Genomic characteristics of *mcr-1* and *bla*$_{\text{CTX-M-type}}$ in a single multidrug-resistant *Escherichia coli* ST93 from chicken in China

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ABSTRACT This study was undertaken to discern the transmission characteristics of *mcr-1* and *bla*$_{\text{CTX-M-type}}$ in one multidrug-resistant *Escherichia coli* LWY24 from chicken in China. The genetic profiles of LWY24 isolate were determined by conjugation, S1-pulsed-field gel electrophoresis, southern blot hybridization, and whole genome sequencing analysis. Meanwhile, co-transfer of plasmids in LWY24 isolate was screened by dual conjugation assays. The LWY24 isolate was identified as ST93, and harbored 3 conjugative plasmids, pLWY24J-3 (*bla*$_{\text{CTX-M-55}}$-bearing IncFII), pLWY24J-*mcr-1* (*mcr-1*-carrying IncI2), and pLWY24J-4 (non-resistance-conferring IncI1), and one nonconjugative plasmid pLWY24 (*bla*$_{\text{CTX-M-14}}$-containing IncHI2/IncHI2A). Numerous resistance genes, insertion sequences (especially IS*26*), and transposons were found in the 4 plasmids, suggesting that horizontal transmission have occurred by plasmid mating, homologous recombination, and transpositions. Under the selection pressure of cefotaxime and colistin or cefotaxime alone, the *mcr-1*-bearing plasmid and the *bla*$_{\text{CTX-M-55}}$-harboring plasmid could be co-transferred at a similar frequency, with 8.00×10^{-4} or 9.00×10^{-4} transconjugants per donor cell, respectively. The specific shufflon region in *mcr-1*-encoding plasmid could generate up to 6 diverse PilV structures, which may further accelerate the horizontal transfer of plasmid. In conclusion, the transmission characteristics of *mcr-1* and *bla*$_{\text{CTX-M-type}}$ in LWY24 isolate could due to clonal spread of ST93, selective pressure of cefotaxime, IS*26*-mediate homologous recombination and transposition, and the specific shufflon region.

Key words: multidrug-resistance, shufflon region, *mcr-1*, *bla*$_{\text{CTX-M-type}}$, IS*26*

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INTRODUCTION

The rapid spread and evolution of the mobile colistin-resistance gene *mcr* has become a global concern. To date, *mcr-1* ∼ *mcr-10* have been widely identified around the world (Liu et al., 2016; Wang et al., 2020), and new genotypes are still being discovered. Numerous studies have confirmed that different classes of plasmids can harbor *mcr* genes, and IncI2, IncHI2, and IncX4 are the major types of plasmids driving the dissemination of *mcr-1* (Li et al., 2016; Matamoros et al., 2017; Wu et al., 2018a, 2018b), which accounted for over 90% of reported *mcr-1*-bearing plasmids (Nang et al., 2019). As compared with IncX4-type plasmid (∼30 kb), the genetic contexts of IncI2- and IncHI2-type plasmids are more variable (Fernandes et al., 2016; Li et al., 2016). The IncHI2-type plasmids (∼200 kb) are bigger, and usually contain 2 copies of IS*Apl1* flanking *mcr* gene, which can form a composite transposon Tn*6330*, while the IncI2-type plasmids (∼60 kb) sometimes also harbor IS*Apl1* (Li et al., 2016; Poirel et al., 2017; Snesrud et al., 2018). In a word, there are more ways of horizontal transfer of *mcr* gene located on the IncHI2 and IncI2 plasmids, which could be either through the plasmid conjugation or transformation, or through homologous recombination and transposition.

Similarly, many conjugative plasmids, such as IncF, IncI, IncK, and IncHI2, play important roles in the global dissemination of extended-spectrum β-lactamase (**ESBL**) genes, which confers resistance to most β-lactam antimicrobials, especially to third-generation cephalosporins (Wang et al., 2018b; Irrgang et al., 2018; Furlan et al., 2020; Grevskott et al., 2020). Studies over the last decade have shown that class A β-lactamases belonging to the CTX-M-type have become the predominant ESBL in many parts of the world, and

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have spread rapidly among clinical populations of *Enterobacterales* (D'Andrea et al., 2013; Furlan et al., 2020). Insertion sequences (**IS**), for example, IS*Ecp1*, IS*CR1*, IS*26,* and IS*903*, were also involved in the mobilization of $bla_{\text{CTX-M-type}}$ genes via transposition or homologous recombination (D'Andrea et al., 2013; Wang et al., 2018b; Zhang et al., 2019).

