ORIGINAL RESEARCH

IS26-Mediated Formation of a Hybrid Plasmid Carrying *mcr-1.1*

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Purpose: The objective of this study was to elucidate the characteristics and mechanism of formation of the fusion plasmid pHNSHP24 carrying *mcr-1.1*.

Materials and Methods: *mcr-1.1*-bearing *Escherichia coli* SHP24 and the corresponding transconjugant were subjected to whole-genome sequencing (WGS) combining the Illumina and MinION platforms to obtain the complete sequences of the fusion plasmid and its original plasmids.

Results: Complete sequence analysis and S1 nuclease-pulsed field gel electrophoresis (S1-PFGE) results indicated that *E. coli* SHP24 carried four plasmids: *mcr-1.1*-harboring phage-like plasmid pHNSHP24-3, F53:A-:B- plasmid pHNSHP24-4, pHNSHP24-1, and pHNSHP24-2. However, the plasmid pHNSHP24 carrying *mcr-1.1* presents in the transconjugant differed from the four plasmids in the donor strain SHP24. Further analysis showed that pHNSHP24 may be the fusion product of pHNSHP24-3 and pHNSHP24-4 and is formed through a replicative transposition mechanism mediated by IS*26* in *E. coli* SHP24.

Conclusion: This study is the first to report the fusion of an *mcr-1.1*-harboring phage-like pO111 plasmid and an F53:A-:B- plasmid mediated by IS*26*. Our findings revealed the role of phage-like and fusion plasmids in the dissemination of *mcr-1.1*.

Keywords: fusion plasmid, *mcr-1.1*, IS*26*, pO111 plasmid

Introduction

The emergence and global transmission of the plasmid-mediated colistin resistance gene *mcr-1.1* poses a great threat to public health.[1,2] More than ten types of *mcr-1.1*-carrying plasmids have been reported, with IncI2, IncX4, and IncHI2 plasmids being the dominant vehicles that mediate the spread of *mcr-1.1* among different Enterobacteriaceae species from various sources worldwide. *mcr-1.1*-bearing phage-like pO111 plasmids have also been reported in animal, human, or environmental original isolates from China and have gradually become an important vehicle that mediates the dissemination of *mcr-1.1*.[3–5] The original pO111 plasmid pO111_2 was identified as a bacteriophage P1-like plasmid and found in enterohemorrhagic *Escherichia coli* of the O111:H1 serotype in Japan in 2001.[6] Thereafter, phage-like pO111 plasmids were isolated from *E. coli, Klebsiella pneumoniae*, and *Salmonella enterica*.[7–9] In addition to the *mcr-1.1* gene, phage-like pO111 plasmids also carried other resistance genes, such as *aadA, oqxAB, floR, bla*CTX-M, *bla*TEM and so on.[8,10,11] Similar to other phage-like plasmids, pO111 plasmids lack conjugative transfer ability.[7–9,12]

However, pO111 plasmids can be inserted into conjugative plasmids, such as IncF33:A-:B-, and IncFIA, and thereby obtaining conjugation capabilities.[7,12] Furthermore, an increasing number of hybrid plasmids have been reported in recent years. Some hybrid plasmids are formed through recombination mediated by insertion sequences, transposons, or

other homologous regions, such as IS*26*, IS*1294*, IS*1216E*, and Tn*1721*.[7,13–16] Some hybrid plasmids are formed through multiple homologous recombinations, such as the IncX3-X4 hybrid plasmid pCQ02-121. The plasmid pCQ02-121 is formed through two recombinations mediated by both IS*26* and *nic* site, which is a specific site cleaved by relaxation enzymes on the origin of transfer.[17,18]

Here, we characterized an *mcr-1.1*-carrying hybrid plasmid formed through the fusion of IncF53:A-:B- and phage-like pO111 plasmids and depicted the possible fusion mechanism.

