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MECHANISMS OF RESISTANCE

IS*Kpn40*-Mediated Mobilization of the Colistin Resistance Gene *mcr-3.11* in *Escherichia coli*

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ABSTRACT The mobile colistin resistance gene *mcr-3* has globally disseminated since it was first reported in 2017 in *Escherichia coli*. *In vitro* mobilization assays in this study demonstrate the functionality of the composite transposon structure IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* in wild-type and *recA*$^-$ *E. coli* strains. These transpositions generated 4-bp duplications at the target sites. This is the first report demonstrating the mobility of the *mcr-3.11* gene by transposition.

KEYWORDS transposition, *mcr-3.11*, IS*Kpn40*, colistin resistance, transposition mechanism

Colistin is one of the last lines of defense against multidrug-resistant (MDR) Gram-negative bacteria. The first plasmid-mediated colistin resistant *mcr-1* gene was identified in November 2015, indicating the capacity of horizontal transfer of colistin resistance (1). Since then, 9 additional *mcr* genes have been identified (*mcr-2* to *mcr-10*) (2–9).

The *mcr-3* gene was first identified in *Escherichia coli* from a healthy pig fecal sample in Shandong Province, China (3). This gene shares 45 and 47% nucleotide sequence homology with *mcr-1* and *mcr-2*, respectively (3). The *mcr-3.11* gene, first identified in an *E. coli* isolate from a swine feedlot, had an upstream IS*Kpn40* insertion (3). IS*Kpn40* is an IS*3*, insertion sequence element family member of 1,213 bp and is flanked by 12-bp inverted repeats (IR) (5′-TGTAATGACCCA-3′). Other IS*3* family members, such as IS*911* and IS*150*, can transpose via circular intermediates (10, 11), and therefore transposition of *mcr-3* via a circular form mediated by IS*Kpn40* has been postulated.

Previous studies have detected a 3,535-bp circle of *mcr-3.1-dgkA*-IS*Kpn40* using inverse PCR (12), suggesting that IS*Kpn40* might be involved in the mobilization of this resistance gene. Moreover, direct repeats (DRs) (5′-CACC-3′) were identified both immediately upstream and downstream of the IS*Kpn40-mcr-3.1-dgkA*-IS*Kpn40* segment in two plasmids, pZR5_*mcr-3* and pZR10_*mcr3* (13). These previous studies suggested the ability of IS*Kpn40* to mobilize *mcr-3.11*; however, its putative role in the mobilization of the *mcr-3.11* gene remains to be determined. In this current work, we aimed to close this gap in knowledge and determine experimentally if IS*Kpn40* can mobilize the *mcr-3.11* gene.

A plasmid pYH01-TraJ, carrying an R6K ori which can only replicate in a *pir*+ host,

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
E. coli MG1655 (wild type)	K-12 strain F− λ− ilvG rfb-50 rph-1	(19)
E. coli MG1655(*recA*::Km)	K-12 strain F− λ− ilvG rfb-50 rph-1 *recA*-	(19)
E. coli WM3064	RP4(tra) in chromosome, DAP-	(20)
E. coli 22FS3-1	Clinical isolate carrying IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40*	This study
Plasmids		
pJS01	Suicide plasmid (R6K replication origin) contains $oriT_{RP4}$ fragment	(15)
pJS05	Suicide plasmid (R6K replication origin) contains and chloramphenicol resistance gene (CmR)	This study
pYH01-traJ	Suicide plasmid (R6K replication origin) contains and IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40*	This study

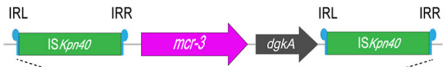
was constructed by cloning the structure IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* along with its flanking sequence into plasmid pJS05. The pJS05 was constructed as follows. First, the $RP4_{oriT}$ conjugation transfer fragment was amplified using primers traj-1 and traj-2 (Table S2 in the supplemental material) using the plasmid pCVD442 (14) as a template. Then the $RP4_{oriT}$ conjugation transfer fragment and pJS01 (15) were both digested with *speI* and SalI and ligated to give rise to the recombinant plasmid pJS05. Next, the IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* element was amplified by PCR using primers pYH-AflII and pYH-BglII, and using *E. coli* 22FS3-1 genomic DNA as the template. *E. coli* 22FS3-1 is a clinical isolate from Foshan City, China carrying IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* confirmed by primers MCR3-F and MCR3-R. (Table S2). PCR primers pJS05-AflII and pJS05-BglII were used to amplify the backbone of pJS05 using pJS05 as the template that contained a conditional replication origin R6K, which relies on π protein encoded by the *pir* gene, Cm^R (chloramphenicol resistance), and an $RP4_{oriT}$ fragment. This suicide plasmid only survives in a bacterial host with the *pir* gene (e.g., *E. coli* WM 3064) and is unable to replicate in other hosts. Ligation of these fragments after digestion with the restriction enzymes noted in the primer names resulted in the recombinant plasmid pYH01-TraJ (Table 1, Fig. S1).