In recent studies, resistant genes $bla_{\text{CTX-M-type}}$ and *mcr-1* have been simultaneously detected in *Enterobacteriaceae* family isolated from humans and animals (Zhang et al., 2016, 2019; Wu et al., 2018b; Shafiq et al., 2019). Although they sometimes appear on the same plasmid (Sun et al., 2016; Zhang et al., 2019), more studies have shown that $bla_{\text{CTX-M-type}}$ and *mcr-1* genes are usually located on distinct plasmids of bacteria (Manageiro et al., 2019; Lv et al., 2020). Our study was initiated by the isolation of one cefotaxime and colistin co-resistant *Escherichia coli* cultured originally from a chicken farm in Henan Province of China in 2016. The aims of the present study were (i) to discern the genetic characteristics of *E. coli* LWY24 by whole genome sequencing (WGS), (ii) to identify the co-transfer mechanisms by dual conjugation assays, and (iii) to identify the resistant plasmids backbones and genetic environments.

MATERIALS AND METHODS

Bacterial Isolates and Antimicrobial Susceptibility Testing

In a survey on antimicrobial-resistant bacterial isolates in China in 2016, an *E. coli* isolate, LWY24, was isolated from healthy chicken feces in Henan Province and identified using the VITEK-2 system (bioMérieux, France). Antimicrobial susceptibility testing was performed using broth microdilution method except for fosfomycin, which was detected by agar microdilution method (CLSI, 2018). The antimicrobial agents tested in this study were provided by Henan Muxiang Veterinary Pharmaceutical Co. Ltd., China, containing cefotaxime, gentamicin, amikacin, oxytetracycline, doxycycline, florfenicol, colistin, olaquindox, enrofloxacin, sulfamonomethoxine/trimethoprim (5/1), and fosfomycin. The results were analyzed according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2018) and the European Committee on Antimicrobial Susceptibility Testing guidelines (http://www.eucast.org/clinical_breakpoints). *Escherichia coli* ATCC 25922 was used as a quality control strain.

Bacteria Genotyping and Screening for $bla_{\text{CTX-M-type}}$ and mcr-1 Genes

Multilocus sequence typing (**MLST**) was performed, as per the method of the *E. coli* MLST database website. Seven housekeeping genes: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA,* and *recA* were targeted by PCR amplification and sequencing (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/). The genomic DNA was extracted from the LWY24 isolate by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Then, the $bla_{\text{CTX-M-type}}$ and *mcr-1* were screened using the primers as described before (Eckert et al., 2006; Liu et al., 2016) and sequenced. Sequencing analysis was performed online using BLAST (www.ncbi.nlm.nih.gov/BLAST/).

Conjugation, S1-Pulsed-Field Gel Electrophoresis, and Southern Blot Hybridization

The mating assay was performed between the LWY24 isolate and rifampicin-resistant *E. coli* C600 as the recipient isolate. Transconjugants, designated as pLWY24J, were selected on MacConkey agar plates (Beijing Aoboxing Bio-Tech Co. Ltd., China) supplemented with 400 mg/L rifampicin, 4 mg/L cefotaxime and 2 mg/L colistin. Antimicrobial susceptibility tests were performed to confirm the plasmids transfer as described above, followed by PCR, S1-nuclease pulsed-field gel electrophoresis, and southern blot hybridization to confirm which plasmids and resistance markers were transferred.

Dual Conjugation Assays

To investigate co-transfer of pLWY24J-3 (which harbors $bla_{\text{CTX-M-55}}$) and pLWY24J-*mcr-1* (which harbors *mcr-1*), the conjugation assay was further performed using the transconjugant pLWY24J as the donor and azide-resistant *E. coli* J53 as the recipient. The selective plates were supplemented with sodium azide (200 mg/L) + cefotaxime (4 mg/L), sodium azide (200 mg/L) + colistin (2 mg/L), or sodium azide (200 mg/L) + cefotaxime (4 mg/L) + colistin (2 mg/L). All transconjugants were confirmed as described above. Dual conjugation experiments were carried out in triplicate. The conjugation frequency was calculated as the number of transconjugants per recipient.