Materials and Methods

Bacterial Strain
The *mcr-1.1*-positive *E. coli* strain SHP24 and its transconjugant C600/pHNSHP24 as well as its transformant *E. coli* DH5α/pHNSHP24 carrying *mcr-1.1* were obtained in our previous study.[5]

S1 Nuclease-Pulsed Field Gel Electrophoresis (S1-PFGE)
The plasmid profiles of SHP24 and its transconjugant were determined using S1-PFGE. Briefly, the DNA of SHP24 and its transconjugant were digested with S1 nuclease (Takara, Japan), and the DNA of the *S. enterica* serotype Braenderuo H9812 was digested with *Xba*I restriction enzymes (Takara, Japan). The digested DNA was subjected to S1-PFGE using the CHEF-MAPPER System (Bio-Rad, USA), and the results were visualized using a gel imaging system (Bio-Rad, USA).

Whole-Genome Sequencing (WGS) Analysis
E. coli SHP24 and its transconjugant *E. coli* C600/pHNSHP24 were subjected to WGS combining the Illumina HiSeq and Oxford Nanopore MinION platforms, and complete sequences were generated through *de novo* hybrid assembly using Unicycler version 0.4.3.[19] Multilocus sequence types (MLST), antimicrobial resistance genes, plasmid replicon types, and plasmid MLST (pMLST) were analyzed using the CGE server (https://cge.cbs.dtu.dk/services/). Insertion sequence (IS) elements were identified using the ISfinder (https://isfinder.biotoul.fr/). The phage sequence was identified using PHASTER.[20] The plasmid sequences were annotated using the RAST server (https://rast.nmpdr.org/). The complete sequence comparison of plasmids was performed using Easyfig.[21]

Analysis of *mcr-1.1*-Bearing Plasmids in the NCBI Database
In the NCBI database, we used the complete sequence of *mcr-1.1* to search for the complete sequences of *mcr-1.1*-bearing plasmids, and a total of 627 complete sequences of *mcr-1.1*-bearing plasmids were obtained. Antibiotic resistance genes and replicon types of *mcr-1.1*-carrying plasmids were retrieved using ABRicate (https://github.com/tseemann/abricate). Information on these plasmids was summarized and statistically analyzed (Tables S1–S3).

Conjugation Frequencies of Fusion Plasmid
The conjugation frequencies of pHNSHP24 were determined by conjugation assays using strain SHP24 or the transformant *E. coli* DH5α/pHNSHP24 as the donors and streptomycin-resistant *E. coli* C600 as the recipient. The experiment was carried out with biological triplicate. Conjugation frequencies were calculated as the number of transconjugants per recipient. Transconjugants were selected on lysogeny broth (LB) agar plates supplemented with colistin (2 μg/mL) and streptomycin (3000 μg/mL). PCR was performed to confirm the presence of *mcr-1* and the corresponding replicon type in the transconjugants (Table S4). In addition, PCR was performed to detect the presence of the fusion plasmid pHNSHP24 in parental strain SHP24 by using two pairs of specific primers Hybrid-1/2-F/R (Table S4).

Nucleotide Sequence Accession Number
The complete nucleotide sequence of the fusion pHNSHP24 was deposited in GenBank under the accession number CP065023.

Results

Identification of the Fusion Plasmid in the Transconjugant C600/pHNSHP24

The *mcr-1.1*-bearing plasmid pHNSHP24 in transconjugant belonged to the IncFII group which was rarely reported to carry the *mcr-1.1* gene.[5] Therefore, we sequenced pHNSHP24 to study the characteristics of this plasmid. Sequence analysis showed that pHNSHP24 is a 174812-bp hybrid plasmid harboring pO111 and F53:A-:B- plasmid replicons (Table 1). However, the S1-PFGE results showed that the size of the plasmid pHNSHP24 carried by the transconjugant was different from that of the plasmids carried by the donor SHP24 (Figure S1).

