

Complete Nucleotide Sequence of an IncI2 Plasmid Coharboring *bla*_{CTX-M-55} and *mcr-1*

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We report the complete nucleotide sequence of a plasmid, pA31-12, carrying *bla*_{CTX-M-55} and *mcr-1* from a chicken *Escherichia coli* isolate. pA31-12 has an IncI2 replicon that displays extensive sequence similarity with pHN1122-1-borne *bla*_{CTX-M-55} and pHNSHP45-borne *mcr-1*. Insertion sequences *ISEcp1* and *ISAp11* are responsible for the mobilization of *bla*_{CTX-M-55} and *mcr-1*, respectively. The colocalization of *mcr-1* with an extended-spectrum β -lactamase gene on a conjugative plasmid may accelerate the dissemination of both genes by coselection.

Infections caused by multidrug-resistant Gram-negative bacteria, particularly extended-spectrum- β -lactamase (ESBL)-producing bacteria or carbapenem-resistant *Enterobacteriaceae* (CRE), have become significant threats to public health (1). The rapid increase in the prevalence of ESBL-producing or CRE isolates has prompted the reconsideration of colistin as a valid therapeutic option (2). However, an increase in the global presence of ESBL/CRE strains has resulted in an increased colistin use, leading to the inevitable risk of emerging resistance. Moreover, the massive use of colistin in animal production facilities has aggravated the problem. Recently, a plasmid encoding colistin resistance (*mcr-1*) was identified, and its importance was quickly recognized (3). This plasmid encodes a phosphoethanolamine transferase responsible for lipid A modification and, concomitantly, decreased colistin susceptibility.

The *mcr-1* gene was initially identified in *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from animals, food products, and clinical patients in China. The presence of this gene was subsequently reported from various isolates with diverse origins worldwide (3–9). The immediate clinical concern was the inevitable appearance of a plasmid cointegrating the *mcr-1* gene with other resistance genes, creating a multidrug-resistant isolate that would reduce therapeutic options. Here, we report a cephalosporin- and colistin-resistant *E. coli* strain that harbors a transferable IncI2 plasmid mediating cotransfer of *bla*_{CTX-M-55} and *mcr-1*.

E. coli isolate A31-12 was recovered from the rectal swab of a chicken with diarrhea from a veterinary clinic in Guangzhou, China, in August 2012. Antimicrobial susceptibility was determined by the agar dilution method by following CLSI guidelines. The breakpoints for each antimicrobial were as recommended by the Clinical and Laboratory Standards Institute (CLSI) (10), veterinary CLSI (11), and DANMAP 98 (olaquinox) (12). The results showed that this isolate was resistant to most antimicrobial drugs tested, including colistin, cefotaxime, ceftazidime, ceftiofur, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, amikacin, gentamicin, and trimethoprim-sulfamethoxazole, and it showed intermediate susceptibility to fosfomycin. This isolate retained sensitivity to meropenem, ceftiofur, olaquinox, doxycycline, and streptomycin (see Table S1 in the supplemental mate-

rial). Multilocus sequence typing (MLST) (13) showed that *E. coli* A31-12 belonged to sequence type 1011 (ST1011).

PCR analysis and DNA sequencing confirmed that this strain carried *bla*_{CTX-M-55} and *mcr-1*, which accounted for the corresponding drug sensitivity phenotype. Filter mating assays (14) revealed that the *bla*_{CTX-M-55} and *mcr-1* genes were able to cotransfer to *E. coli* C600 (streptomycin resistant). Compared with the MICs for the recipient strain, those for the transconjugant increased 256-fold for cefotaxime (from 0.125 to 32 μ g/ml) and 64-fold for colistin (from 0.0625 to 4 μ g/ml). S1 pulsed-field gel electrophoresis and Southern blot analysis (15) showed that both genes in donor clinical isolate A31-12 and recipient strain C600 were located on a single plasmid of approximately 65 kb (see Fig. S1 in the supplemental material), and this plasmid was named pA31-12 in this study.