Whole Genome Sequencing and Analysis

The genomic DNA was subjected to WGS using Illumina Nextseq 500 and the Oxford Nanopore Technologies MinION platforms as described before sequencing reads including short-read and long-read data were assembled with unicycler 0.4.4 with the hybrid strategy (Wick et al., 2017; He et al., 2019). The plasmid sequences were initially annotated using the RAST server (http://rast.nmpdr.org) and corrected manually. The plasmid replicon genotype was identified using PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/). Insertion sequence elements were identified using ISfinder (https://isfinder.biotoul.fr/). The comparative analysis and plasmid maps were generated using Easyfig 2.2.3.

Nucleotide Sequence Accession Numbers

The complete nucleotide sequences of 4 plasmids and chromosome in LWY24 isolate have been submitted to GenBank with the following accession numbers:

pLWY24 (MT318677), pLWY24J-*mcr-1* (MN689940), pLWY24J-3 (MN702385), pLWY24J-4 (MN702386), and LWY24 (CP054556).

RESULTS

Characterization of LWY24 Isolate

The LWY24 isolate was resistant to cefotaxime (512 mg/L), gentamicin (>512 mg/L), amikacin (512 mg/L), oxytetracycline (64 mg/L), doxycycline (32 mg/L), florfenicol (512 mg/L), colistin (8 mg/L), enrofloxacin (64 mg/L), fosfomycin (>512 mg/L), and sulfamonomethoxine/trimethoprim (>512/102.4 mg/L) (Supplementary Table 1). In accordance with the MLST result, the LWY24 isolate belonged to ST93. This ST acts as a common *mcr-1* and $bla_{\text{CTX-M}}$ carrier to clonal spread in *E. coli* (Maluta et al., 2014; Wang et al., 2018a), indicating that it may be one of the reasons accounting for *mcr-1* and $bla_{\text{CTX-M}}$ transmission rapidly.

S1-pulsed-field gel electrophoresis exhibited that there were 4 plasmids, designated as pLWY24 (~120 kb), pLWY24J-*mcr-1* (~60 kb), pLWY24J-3 (~70 kb), and pLWY24J-4 (~90 kb) in the parental isolate LWY24. In the transconjugant pLWY24J, the other plasmids were detectable except the biggest plasmid pLWY24 (Supplementary Figure 1). Southern blot hybridization confirmed that the *mcr-1* gene was located in the ~60 kb plasmid pLWY24J-*mcr-1*, the $bla_{\text{CTX-M-55}}$ gene was located in the ~70 kb plasmid pLWY24J-3, and the pLWY24-4 did not carry *mcr-1* or $bla_{\text{CTX-M-type}}$ (Supplementary Figure 1).

Whole genome sequencing indicated that the quinolone resistance genes, *gyrA* mutant (L83S, Y87D, V863A) and *gyrB* mutant (N492S, T618A), were located in the chromosome, *mcr-1* was in plasmid pLWY24J-*mcr-1*, $bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-1b}}$, and *rmtB* were in plasmid pLWY24J-3. Plasmid pLWY24 carried many resistance genes, such as $bla_{\text{CTX-M-14}}$, *fosA3*, *floR*, *cmlA1*, *aac(3)-IVa*, *Δaac(3)-IIa*, *aph(4)-Ia*, *aph(3′)-Ia*, *ant(3″)-Ia*, *ant(3″)* family, *sul2*, *sul3*, *dfrA12*, *qac*L, *mef*(B), and *lnu*(F). Meanwhile, plasmid pLWY24J-4 did not harbor any resistance genes.

Co-selection of $bla_{\text{CTX-M-type}}$ and Mcr-1

Under the selection pressure of colistin, transconjugants harbored plasmid pLWY24J-*mcr-1* with an average of 3.87×10^{-2} transfer rates. Meanwhile, plasmids pLWY24J-*mcr-1* and pLWY24J-3 co-transferred via conjugation (8.00×10^{-4} transconjugants per donor cell) when transconjugants were selected by cefotaxime and colistin, the transfer frequencies of which were 48.38-fold lower than those of single plasmid pLWY24J-*mcr-1*. Interestingly, there were 2 types of transconjugants on MacConkey agar containing cefotaxime, one harbored plasmid pLWY24J-3, with 3.60×10^{-3} transconjugants per donor cell, and the other harbored plasmids pLWY24J-*mcr-1* and pLWY24J-3, with frequencies of 9.00×10^{-4}. The results demonstrated that plasmids pLWY24J-*mcr-1* and pLWY24J-3 could co-transfer under the selection pressure of cefotaxime, but not under the selection of colistin.