The pYH01-TraJ plasmid was electroporated into *E. coli* WM3064 (16) and transformants were selected through plating on LB agar supplemented with 25 μg/ml chloramphenicol. The integrity of both IS*Kpn40* elements and *mcr-3.11* were confirmed by DNA sequencing. *E. coli* WM3064 is a pir+ diaminopimelic acid auxotroph (DAP) and contains the RP4 transfer machinery necessary for conjugation. Next, the suicide plasmid was conjugated into two recipient strains, *E. coli* MG1655 (wild type) and *E. coli* MG1655 (*recA*::Km). The survival of the transconjugants was contingent upon transposition of the selectable marker into the host genome. The transposition frequencies of pYH01-TraJ into the two *E. coli* strains were 2.85×10^{-6} and 2.53×10^{-6} per transformed cell, respectively.

Arbitrary primed PCR-based analyses (17) revealed 23 integration sites of transposon IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40*. The insertion locations of the *mcr-3.11* gene were further confirmed by PCR and Sanger sequencing. All primers are listed in Table S1. The results showed that all transposition events were separated by 4-bp direct repeats (DRs) at the insertion sites (Fig. 1). The mean AT content extending in each direction from the 4-bp target sites (−46 to −1 bp and +1 to +46 bp) were 60 and 56%, respectively (Fig. 1A). In addition, the AT content was higher in the sequences closer to the target site, and was 92% at positions −2 and −1 and 85% at positions −6 and +6. At the duplicated target site positions (C1, C2, C3, and C4), the AT content was 63%, 54%, 71%, and 50%, respectively (Fig. 1B).

To further characterize the distribution of IS*Kpn40*-mediated *mcr-3.11* transposition, we determined the insertion sites for 23 transposon events in *E. coli* MG1655. We found that 18 IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* sites were randomly located into nonessential genes and 5 were inserted between two nonessential genes in the bacterial chromosome (Table S5). That this study only found insertions into nonessential genes may be because insertions into essential genes are deleterious and/or may negatively impact