To determine the complete nucleotide sequences of the plasmid, the total genomic DNA from the transconjugant C600/pA31-12 was extracted and sequenced using an Illumina MiSeq system. After filtering C600 chromosomal DNA data and assembling the remaining reads, we obtained a single contig for pA31-12 that was manually closed into a circle. The complete plasmid structure was further confirmed and verified by long-range PCR. The plasmid pA31-12 is 67,134 bp in length and has an average G+C content of 46.06%. Gene prediction and annotation were performed using the RAST tools. Sequence comparison and map

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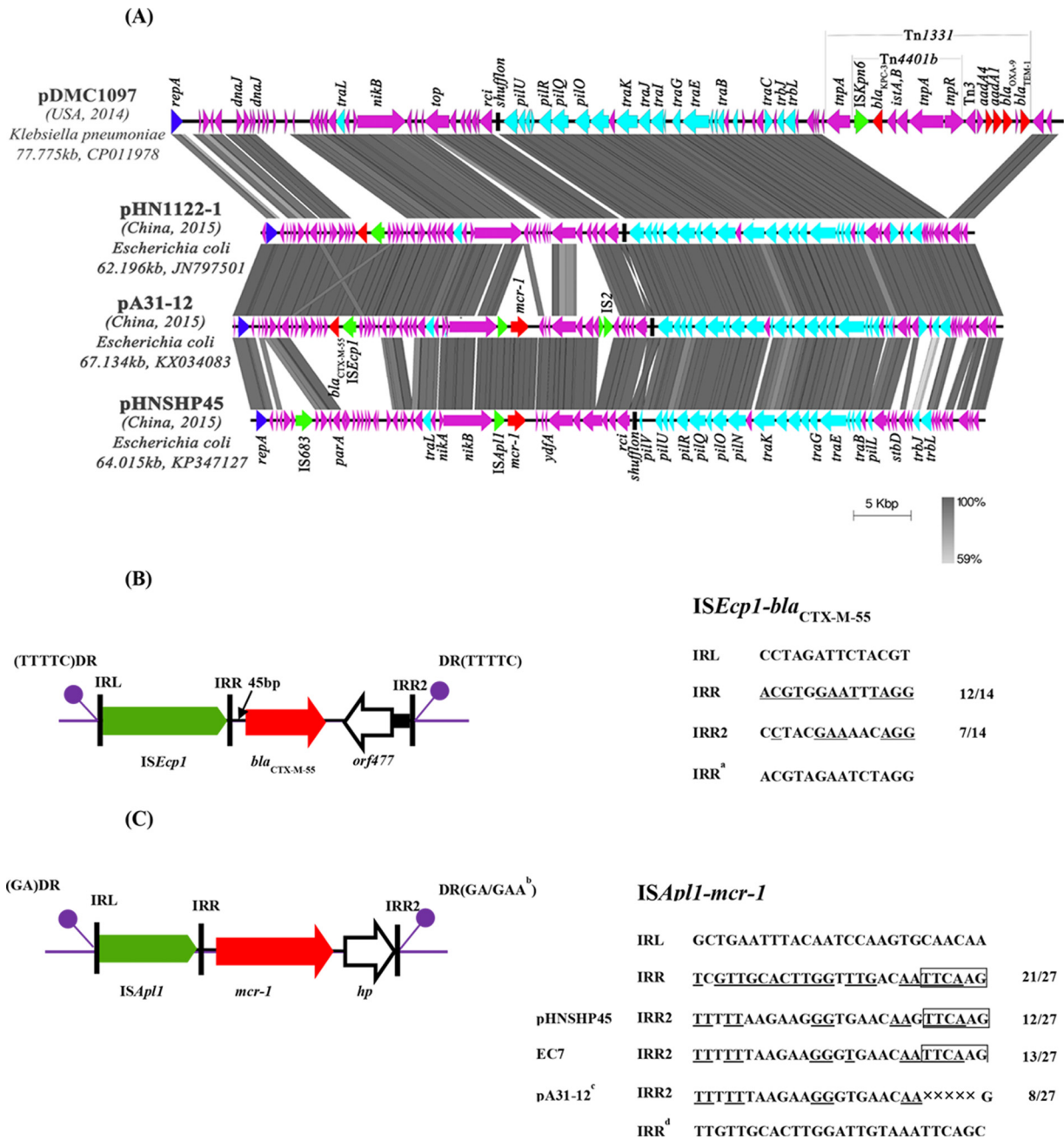