Sequence Analysis of Plasmids in LWY24 Isolate

The 68,718-bp plasmid pLWY24J-3 carrying $bla_{\text{CTX-M-55}}$ consisted of average guanine and cytosine (**GC**) content of 51.2% and contained 94 open reading frames (**ORF**), which was an epidemic IncFII-F33: A-: B- plasmid possessing the typical structure of F33: A-: B- plasmids, and showed high gene synteny to the reported $bla_{\text{CTX-M-55}}$-harboring F33: A-: B- plasmids (pHNGD4P177 and pHN04NHB3) in *E. coli* isolates of different animal origins in China, with 99% identity (Figure 1). The multidrug resistance region (MRR) of plasmid pLWY24J-3, IS*26*-Δ*TnpR*-$bla_{\text{TEM-1b}}$-*rmtB*-IS*26*-*orf477*-$bla_{\text{CTX-M-55}}$-IS*26*, showed a high similarity to that of plasmid pHN04NHB3. The main difference was that the $bla_{\text{TEM-1b}}$, *rmtB*, and $bla_{\text{CTX-M-55}}$ in plasmid pLWY24J-3 were bordered by 2 IS*26* copies in the same orientation and formed a composite transposon, which could form a circular intermediate product and excise from pLWY24J-3 (Figure 1), indicating that IS*26*-mediated insertion in pLWY24J-3 may have occurred not only by homologous recombination, but also by transposition.

The plasmid pLWY24J-*mcr-1* was 62,009-bp in size and contained 80 ORF with an average GC content of 43.0%. It belongs to the plasmid incompatibility group IncI2 and has a typical plasmid backbone region responsible for replication, maintenance, and transfer (Figure 2). The IncI2 plasmid was one of the most popular plasmids involved in the dissemination of *mcr-1* (Li et al., 2016; Liu et al., 2016; Matamoros et al., 2017; Wu et al., 2018a, 2018b). The plasmid pLWY24J-*mcr-1* did not carry any additional known antimicrobial resistance gene but *mcr-1*. In addition, no IS*Apl1* elements were found to flank the *mcr-1* gene, which contributed to improve the stability of *mcr-1* in plasmids (Snesrud et al., 2016, 2018). Further alignments revealed that plasmid pLWY24J-*mcr-1* showed almost identical nucleotide sequences with previously reported IncI2 plasmid pColR644SK1 (MF175188) of *E. coli* from humans (Zurfluh et al., 2017), and differed by the addition of an IS*903* mobile element downstream of *mcr-1* cassette and a specific shufflon region (Figure 2).

The pLWY24, which was $bla_{\text{CTX-M-14}}$-bearing IncHI2/IncHI2A plasmid, was 195,614 bp and included 256 ORF, comprised with average GC content of 47.0%. The backbone sequences of pLWY24 were highly similar to that of plasmid pHYEC7-IncHI2 (KX518743), except that an IS*2* was inserted at 25,869 bp of the transfer region of plasmid pLWY24 (Figure 3) which may make it difficult for plasmid pLWY24 to transfer horizontally. Indeed, no transconjugant or transformant carrying