Analysis of Plasmids in SHP24

To further explore the source of plasmid pHNSHP24, the complete sequence of SHP24 was also obtained using WGS combining the Illumina and MinION platforms. Sequence analysis showed that the *E. coli* strain SHP24 belonged to ST6913 and harbored four plasmids, namely pHNSHP24-1 (IncHI2, 259,017 bp), pHNSHP24-2 (IncFII(pCoo), 186,621 bp), pHNSHP24-3 (pO111, 99,354 bp), and pHNSHP24-4 (IncF53:A-:B-; 76,121 bp). The IncHI2 plasmid pHNSHP24-1 harbored the resistance genes *aac(3)-IV, aac(6')-Ib-cr, aadA1, aadA2b, aph(3')-Ia, aph(4)-Ia, arr-3, bla*$_{OXA-1}$*, catB3, cmlA12, dfrA12, floR, oqxAB, sul1, sul2,* and *sul3*. The InFII(pCoo) plasmid pHNSHP24-2 and IncF53:A-:B- plasmid pHNSHP24-4 both harbored no resistance genes, and the colistin resistance gene *mcr-1.1* was carried by the phage-like pO111 plasmid pHNSHP24-3 (Table 1). Although the WGS data of the original strain SHP24 lack the sequence of the hybrid plasmid, we detected it in SHP24 by PCR.

The pO111 plasmid pHNSHP24-3 encodes 159 predicted open reading frames (ORFs), most of which are related to phage-related genes. Plasmid pHNSHP24-3 exhibited high homology to *Escherichia* phage P1 (AF234172) and P7 (AF503408), *mcr-1.1*-positive phage-like IncY plasmid pHYEC7-mcr1 (KX518745), *mcr-1.1*-carrying phage-like pO111 plasmids pPC6-mcr1 (CP080256) and pMCR_SCKP-LL83 (MF510496), which originated from *E. coli* and *K. pneumoniae* in China, with 97–99% identity and 69–92% coverage (Figure 1).

The F53:A-:B- plasmid pHNSHP24-4 had a typical IncFII plasmid conjugative transfer region structure, including *tra, trb*, and *finO* genes, and exhibited high homology to the *mcr-1.1*-bearing IncFII plasmid pCAU16175_3 (CP047381) and *floR*-harboring IncFII plasmid pCP8-3-IncFII (CP053737), originating from *E. coli* in China, with 94–99% identity and 68–77% coverage (Figure 1).

Table 1 Characterization of *E. coli* Strain SHP24 and Its Transconjugant Used in This Study