A



NO1	5'-AACCTTGCTGCGACTGTTTAATCTCCGATTCGGCTTGCGCAATATTTCGC	TCGCTTCATTGCTTCCATTTCCGCAATACGACTCGCAGCCCCGGCCTGAA-3'
NO2	5'-TGCCTGGAAATATATCTACAAAAAGCTGTGTAATACGCAATGCGGCGCAG	GCAGGCCTTAACTGACGATGGAACTCTATACGGAGCCACAATTCTTGGCG-3'
NO3	5'-TTTTACCATGGGCTACCAACGGCAAGTAACCGAAGATACTTACGAATATT	TATTTGCTGCTAAATGGTGAGGAATCTTTTAACGATCTCGGCGAGCCAGA-3'
NO4	5'-GTATTAGCTTTCGCTCTGCGGAGGGTGAGGAAAGTCAGTTAATGTAATGC	ATGCCTCCTACTGACCAAAGAATACTTGCACTTAAGGTTCAGTATAAAAG-3'
NO5	5'-TCATGTCATTGCCTCTCATTGAAGTATGATGGCTATTTGACACTATCCTT	CCTTTACCCACGCTCAACAGTTTAATAACCTGCCAGCAATAAGGGATGTT-3'
NO6	5'-AGACTTGTGCAGAAATCCTCCAGCCTGCCTTCTTCTGATATATATTAAAT	AAATAAGAATAAGATGTAGCGGAGTTGTTTTTGTGTTTACAAACAATGGC-3'
NO7	5'-CGCAACTGGAACGCCTCTCTCACCGCGTTCCATTACGGGCAATGAAAAAT	AAATGGTGTTACAGGCAACAGTGCTCAGGCTTATGTTCGCTGGTATCAAA-3'
NO8	5'-CTTCAACAACACCTTTACCCTCTTTCTCCATGAGATCTTTAGCGAACTCA	CTCAACGATCAAAATAGCGTTTTTGGCCGACAAGCCAATTGTCGTTAGCA-3'
NO9	5'-AAGGTTATCAACGTTATCAATGGCAAAACGGATACGTTGTTAGAAATCTC	TCTCTGTATTACCGTTGGATAGCTATGCGAAGCCTGATATTGAAGCCAAC-3'
NO10	5'-GGATACTTATCTGGATTTAACGATAAATAACTTTTATCTGTTGCTGCCCC	CCCCCGTGGAATTAAGAATGTTAAATACAGATTCACTCGTCACTTCTGCC-3'
NO11	5'-CGGTTATCTTTAACCGATTAATTTGATTTAGATCGCAATTTGCGATTTAA	TTAAACACAAATCTAATTCCTTGATTTAAAATACTTTCACTCTGTTACTA-3'
NO12	5'-TGTATTCACAGTATGCGCCGTTTCGGTTCTGCAGAGCAGCTACGTAAAAC	AAACGGCAGAGAGCACCCTGGAAACGGGTGATCCCGCCTATGCCCTGGCG-3'
NO13	5'-GCTTTAGTATCAGTTTTATCCGTAAACATCCGGGGCTGTTTATCGGTATG	TATGTACGTTGCTTTTTTTGCCACCCTGGCGGTGATGTTGCAGTCCGAAA-3'
NO14	5'-TATCACCTGCGTGATATAACCCTGCGCGCGAGCAGATTTCACGGAATAAT	TAATTTCACCAGACTTATTCTTAGCTATTATAGTTATAGAGAGCTTACTT-3'
NO15	5'-TTTTGAAAGACAAAGATTCTCTAGCGAGTAAAGCCCATAGAATCATCTAC	CTACACTGGACCCATTGATCAGTACTTCGACTATAGGTTTGGAGCGTTAG-3'
NO16	5'-ATAATGCGCCTCCGTTGAGACGACAACGTGAAACACTTCACAGGATGGTC	GGTCGGAACAACGAAGAGAAAAAAATCCTGAAATTCAGGGTTGACTCTGA-3'
NO17	5'-ACTAGTGCAGTTGTGGCGTGGCAAGATGACAATCATCATTTCCGTCATTG	ATTGTGGCTATTGCCCTGGCTATTGGATATTTGGCAGTAGCGAAGGAGAA-3'
NO18	5'-TAGTAATTTTCGCGAATGTCAGATTAGAAATTCAAACTTCGATAATTGTT	TGTTATCTTTACGCTTCGCACTTCACCAGAGCAGAGTTTCTGTCTGCCAA-3'
NO19	5'-CCGCCTCGCCGAATTGACCATGAATAAACTGTACGACAAGATCCCTTCCT	TCCTCAGTATGGAAATTTATTCGCTAATAAATAATTCGCTTTCGGAGCTA-3'
NO20	5'-ATCGCTGCGCTGGAAAAAGCGCACCCGGACGTCGAACTGTATACCGCATC	CATCGATTGATCAGGGACTGAACGAGCACGGATACATTATTCCGGGCCTC-3'
NO21	5'-GGTAAAAAACTTCGCCACCAGAAAGATAATCCCGCCATAGTAAGCAGGCA	GGCATCCCTAATTCATCGGTATAAAATTTGAGGAGATACAGAGTACCGAT-3'
NO22	5'-TCTATCGAAATAACTGAACAGGGCGCGGAGTTCCGCGCTCTTTTTAACGA	ACGATACAGTATCTCTCCATAAGATAAAATATTGCTATATTCTGCTGGGT-3'
NO23	5'-ACTTTAATGCCTTCTTGTTTGGCTTCTTTTACAACGTTACTTAATACCTG	CCTGACCGTTATACGGAATAATGACAAGAACATCGACACCCCGGTTTATC-3'

