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In Vitro Study of IS*Apl1*-Mediated Mobilization of the Colistin Resistance Gene *mcr-1*

Laurent Poirel,[a,b,c] Nicolas Kieffer,[a,b,c] Patrice Nordmann[a,b,c,d]

Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland[a]; French INSERM European Unit, University of Fribourg (LEA-IAME), Fribourg, Switzerland[b]; Swiss National Reference Center for Emerging Antibiotic Resistance, Fribourg, Switzerland[c]; University of Lausanne and University Hospital Center, Lausanne, Switzerland[d]

ABSTRACT The plasmid-mediated *mcr-1* gene encodes a phosphoethanolamine transferase that confers resistance to polymyxins. The *mcr-1* gene is associated with insertion sequence IS*Apl1* (IS*30* family). *In vitro* mobilization assays demonstrated the functionality of the composite transposon structure IS*Apl1*-*mcr-1*-IS*Apl1*. Transposition generated a 2-bp duplication and occurred in AT-rich DNA regions. This is the first report demonstrating the mobility of the *mcr-1* gene by transposition.

KEYWORDS IS*Apl1*, composite transposon, *mcr-1*, plasmid, polymyxins, transposition

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Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

L.P. and N.K. contributed equally to this work.

Since its discovery by the end of 2015 (1), the occurrence of the plasmid-mediated colistin resistance gene *mcr-1* has been reported worldwide. This gene has now been reported in a variety of enterobacterial species, mostly in *Escherichia coli*, from human, environmental, and animal samples (2), and also from retail food (3). Retrospective studies reported MCR-1-producing colistin-resistant isolates as early as in the late 1980s (4), but several studies suggest that spread of the *mcr-1* gene is on a rising trend (5).

Various plasmids may carry the *mcr-1* gene, including those belonging to the incompatibility groups IncX4, IncI2, InHI2, IncF, IncY, and IncP (6–10). This gene is often identified in association with the insertion sequence IS*Apl1*, which may play a major role in its mobilization (11–13).

IS*Apl1* belongs to the IS*30* family and was first identified in *Actinobacillus pleuropneumoniae* (14), a Gram-negative rod of the *Pasteurellaceae* family that is a causative agent of porcine necrotic pleuropneumonia. It is a 1,070-bp-long mobile element possessing a 924-bp open reading frame (ORF) encoding a 307-amino-acid transposase that contains a DDE domain containing the carboxylate residues believed to be responsible for coordinating metal ions needed for catalysis. IS*Apl1* is flanked by two imperfect 27-bp inverted repeats (IRs) exhibiting 6 base pair mismatches. In a recent study (11), an intermediate circular form of IS*Apl1* associated with *mcr-1* was detected, suggesting that IS*Apl1* might be involved in the mobilization of this resistance gene. Moreover, a ca. 790-bp open reading frame has been identified downstream of the *mcr-1* gene in most of the MCR-1 producers. This sequence is not believed to play any role in colistin resistance (15); nevertheless, its putative role in the mobilization of the *mcr-1* gene remains to be determined. Recent works (6, 16) showed that *mcr*-1 is part of a 2,600-bp cassette containing promoter sequences for *mcr*-1 expression and is bracketed in most cases by two direct copies of IS*Apl1*, suggesting that it may constitute a composite transposon element. Therefore, the aim of our study was to determine experimentally whether IS*Apl1* could actually mobilize the *mcr-1* gene.

Since preliminary experiments showed that cloning the *mcr-1* gene in regular recombinant vectors is difficult, likely due to a toxic effect of MCR-1 once overproduced