| Strain	Isolate	Plasmid	Size (bp)	Replicon Type	Resistance Genes	References
Parental strain	*E. coli* SHP24	pHNSHP24-1	259,017	IncHI2	*aac(3)-IV, aac(6')-Ib-cr, aadA1, aadA2b, aph(3')-Ia, aph(4)-Ia, arr-3, bla*$_{OXA-1}$*, catB3, cmlA1, dfrA12, floR, oqxAB, sul1, sul2, sul3*	[5]
		pHNSHP24-2	186,621	IncFII(pCoo)	No resistance gene	
		pHNSHP24-3	99,354	pO111	*mcr-1.1*	
		pHNSHP24-4	76,121	IncFII(F53:A-:B-)	No resistance gene	
Transconjugant	*E. coli* C600/ pHNSHP24	pHNSHP24	174,812	pO111-IncFII(F53: A-:B-)	*mcr-1.1*	[5]
Transformant	*E. coli* DH5α/ pHNSHP24	pHNSHP24	174,812	pO111-IncFII(F53: A-:B-)	*mcr-1.1*	[5]
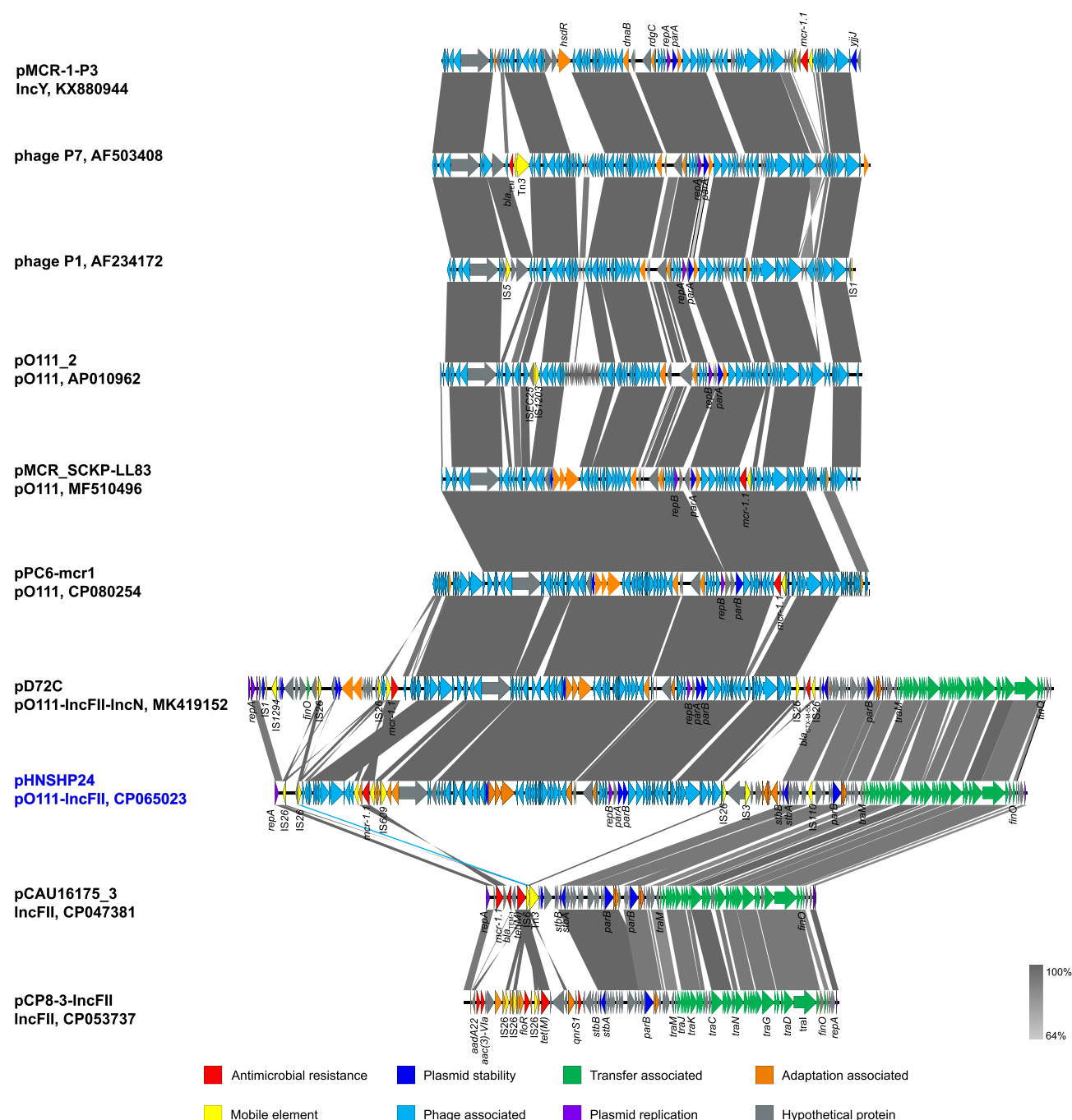
Figure 1 Complete sequence comparison of pO111-IncFII plasmid pHNSHP24 with phage-like plasmid, IncFII plasmid, *Escherichia* phage P1 (AF234172), and *Escherichia* phage P7 (AF503408). pMCR-1-P3 (KX880944), pO111_2 (AP010962), pMCR_SCKP-LL83 (MF510496), pPC6-mcr1 (CP080254), and pD72C (MK419152) are phage-like plasmids. pCAU16175_3 (CP047381) and pCP8-3-IncFII (CP053737) are IncFII plasmids. Functions encoded by different genes are represented in different colors, as shown in the square.