B

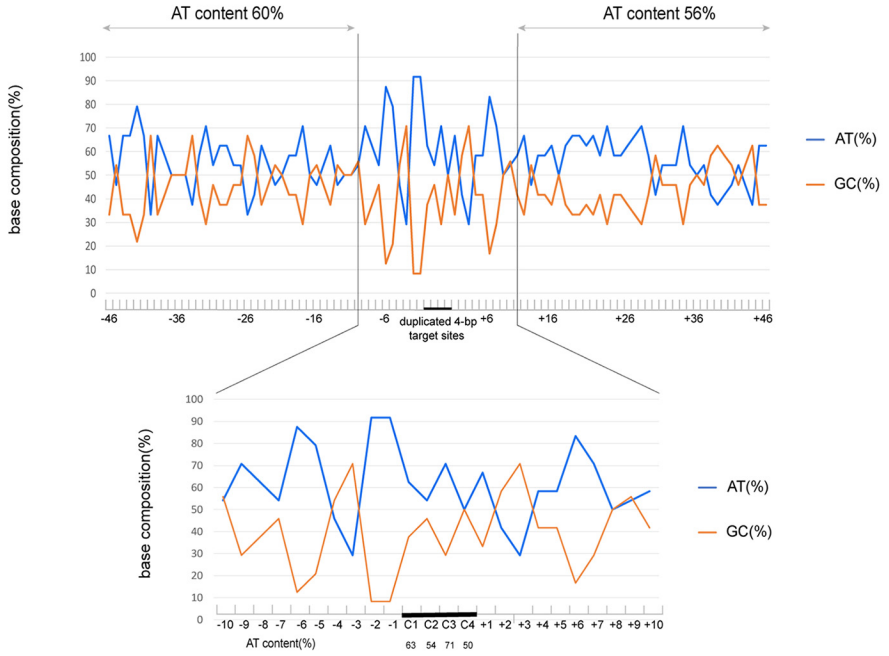


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FIG 1 Target site analyses of 23 transposition events. (A) Molecular characterization of 23 transposition events of transposons in *E. coli* MG1655 (*recA*::Km). The duplicated 4-bp target sites are underlined in the context of the surrounding 46 nucleotides upstream and downstream of the target sites. (B) Statistical analyses of the 23 transposition sites. The percentage of AT and GC at each position from 46 nucleotides upstream to 46 nucleotides downstream of the target sites are shown. The 4-bp duplicated target sites (C1, C2, C3, and C4) are indicated by black bars. The AT and GC percentages of regions spanning positions −46 to −1 bp and positions +1 to +46 bp and that of the region spanning positions −10 to +10 bp are indicated in the upper and lower graphs, respectively. (C) Pictogram showing the relative frequencies of each A, T, C, and G at the target site deduced from the 23 experimental transposition events.

growth, and therefore cannot be selected on plates to be identified. Finally, we verified those sites containing the whole IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* genetic structure in the genome of *E. coli* MG1655 by PCR with primers (Table S3) binding the upstream and downstream sequences, respectively.

In this study, we demonstrated the functionality of IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* transposition from plasmids where cell survival was dependent on transposition of the *mcr-3.11* selective marker. This IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* structure from plasmid pYH01-TraJ can transpose efficiently and randomly into the *E. coli* chromosome. Notably, several transposases and IS elements, including IS*4321*, IS*26*, ΔTnAs2, or the IS*Kpn40* have been identified in the flanking regions of *mcr-3.1* (13). IS*Kpn40* is present in *E. coli*, *Salmonella enterica*, *Aeromonas caviae*, *A. veronii*, and *Klebsiella pneumoniae* (Table S4). The IS*Kpn40*-mediated translocation of *mcr-3.11* may accelerate transmission of *mcr-3.11* among these species, as IS*Kpn40* can efficiently mobilize *mcr-3.11* (this study). Conversely, the transposition of *mcr*-1 has always been associated with IS*Apl1*, and no other insertion sites were identified in flanking regions (15, 18).

The association of multiple insertion elements in *mcr-3.11* also suggests the possibility that the new mobile colistin resistance gene *mcr-3.11* may utilize different transposons to mobilize. In this study, we confirmed the functionality of IS*Kpn40*; however, whether and how other IS elements and transposons contribute to the mobility of *mcr-3* in different species or genera has yet to be determined. This work demonstrates the effective mobilization of the *mcr-3.11* gene into the *E. coli* chromosome mediated by IS*Kpn40*. Interestingly, the IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* structure was incorporated randomly in or near nonessential genes with no obvious preference for GC- or AT-rich DNA domains.

In summary, we verified that transposition of *mcr-3.11* is mediated by IS*Kpn40*. Our work demonstrates that IS*Kpn40* can transpose *mcr-3.11* in *E. coli*. This is especially important for other clinically relevant bacterial species in the *Enterobacteriaceae* family, in which IS*Kpn*40 is present, for possible translocation of *mcr-3.11*. Future studies will focus on the regulatory mechanisms of IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* transposition. Additional future work will focus on the other routes of *mcr-3.11* gene transmission to elucidate these pathways and help to control the spread of *mcr-3.11* and colistin resistance.

MATERIALS AND METHODS

Data availability. The nucleotide sequence of amplicon IS*Kpn40-mcr-3.11-dgkA*-IS*Kpn40* has been deposited in GenBank under accession number MT561503.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We declare that we have no competing interests.

J.S. designed this project. Y.-Z.H., T.-F.L., C.-P.C., and B.H. performed the experiments. Y.-Z.H., X.-P. Li, L.C., and J.S. analyzed the data. Y.-Z.H. made the figures. Y.-Z.H. wrote the manuscript. X.-P. Liao, and J.S. edited and revised the manuscript. Y.-H.L. coordinated the whole project.

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