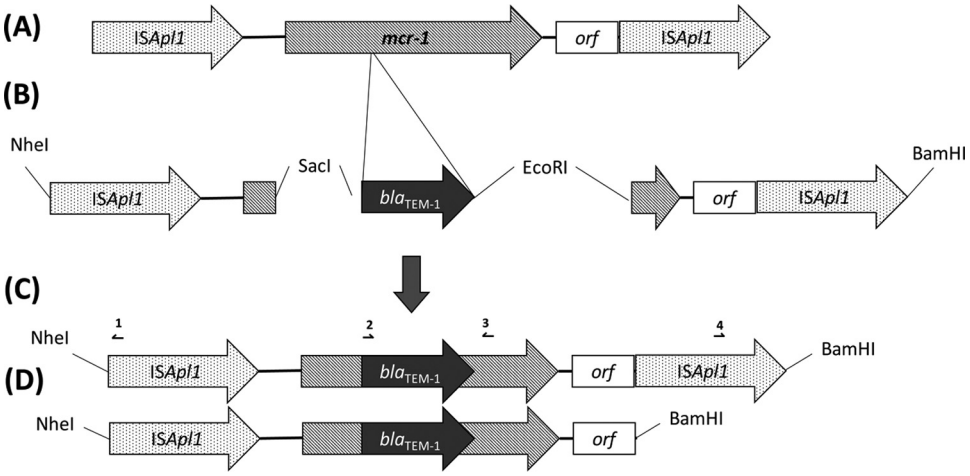

FIG 1 Schematic map of the different constructs performed for the transposition study. (A) corresponds to the original transposon IS*Apl1-mcr-1-orf*-IS*Apl1* identified in clinical isolates; (B) shows the different fragments generated by PCR (with corresponding restriction sites indicated) and used as templates for ligation and subsequent genesis of (C) IS*Apl1-mcr-1–bla*$_{TEM-1}$-*orf*-IS*Apl1* or (D) IS*Apl1-mcr-1–bla*$_{TEM-1}$-*orf* genetic structures. Locations of primers used for the inverse PCR strategy (as listed in Table 2) are indicated by small half arrows. Restriction sites of endonucleases used for cloning are indicated (NheI, SacI, EcoRI, and BamHI).

in *E. coli* (data not shown), a truncated form of *mcr-1* was created by inserting the *bla*$_{TEM-1}$ gene into the coding sequence of *mcr-1*, as shown in Fig. 1. Then, two structures were analyzed, the entire composite transposon bracketed by two copies of IS*Apl1* and encompassing the *mcr-1–bla*$_{TEM-1}$ gene, and the same structure deleted from the right-hand copy of IS*Apl1*. Those two different genetic structures, namely, IS*Apal1-mcr-1–bla*$_{TEM-1}$-*orf*-IS*Apl1* and IS*Apl1-mcr-1–bla*$_{TEM-1}$-*orf*, respectively, were obtained by PCR and then ligated and cloned into plasmid pNK1 (p15A-pTOPO-ΔlacP-Kan^r), giving rise to recombinant plasmids pNK31 and pNK45, respectively. Those plasmids were transformed into *E. coli* TOP10 (InvitroGen, Thermo Fisher Scientific, Ecublens, Switzerland) and selected onto Luria-Bertani (LB) agar plates supplemented with 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. Plasmids pNK31 and pNK45 were then transformed into the *E. coli* strain RZ211 containing the transfer-proficient pOX38 F plasmid carrying a gentamicin resistance gene (17). Plasmid pOX38 is a self-conjugative and IS-free plasmid encoding resistance to gentamicin that serves as a target for transposition events that may be searched after 24 h of growth, as described previously (18). By conjugating the pOX38 plasmid into another *E. coli* recipient strain using gentamicin as selective marker, it is therefore possible to isolate and identify putative transposition events. The strains used in this study are listed in Table 1.

Clones were selected onto LB agar plates supplemented with ampicillin (100 μg/ml), kanamycin (25 μg/ml), and gentamicin (10 μg/ml). *E. coli* RZ211-harboring recombinant plasmid pNK31 or pNK45 was used as a donor for conjugation experiments with azide-resistant *E. coli* strain J53. Briefly, the donor and recipient strains were separately cultured overnight and then subcultured for 5 h in order to reach the exponential phase. Mating-out assays were performed on solid medium using filters with a 1:10 donor to recipient ratio. After 5 h of incubation, filters were resuspended in NaCl 0.85% and bacterial mixtures were plated onto agar plates supplemented with gentamicin (10 μg/ml) and sodium azide (100 μg/ml) or onto agar plates supplemented with genta-