Sequence Comparison and Proposed Formation Mechanism of the Fusion Plasmid pHNSHP24

The complete sequence of pHNSHP24 was 174,812 bp with a G+C content of 49%, and only harbored the colistin resistance gene *mcr-1.1*. BLASTn results showed that pHNSHP24 was highly similar to the *mcr-1.1*-bearing pO111-IncN-IncF33:A-:B- plasmid pD72C (MK419152) with 81% coverage and 98% identity (Figure 1). Further analysis indicated that the fusion plasmid pHNSHP24 consisted of pHNSHP24-3 and pHNSHP24-4 which were bound to two copies of

IS26. (Figure 2). In addition, the fusion plasmid pHNSHP24 was both identified in the transconjugant and the parental strain SHP24. Thus, the hybrid plasmid pHNSHP24 carrying *mcr-1.1* may be formed before conjugation and may be the fusion product of pHNSHP24-3 and pHNSHP24-4.
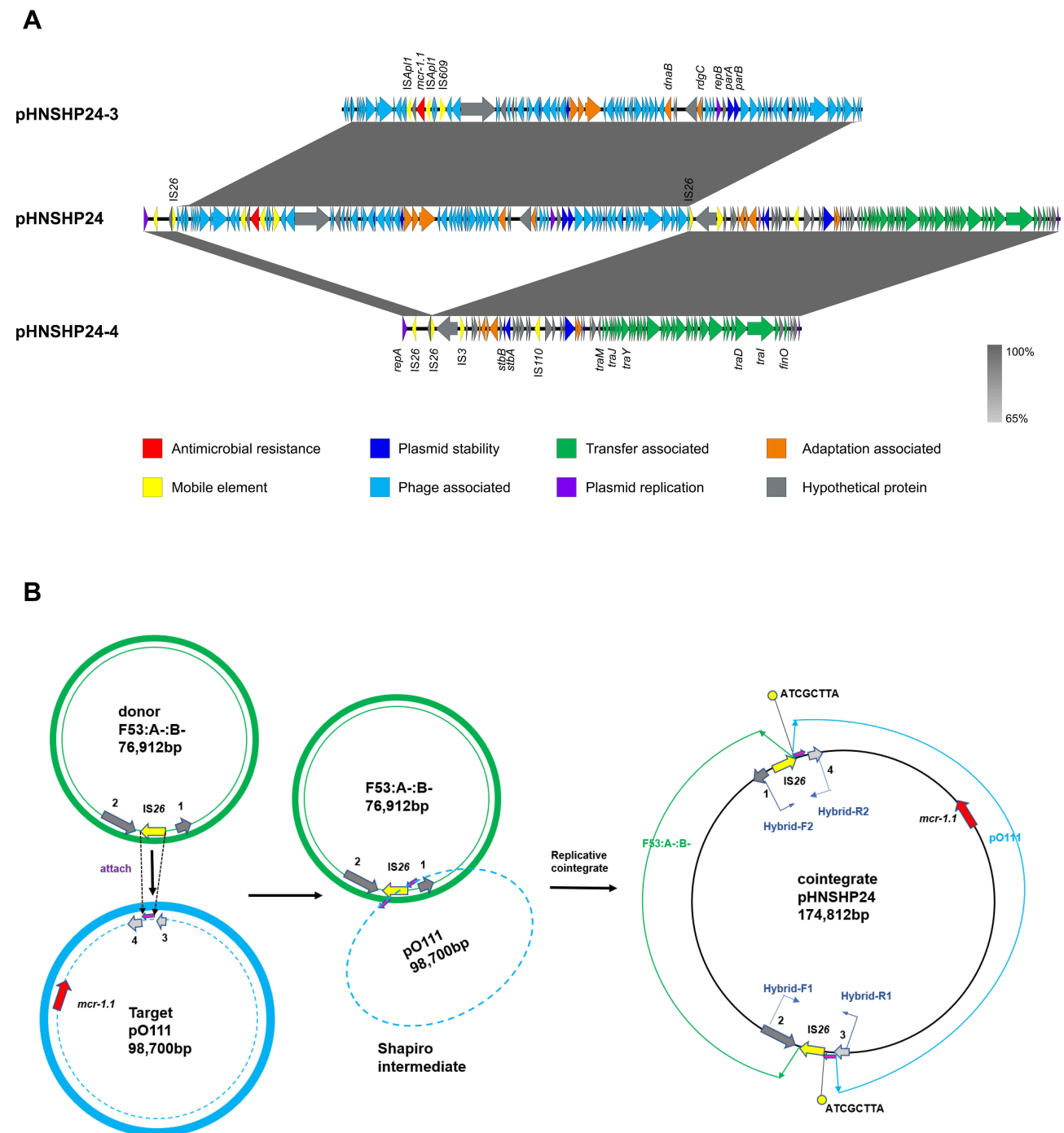
Figure 2 The proposed mechanism of the plasmid fusion. (**A**) Complete sequence comparison of fusion plasmid pHNSHP24 with daughter plasmids pHNSHP24-3 and pHNSHP24-4. Functions encoded by different genes are represented in different colors, as exhibited in the square. (**B**) A proposed model for the IS26-mediated formation of the fusion plasmid. Green lines and annulus represent donor plasmid IncF53:A-:B-. Blue lines and annulus represent target plasmid pO111. Red arrows represent the resistance genes. Yellow arrows represent the mobile element. Pink arrows represent the 8-bp target site (ATCGCTTA). Dark grey arrows labeled numbers 1 and 2 represent recombinase family gene (named *refP*) and autotransporter outer membrane gene (named *aomP*), respectively. Light grey arrows labeled numbers 3 and 4 represent phage-associated genes. Blue arrows represent the two pairs of hybrid primers spanning the regions of the fusion plasmid. The IS26 in plasmid pHNSHP24-4 (F53: A-:B-) attached the TSD in plasmid pHNSHP24-3 (pO111), causing the formation of a Shapiro intermediate. DNA replication at the intermediate branch generated a cointegrate containing a duplication of IS26 and 8-bp TSD.

