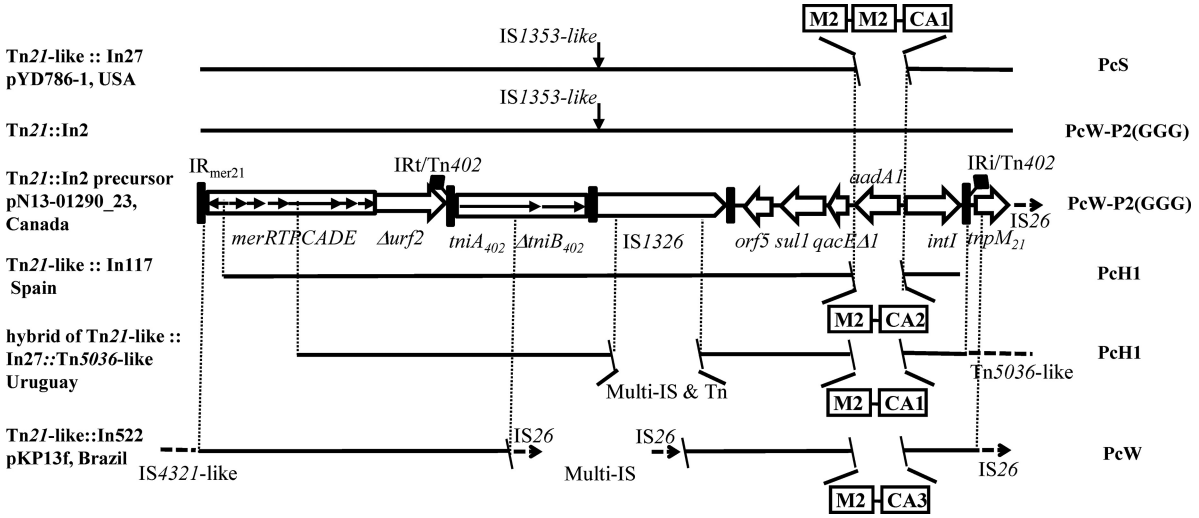

FIG 3 Schematic diagram of the Tn*21*::In2 transposon and its derivatives bearing IS*CR1*-$bla_{CTX-M-2}$. Tn*2411* in pN13-01290_23, a precursor of Tn*21*::In2, was used as the template. Solid lines indicate sequences identical to the template. Vertical arrows indicate the insertion site of the IS*1353*-like element. Sequences between the double slash were exchanged. The Pc promoters of the integrons are indicated on the right. M2, $bla_{CTX-M-2}$ module as shown in Fig. 2A. "CA" designations indicate the gene cassettes, as follows: CA1, *aadA2-gcuF-dfrA12*; CA2, *aadA1-estX*; and CA3, *aadA2-cmlA1-dfrA15*. The sequences used in the diagram are available under the following GenBank accession numbers: pYD786-1, KU254578.1; Tn*21*::In2, AF071413.3; pN13-01290_23, CP012931.1; In117, DQ125241.2; Tn*21*-like::In27::Tn*5036*-like, EU780013.1; and pKP13f, CP004000.1.

2176 from Haiti (18), contains a structure closely related to that of Tn*1696*-like::In35::IS*CR1*::$bla_{CTX-M-2}$, although *arr-3-dfrA27-aadA16* gene cassettes in In152 (PcH1) replaced those in In35, indicating their coevolution in this lineage.

It is worth noting that Tn*21* derivatives, which are involved in the formation of MRRs in plasmids, including pYD786-1, pN13-01290_23, and pHK0653 (Fig. 1), are also associated with the IS*CR1*-$bla_{CTX-M-2}$ module. pN13-01290_23 from Canada harbors a precursor of Tn*21*::In2, Tn*2411* (26), but lacks IS*1353* (Fig. 3). Similar derivatives of Tn*21*::In2-like transposons bearing the IS*CR1*-$bla_{CTX-M-2}$ module have been identified in at least 4 strains from North America, South America, and Europe (Fig. 3).