TABLE 1 *E. coli* strains used in this study

Strain	Feature
Af31	MCR-1-producing clinical isolate carrying two copies of IS*Apl1*
Af45	MCR-1-producing clinical isolate carrying only one copy of IS*Apl1*
RZ211	Isolate carrying the pOX38-Gen plasmid
J53	Azide-resistant isolate used for mating-out experiments

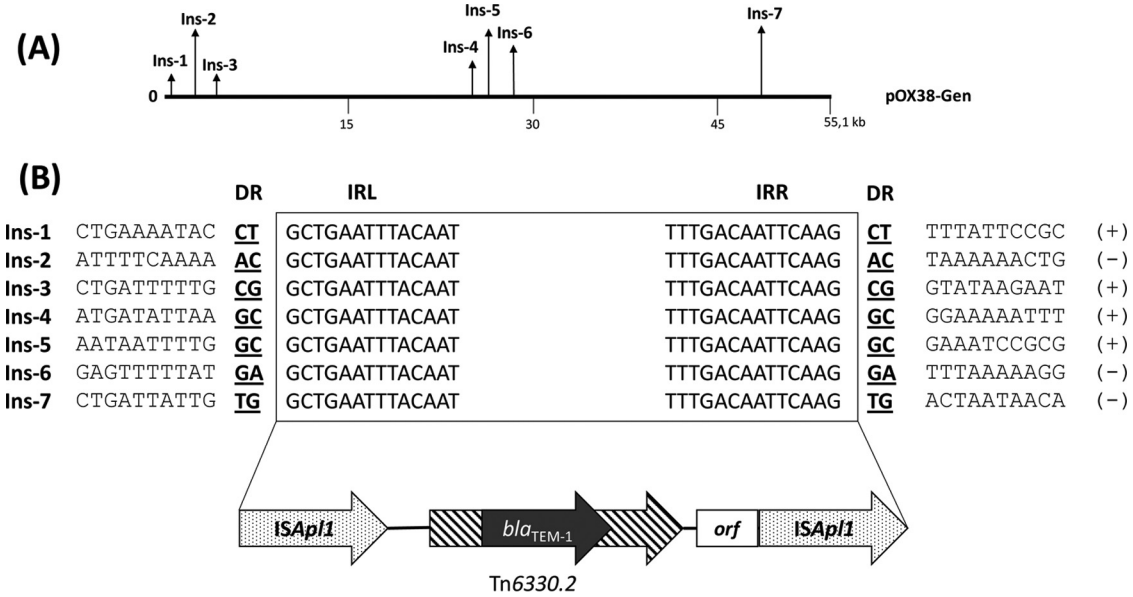

FIG 2 Target sites of the IS*Apl1-mcr-1–bla*$_{TEM-1}$*-orf*-IS*Apl1* composite transposon. (A) Map positions of IS*Apl1-mcr-1–bla*$_{TEM-1}$*-orf*-IS*Apl1* composite transposon in plasmid pOX38-Gen. Insertions of the tagged insertion sequence (Ins-1 to −4) are indicated by a vertical arrow. (B) Nucleotide sequence alignment of the three IS*Apl1-mcr-1–bla*$_{TEM-1}$*-orf*-IS*Apl1* transposon events identified into pOX38-Gen. Nucleotide sequences of the end regions of transposon are boxed. Boldfaced letters indicate target site sequences duplicated upon transposition. Orientations of the insertion sequences are indicated by (+) and (−).

micin, sodium azide, and ampicillin (100 μg/ml). All Gen^{r}Azide^{r}Amp^r colonies were screened for kanamycin susceptibility to exclude the spontaneous *E. coli* RZ211 azide-resistant mutants or possible cointegration events that might not correspond to transposition events. The transposition frequency was obtained by dividing the number of Gen^r Azide^r Amp^r Kan^s colonies by the number of Gen^{r}Azide^r transconjugants. In total, we randomly selected 100 Gen^r Azide^r Amp^r Kan^s transposants recovered from the conjugation experiment, using RZ211-pNK31 as donor, and identified seven distinct transposition events (Fig. 2). No transposant was found with the donor strain RZ211-pNK45 (only a single copy of IS*Apl1*). The transposition frequency determined in *E. coli* J53 with pNK-31 as donor plasmid was estimated to be 2.2×10^{-8}, which is relatively low. The insertion sites of the IS*Apl1-mcr-1–bla*$_{TEM-1}$*-orf*-IS*Apl1* cassette were determined by using an inverse PCR strategy. Briefly, DNA from the transposants was extracted using the GenElute bacterial genomic DNA kit (Sigma-Aldrich) and digested by the PstI restriction enzyme (InVitrogen). Digested fragments were self-circularized by ligation and used as templates for reverse PCR using outward primers, as listed in Table 2 and indicated on the figures. Since two PstI restriction sites were located into the *bla*$_{TEM-1}$ and *mcr-1* genes, respectively, two PCR amplifications per transposant were performed. The first PCR amplification was performed using primers IS*Apl1*-SP3 and