Based on the complete sequence analysis of the fusion plasmid and two daughter plasmids, we proposed a formation model for the fusion plasmid (Figure 2B). In this model, IS*26* adjacent to the autotransporter outer membrane gene (named *aomP*) in pHNSHP24-4 (F53:A-:B-) recognized and attached the target site duplication (TSD) (ATCGCTTA) in the non-conjugative plasmid pHNSHP24-3 (pO111), thus forming a replicative cointegrate intermediate. Subsequently, pHNSHP24-3 was incorporated into pHNSHP24-4, producing a cointegrated pHNSHP24, accompanied by an 8-bp direct repeat (ATCGCTTA), and an additional IS*26* copy appeared upstream of the phage tail fiber gene (named *ptfP*).

The Conjugation Frequencies of the Fusion Plasmid pHNSHP24

The conjugation frequency of the fusion plasmid pHNSHP24 from parental strain *E. coli* SHP24 to recipient *E. coli* C600 was $2.95 \pm 0.36 \times 10^{-7}$, while the conjugation frequency of pHNSHP24 from the transformant *E. coli* DH5α/pHNSHP24 to recipient *E. coli* C600 was $2.66 \pm 1.44 \times 10^{-4}$.

Discussion

Multiple previous studies have reported the discovery of phages carrying multiple antibiotic resistance genes (ARGs).[22,23] However, the role of phages in the transmission of antibiotic resistance genes remains a controversial problem.[24] Nevertheless, some studies have shown that phages are capable of transferring resistance genes through phage-mediated transduction and promoting horizontal gene transfer of plasmids through transformation.[25,26]

In the present study, *mcr-1.1* was found in the phage-like plasmid pHNSHP24-3 which showed highly similar to other *mcr-1.1* positive phage-like plasmids from animal, human and environmental samples (Table S2), indicating that phage-like plasmids play significant vehicle role in the dissemination of *mcr-1.1*.[8,9,27] IncI2, IncX4 and IncHI2 plasmids are predominant *mcr-1.1*-carrying plasmids with self-transmissible capacity, high stability and fitness advantage.[5] Contrastingly, the phage-like plasmids have fitness disadvantages and were non-conjugative. However, they can be integrated with conjugative plasmids to form fusion plasmids, such as pD72C and pHNSHP24, thus acquiring conjugative transfer ability, which would accelerate the spread of resistance genes.[5,7–9,28]

pHNSHP24 was a cointegrate plasmid formed by an IncF53:A-:B- conjugative plasmid and a non-conjugative *mcr-1.1*-carrying phage-like pO111 plasmid, mediated by IS*26* through a replicative transposition mechanism.[29] Previous studies also reported the formation of some hybrid plasmids mediated by IS*26*, such as pD72C (fusion of IncN1-F33:A-:B- and phage-like pO111 plasmid), pS13D (fusion of IncN1-F33:A-:B- and IncFI:A-:B- plasmid), pSE380T (fusion of IncHI2 and virulence-carrying IncFIA plasmid), pL53T (fusion of IncX3 and IncFII plasmid), and pSL131 (fusion of IncA/C and IncX3 plasmid).[7,8,30–32] It is noteworthy that the formation of cointegrate plasmids pHNSHP24, pD72C (MK419152), and pSE380T (KY401053) was because of the IS*26* in the conjugative plasmids which attack the TSD of the non-conjugative plasmids.[7,8,32] The fusion of plasmids enables non-conjugative plasmids to obtain self-transmissible capacity, promoting the dissemination of the ARGs carried by the non-conjugative plasmids.

The number of *mcr-1.1*-bearing hybrid plasmids is currently increasing (Table S1). Of the 627 *mcr-1.1*-bearing plasmids, 51 were hybrid plasmids, including IncHI2-IncN (n = 25), IncHI1-IncFIA (n = 7), pO111-IncFIB (n = 1), pO111-IncFII-InN (n = 1), and IncY-IncHI2-IncN (n = 1) (Table S3). These hybrid plasmids typically carry multiple antibiotic resistance genes (Table S3). Interestingly, the *mcr-1.1*-carrying hybrid plasmids pHNSHP24 and pD72C both were found under antimicrobial selection pressure during conjugation assays.[7] The same phenomenon was also occurred in the formation of hybrid plasmids pS13D (CP047094), pC21-F1 (MT554516), and pC21-F2 (MT554517).[8,14] pHNSHP24-3 (pO111) carrying *mcr-1* was a non-conjugative plasmid,[7,8,14] indicating the formation of the fusion plasmid pHNSHP24 (pO111-FII) might has occurred before conjugation, and we confirmed the existence of the fusion plasmids in the original strain SHP24. But the S1-PFGE and WGS data of the original strain SHP24 failed to detect the fusion plasmid, indicating the abundance of the fusion plasmid in SHP24 is low. During conjugation experiments, fusion plasmids transferred to the recipient and transconjugants carrying fusion plasmids may be enriched under the antimicrobial selective pressure. In contrast, some previous studies suggested that the fusion plasmid may be formed during conjugation.[31,33] Thus, the fusion plasmid formed before or during conjugation still remain controversial. Nevertheless, it seemed that the antimicrobial pressure promoted the selection and dissemination of fusion plasmids. Therefore, we must charily use antibiotic drugs to avoid the emergence and transmission of fusion plasmids.

Conclusion

This study firstly reported the fusion of an IncF53:A-:B- conjugative plasmid with a non-conjugative *mcr-1.1*-bearing phage-like pO111 plasmid mediated by IS*26*. The formation of conjugative fusion plasmids may stimulate the spread of *mcr-1.1* and other resistance genes, and more attention should be paid to fusion plasmids derived from phage-like plasmids and conjugative plasmids.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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