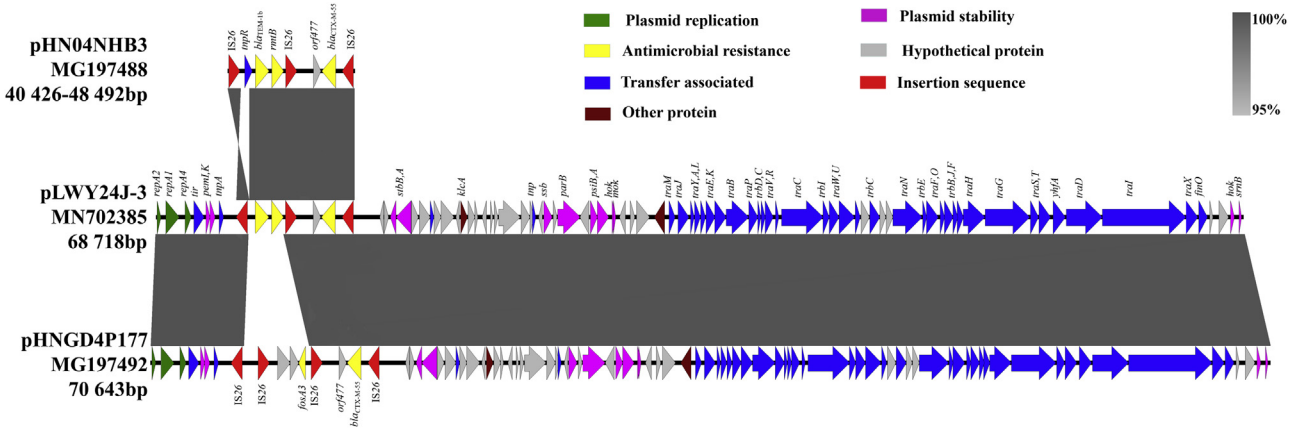

Figure 1. Linear comparison of complete plasmid sequences of pLWY24J-3 (this study, MN702385), pHNGD4P177 (MG197492), and pHN04NHB3 (MG197488). Regions of >99% homology are marked by gray shading. Open reading frames (ORF) are indicated by thick arrows and are colored in accordance with their putative functions as described in the figure legend.

$bla_{\text{CTX-M-14}}$ has been obtained, despite repeated attempts.

The modular MRR of pLWY24 carried 16 antimicrobial resistance genes, including $bla_{\text{CTX-M-14}}$, *fosA3*, *floR*, *cmlA1*, *aac(3)-IVa*, *Δaac(3)-IIa*, *aph(4)-Ia*, *aph(3′)-Ia*, *ant(3″)-Ia*, *ant(3″)* family, *sul2*, *sul3*, *dfrA12*, *qac*L, *mef*(B), and *lnu*(F), which conferred resistance to β-lactam, aminoglycoside, amphenicol, fosfomycin, sulfonamides/trimethoprim, quinolone, macrolide, and lincosamides (Figure 3). Meanwhile, the MRR region also contained numerous complete or truncated IS and transposons, especially including 7 copies of IS*26*. In accordance with the homology, the MRR region could be divided into 3 segments, suggesting that the structure had undergone multiple rearrangements. The right segment was >98% identical to that of plasmid pMUP050 (AY522431) and was flanked by 3 IS*26* elements in the same direction, which could form 2 circular intermediate products to dissemination. The medium segment was >99% identical to that of plasmid pPE15-IncF (CP041629) and contained a composite transposon and a class I integron. The left segment was the most complex structure, which was >99% identical to that of plasmid pT28R-2 (CP049355) and carried 7 antimicrobial resistance genes, multiple IS, and transposons.

DISCUSSION

Dual conjugation assays showed that plasmids pLWY24J-*mcr-1* and pLWY24J-3 could co-transfer under the selection pressure of cefotaxime, or cefotaxime + colistin, but not under the selection of colistin. Similarly, Zhang et al. (2019) also found that during the conjugation experiments, ESBL, and mcr-1 genes could be co-selected by cefotaxime, while colistin only selected the mcr-1-carrying plasmids. Wu et al. (2018a) considered that the widespread use of the third generation cephalosporins not only resulted in the fast rising of ESBL prevalence, but also increased the selective pressure of colistin resistance. So far, the reason is unclear. The interaction between the 2 plasmids maybe is one of the reasons; further research is still in progress in our laboratory.