TABLE 2 Sequences of primers used in this study

Primer	Sequence (5′ → 3′)[a]	Position in Fig. 1
ISApISP3	CAGGCTGCTCTAATTTGCGC	1
IS*Apl1*-3′-Fw	AGACATCAATCAGTGGAGCG	4
mcr-south-Rv	GATAGACACCGTTCTCACCC	3
Nhe-ISApl1	GATGATGCTAGCGCTGAATTTACAATCCAAGT	
SacI-Δmcr-1	GATGATGAGCTCGTAGGGCATTTTGGAGCATG	
Sac-I-TEM-1	GATGATGAGCTCGTATCCGCTCATGAGACAATA	2
EcoRI-TEM-1	GATGATGAATTCTCTAAAGTATATATGAGTAAACTTGGTCTG	
EcoRI-Δmcr-1	GATGATGAATTCCCGAGACCAAGGATCTATTA	
BamHI-Cass	GATGATGGATCCGTTATTTCTGTTTGGGGTTG	
BamHI-ISApl1	GATGATGGATCCCATTGCGCAATCCCATACTG	

[a]Underlining indicates the restriction sites.

TEM-Fw, and a second PCR was performed using primers mcr-south-Rv and IS*Apl1*-3′-Fw, in order to characterize the 5′ and 3′ genetic contexts of the insertions, respectively. Sequencing of the corresponding amplicons revealed that transposition events occurred in seven different sites, namely, Ins-1 to Ins-7 (Fig. 2A), the whole mobilized transposon being always 5,699-bp in size. Each transposition event generated 2-bp direct repeats at the insertion site (Fig. 2B). High AT-rich DNA sequences were identified on the two flanking regions of all insertion sites (Fig. 2). Our data are in accordance with previous studies showing that IS*Apl1* like other IS*30*-like elements targets preferentially AT-rich sequences (14). Noteworthy, *in silico* analysis showed that in most of the sequenced plasmids, the *mcr-1* gene is flanked by AT-rich regions.

Our results therefore support the hypothesis made by Snesrud et al. suggesting that the mobilization of *mcr-1* is mediated by a composite transposon (12). The fact that the *mcr-1* gene was associated with only a single copy of IS*Apl1* at its 5′ extremity in some studies might be explained by the characteristic of IS*30* family members to excise one copy of the IS element by transposition or by illegitimate recombination events after transposition of the original composite transposon, in order to stabilize the genetic structure once integrated (19, 20). This hypothesis agrees with the lack of transposition events observed using the pNK45 construct.

Here, we demonstrate the effective mobilization of the *mcr-1* gene located into a composite transposon named Tn*6330.2*, based on *in silico* comparison with Tn*6330* (11). This work confirms previous hypotheses (12) that the *mcr-1* gene had been initially mobilized by two copies of IS*Apl1* from an unknown progenitor, targeting broad-host range plasmid(s) that subsequently transferred this resistance gene into Enterobacteriaceae. Interestingly, our very recent work showed that *Moraxella* species are natural sources of *mcr*-like genes, and may harbor IS*Apl1* elements (21). Therefore, mobilization of the *mcr-1* gene might have occurred into a *Moraxella* species (still to be precisely identified) through an IS*Apl1*-mediated transposition process. Further studies are being conducted to reproduce mobilization of *mcr*-like genes from such bacterial sources by IS*Apl1*-mediated transposition.

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