FIG 1 (A) Linear comparison of complete plasmid sequences of pA31-12 (this study, GenBank accession number [KX034083](#)), pDMC1097 (accession number [CP011978](#)), pHN1122-1 (accession number [JN797501.1](#)), and pHNSHP45 (accession number [KP347127.1](#)); (B) schematic representations of the genetic organization surrounding *bla*_{CTX-M-55} on pA31-12 and pHN1122-1; (C) schematic representations of the genetic organization surrounding *mcr-1* on pA31-12, pHNSHP45, and a fragment from *E. coli* EC7 (GenBank accession number [NZ_JWKG01000081.1](#)), which has identical IncI2 plasmid backbones. The arrows represent the positions and transcriptional directions of the ORFs. Regions of >99% homology are marked by gray shading. Genes associated with the *tra* and *pil* loci are indicated by light-blue arrows, while replication-associated genes are indicated by dark-blue arrows. Resistance genes are indicated by red arrows, while accessory genes are indicated by purple arrows. Insertion sequences are indicated by green arrows. DR, direct repeats; IRL, terminal inverted repeats at the left; IRR, terminal inverted repeats at the right. Footnotes: a, perfect IRR of ISEcp1; b, the DR of the ISApI1-*mcr-1* transposition unit are varied in IncI2 plasmids (GA in pHNSHP45 but GAA in EC7 or pA31-12); c, TTCAA was lost in pA31-12 compared to EC7; d, perfect IRR of ISApI1. Underlined nucleotides in the alternate IRR elements are identical to those of the perfect IRR. Boxed nucleotides indicated the six conserved nucleotides (TTCAAG) located at the 3' end of alternate IRR.

generation were performed using BLAST (<http://blast.ncbi.nlm.nih.gov>) and Easyfig version 2.1.

The plasmid pA31-12 (GenBank accession number [KX034083](#)) is an IncI2-type plasmid and encodes 91 open read-

ing frames (ORFs). A plasmid comparison based on a full-plasmid BLAST query revealed that pA31-12 is closely related to the other IncI2 plasmids in GenBank (Fig. 1), including pDMC1097 (accession number [CP011978](#)), pHN1122-1 (ac-

cession number JN797501.1), pCTXM64_C0967 (accession number KP091735.1), pCTXM132_P0421 (accession number KP198615.1), pHNY2 (accession number KF601686.2), and pHNSHP45 (accession number KP347127.1) from *E. coli*; pSTH21 (accession number LN623683.2) and pSH146_65 (accession number JN983044.1) from *Salmonella enterica*; p1081-CTXM (accession number KJ460501.1) from *Shigella sonnei*; and pBK15692 (accession number KC845573.1) from *K. pneumoniae*. Like the other IncI2 plasmids, pA31-12 has a typical plasmid backbone set of sequences that are responsible for plasmid replication, maintenance, and transfer (16) (Fig. 1A).

The RepA replicon protein of pA31-12 shows 100% amino acid identity with RepA of pHN1122-1 (17). The genes encoding plasmid stability functions, including *mok*, *hok*, *yafA*, and *yafB*, are also present in pA31-12. pA31-12 contains two pilus-encoding genes (*pil* and *tra*) which are conserved in all IncI2 plasmids and are responsible for plasmid transfer. In addition, pA31-12 contains a plasmid-borne site-specific recombinase (*Rci*) gene and a shufflon region (18). Four different PilV proteins with the same N-terminal sequence but varied C-terminal sequences were most likely created by four imperfect 19-bp repeat sequences (19). In comparison with plasmid R721, segments A and B'/D' are conserved but reversed, while segment C is missing. This suggests that pA31-12 undergoes complex rearrangements in the shufflon region mediated by the recombinase (Fig. 1A).