IS*26* has been regarded as a predominant mobile element related to the spread, clustering, and remodeling of resistance determinants (He et al., 2015, 2019; Wong et al., 2017; Wang et al., 2018b). In this work, numerous

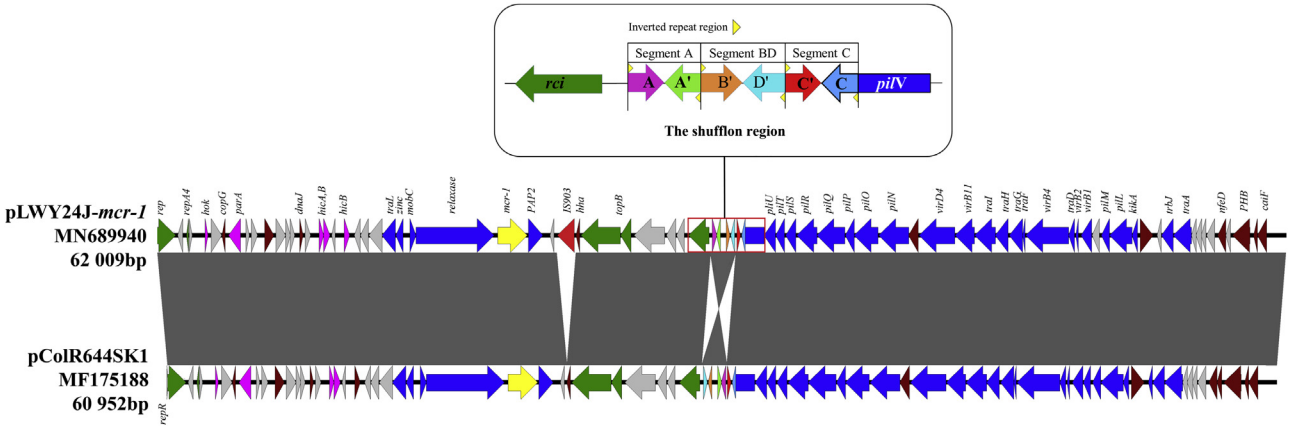

Figure 2. Linear comparison of complete plasmid sequences of pLWY24J-*mcr-1* (this study, MN689940) and pColR644SK1 (MF175188).