In117, which is embedded in Tn*2411*, was identified in an *E. coli* strain from the feces of a healthy volunteer in Spain in 2003 (34). Compared to In2, In117 acquired an *estX* cassette, thus generating the *estX-aadA1a* cassette array and an intermediate Pc promoter (PcH1). A hybrid transposon in *Klebsiella pneumoniae* strain 12836, isolated in Uruguay in 2005, contains an integron identical to In27 in pYD786-1, except for a nucleotide difference in Pc (PcH1 versus PcS) (6). The hybrid likely underwent multiple recombination events, causing the loss of a large part of IS*1326* in the middle but still leaving an intact left inverted repeat (IRL) as evidence of the lack of transposition of IS*1353*. Moreover, the Tn*21 tnp* region was replaced by that of a Tn*5036*-like transposon, presumably via homologous recombination, and formed a hybrid of Tn*21*-like and Tn*5036*-like transposons (6). In the IncH plasmid pKP13f, harbored by a KPC-producing *K. pneumoniae* strain in Brazil (35), the Tn*21*-like transposon is interrupted by multiple copies of IS*26*, which is suggestive of extensive genetic exchange resulting in a complicated MRR and a partial sequence of IS*1326*. The *dfrA15-cmlA1-aadA2* gene cassettes comprise the variable region of In522 in pKP13f.

Overall, we observed several instances where the IS*CR1*-$bla_{CTX-M-2}$ module was linked to an ancestral *tnp-mer* transposon backbone of Tn*21*::In2 or its precursor, suggesting an early combination of three types of mobile genetic elements (MGEs) in association with $bla_{CTX-M-2}$. Frequent exchanges in the variable regions of class 1 integrons with various Pc promoters suggest the contributions of functional gene cassettes to the dissemination of resistance genes. $bla_{CTX-M-2}$-bearing APEC-O1-R-type IncHI2 plasmids have been identified from human and poultry *Salmonella* strains in Europe, suggesting transmission via the food chain (8). pYD786-1 is likely a descendant of the APEC-O1-R-type plasmid pN13-01290_23, which is also carried by a *Salmonella* strain, providing another example of foodborne pathogens serving as a potential source of resistance genes and MGEs.

More recently, sequences for another 9 APEC-O1-R-type plasmids were released (as of 19 July 2016), including plasmid 180-PT54, carried by a foodborne *E. coli* O157 strain from the United Kingdom (ST4-IncHI2) (accession no. CP015833.1), and 3 MCR-1-encoding plasmids, identified from foodborne *E. coli* strains in China (pHNSHP45-2; ST3-IncHI2) (20) and Italy (pS38; ST4-IncHI2) (21) and from a human *E. coli* strain in Saudi Arabia (pSA26-MCR-1; ST4-IncHI2) (accession no. KU743384.1). pHNSHP45-2 is almost identical to pHK0653 (coverage, 95%; identity, >99%), except for 3 mosaic modules, IS*ApI1-mcr-1*, ΔIS*Ecp1*-$bla_{CTX-M-14}$-IS*903B-fosA3*, and *floR-orf*-IS*CR2* in the MRR, probably generated by 3 genetic events (20). pS38 is genetically closest to 180-PT54 (coverage, 95%; identity, >99%), including an almost identical Tn*21*-like transposon. Like pN13-01290_23, the 180-PT54 plasmid also harbors a precursor of Tn*21* with an intact IS*1326* element which is lost in the Tn*21*-like transposon in pS38. Besides pYD786-1, pS38 and the 180-PT54 plasmid are genetically closest to pN13-01290_23 (coverage, 83 to 85%; identity, >99%), providing more evidence of global dissemination of this lineage of plasmids by the food chain.

Conclusions. We identified a duplication of the $bla_{CTX-M-2}$ module in an ST2-IncHI2 plasmid, captured by an ancient Tn*21*::In2 transposon which likely underwent subsequent exchanges of gene cassettes and acquisition of various insertion sequences

and transposons. Alignment of the $bla_{CTX-M-2}$ modules carried by different Tn*21*::In2 progenitor derivatives identified in Europe and South America suggests an early dissemination of IS*CR1*-$bla_{CTX-M-2}$ by this vehicle, together with Tn*1696*-like:: In35::IS*CR1*::$bla_{CTX-M-2}$ elements or their homologues.

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