Two acquired resistance gene regions were identified in pA31-12. As with other pHN1122-1-like IncI2 plasmids, *bla*_{CTX-M-55} is located in a 3.084-kb *ISEcp1* transposition unit (16, 17). It is a typical *ISEcp1*-*bla*_{CTX-M-55} transposition unit containing *ISEcp1*, *bla*_{CTX-M-55}, *orf477*, and a 112-bp fragment of the *IncA/C* backbone. *ISEcp1* is able to mobilize several *bla*_{CTX-M} genes and their adjacent sequences by recognizing its own left inverted repeat (IRL) and the closest downstream sequence that resembles its right inverted repeat (IRR) (20, 21). This whole structure is inserted near *yajA*, generating a 5-bp duplication of the target sequence (GAAAA) (Fig. 1B). The identical configuration, which was observed not only in plasmids harboring *bla*_{CTX-M-55} (pHN1122-1 and pSTH21) but also in other IncI2 plasmids (pHNY2 carrying *bla*_{CTX-M-15}, pCTXM64_C0967 carrying *bla*_{CTX-M-64}, and pCTXM132_P0421 carrying *bla*_{CTX-M-132}), infers that such IncI2 plasmids have a common plasmid ancestor (16).

A plasmid-mediated colistin resistance gene, *mcr-1*, was also identified in pA31-12. Like the original *mcr-1*-positive plasmid pHNSHP45, pA31-12 has an *ISApI1* mobile element that flanks the 5' *mcr-1* gene (3). *ISApI1* is a member of the IS30 family, which was initially identified in *Actinobacillus pleuropneumoniae* (22). A potential linked transposition element was found in pHNSHP45-like plasmids that initiated at the 27-bp IRL sequence of *ISApI1* (5'-GCTGAATTTACAATCCAAGTGAACAA-3') but ended at a fixed position 0.8 kb downstream from the *mcr-1* stop codon by recognizing a similar IRR. Intriguingly, the IRR-like sequences seemed flexible and shared only 12 or 13 bp of the 27 bp of the perfect IRR sequence. Nevertheless, the 6 nucleotides (TTCAAG) located at the 3' end of these IRRs was always found, indicating that these were necessary for transposition. Compared with *E. coli* EC7 (NZ_JWKG01000081.1), TTCAA was lost in pA31-12, implying a new reorganization event (Fig. 1C). These data indicated that *ISApI1* was involved in a transposition process with weakly related IRRs that was similar to the transposition process in other insertion sequences, such as *ISEcp1* (21).

This transposition process resulted in an ~3.7-kb fragment (Fig. 1C) encoding a hypothetical protein in addition to *mcr-1*. Though its exact function is unknown, the hypothetical protein encodes a phosphoesterase and may be involved in colistin resistance. However, further experiments are needed to assess this hypothesis. Consistently with what occurred with the other IS30 family mobile elements, the insertion of *ISApI1*-*mcr-1* resulted in the duplication at the target insertion site of 2 or 3 base pairs (22) (Fig. 1C).

To summarize, this study reports for the first time the identification of a transferable IncI2 plasmid harboring both *bla*_{CTX-M-55} and *mcr-1* in an *E. coli* isolate derived from a chicken. Comparative analysis of the IncI2 plasmids provided information on their evolutionary history, suggesting that *ISApI1* might play a key role in transferring the *mcr-1* gene to an IncI2 backbone. Given the fact that colistin is the last-resort antibiotic for treating human infections due to multidrug-resistant *Enterobacteriaceae*, cotransfer of *mcr-1* with *bla*_{CTX-M-55} by a single mobile plasmid constitutes a potentially serious threat to clinical treatment regimens. Therefore, enhanced surveillance and efforts to limit the spread of such multidrug-resistant plasmids is urgently needed.

Nucleotide sequence accession number. The annotated sequence of plasmid pA31-12 from strain A31-12 has been submitted to GenBank accession number KX034083.

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