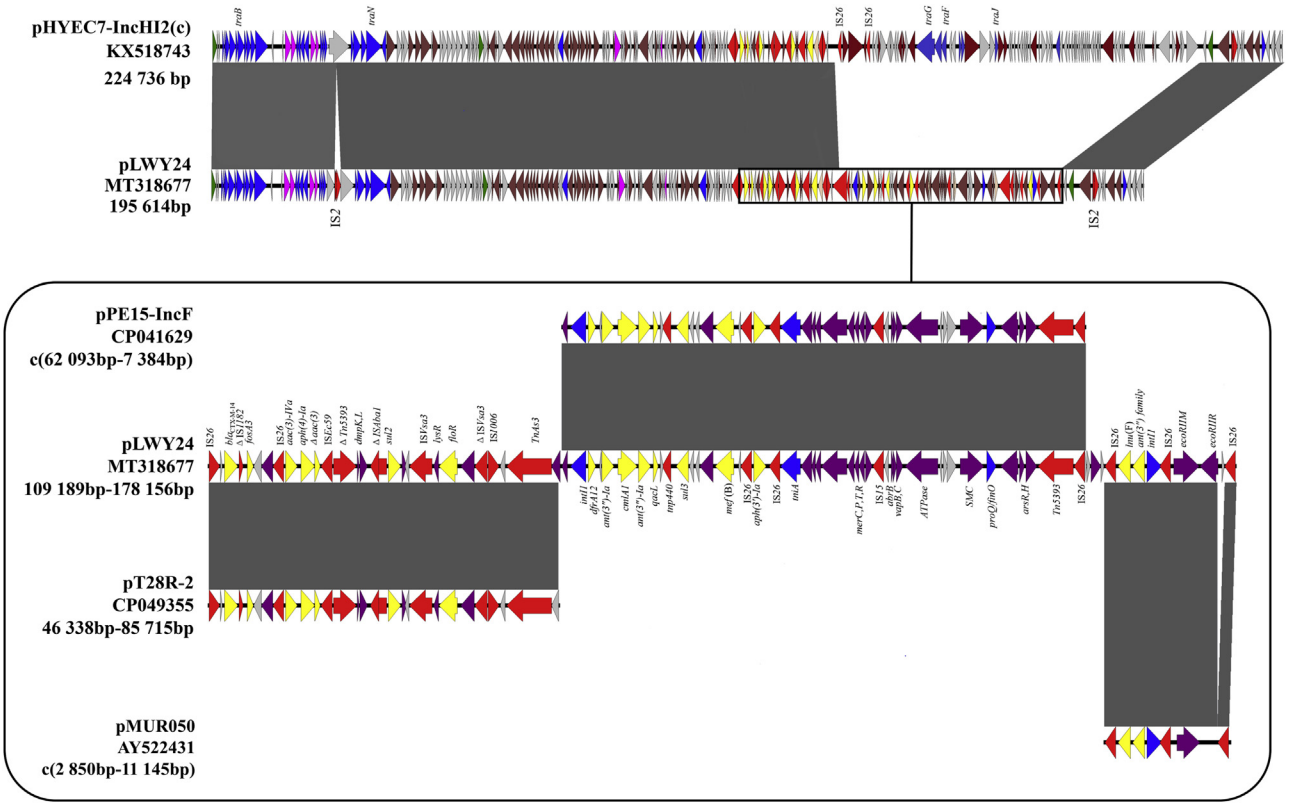

Figure 3. Linear comparison of complete plasmid sequences of pLWY24 (this study, MT318677) pHYEC7-IncHI2(c) (KX518743). In the box, the comparison of multiresistance regions (MRR) of plasmid pLWY24 with pPE15-IncF (CP041629), pT28R-2 (CP049355), and pMUR050 (AY522431), respectively.

IS, especially IS*26*, were found in the plasmids of LWY24 isolate, suggesting that the isolate had obtained multiple resistance genes via extensive homologous rearrangements and transpositions. He et al. (2019) in the exploration of the emergence of a hybrid plasmid process found that IS*26* played a critical role in the fusion of an IncN1-F33: A-: B- plasmid and a *mcr-1*-carrying phage-like plasmid. In this study, multiple plasmids not only co-exist in a single bacterial but also carry many IS*26* elements simultaneously, which makes it possible to recombine plasmids.

The shufflon was a clustered inversion region and located between the site-specific recombinase gene, *rci*, and one of the pilus genes, *pilV* (Komano et al., 1987; Brouwer et al., 2015). To date, the shufflon region has been identified in IncI1, IncI2, IncK, and IncZ plasmids (Sekizuka et al., 2017; Irrgang et al., 2018). Differed from that of IncI1 plasmids, the shufflon region of IncI2 plasmids consists of 3 (A, BD, and C) segments, and each segment includes 2 different ORF, that is ORF A and ORF A′, ORF B′ and ORF D′, ORF C and ORF C′, respectively. The segments are commonly rearranged by Rci (Sekizuka et al., 2017). Then, the rearranged segments can generate variants of *pilV* (Sekizuka et al., 2017; Irrgang et al., 2018). The number of variants of *pilV* is closely related to the number of segments, one segment can produce 2 types of *pilV*, and 2 can form 4 types, whereas 3 segments can generate the most *pilV* types, reaching 6. The variants of *pilV* encode different PilV tip adhesins, which can specifically recognize lipopolysaccharide structures on the surface of recipient cells during liquid mating (Ishiwa and Komano, 2003). So, variants of PilV are more, the scope of IncI2 plasmid horizontal transfer is wider (Brouwer et al., 2015; Sekizuka et al., 2017).

It has previously been proved that the number of shufflon segments is variable in IncI2 plasmids of bacteria (Chen et al., 2013; Ma et al., 2018; Li et al., 2020). In 2020, Li et al. (2020) reported that there were 3 kinds of shufflon regions in 30 IncI2 plasmids, which were composed of 1, 2, and 3 segments, respectively. In the present study, the shufflon region of pLWY24J-*mcr-1* consisted of 3 segments (A, BD, and C) and could present 6 diverse PilV structures via rearrangement, suggesting that the specific shufflon region could increase the likelihood of the widespread dissemination of *mcr-1* via plasmid mating.

In conclusion, the LWY24 isolate contained 4 plasmids and harbored numerous resistant genes, such as *mcr-1*, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-14}}$. The rapid transmission of *mcr-1* and $bla_{\text{CTX-M-type}}$ may be due to clonal spread of ST93, selective pressure of cefotaxime, IS*26*-mediate homologous recombination and transposition, and the specific shufflon region of IncI2-type plasmid.

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Ethical Approval: Not required.

DISCLOSURES

None.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2021.101074.

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