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Functional Characterization of a Miniature Inverted Transposable Element at the Origin of *mcr-5* Gene Acquisition in *Escherichia coli*

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ABSTRACT Plasmid-mediated colistin resistance of the mobile colistin resistance (MCR) type is a growing concern in *Enterobacteriaceae* since it has been described worldwide in humans and animals. Here, we identified a series of MCR-producing *Escherichia coli* isolates corresponding to two different clones (represented by isolates PS1 and PS8b) producing MCR-1 and MCR-5, respectively, obtained from pig fecal samples in France. Plasmid analysis showed that the plasmid carrying the *mcr-1* gene (pPS1) possesses an IncHI2 backbone, whereas the *mcr-5* gene was carried onto a 6,268-bp nontypeable non-self-conjugative plasmid (pPS8b). Detailed analysis of plasmid pPS8b revealed a 3,803-bp-long cassette containing the *mcr-5* gene that was bracketed by two inverted-repeat (IR) sequences with 5-bp-long direct repeats at each extremity, similarly to an insertion sequence, but with the exception that no transposase gene was identified within this cassette. By performing *in vitro* transposition experiments, we showed that the *mcr-5* cassette could be mobilized by the Tn*As1* transposase provided in *trans*, displaying a mobilization mechanism similar to that of miniature inverted-repeat transposable elements (MITEs).

KEYWORDS MCR, MITE, colistin, mobilization, pig, resistance, transposition

Mobile genetic elements represent a key feature in Gram-negative bacteria to adapt to their environment, and particularly, to selective pressures. Acquisition of antibiotic resistance may be either due to mutations in chromosomal genes being intrinsic to the species or to the acquisition of foreign DNA through horizontal gene transfer. Acquisition and horizontal transfer of antimicrobial resistance genes are mainly mediated by transposons, integrons, and plasmids (1, 2). Altogether, those genetic structures contribute to the occurrence of multidrug-resistant isolates and to the large spread of resistance determinants, not only on a geographical point of view, but also within bacterial species.

Plasmid-mediated colistin resistance genes have been recently widely identified among enterobacterial isolates recovered from humans and animals (3). Since the identification of the first colistin resistance determinant, namely, mobile colistin resistance 1 (MCR-1), seven other different MCR-type enzymes have been characterized (4–11). Those enzymes are phosphoethanolamine transferases being responsible for adding phosphoethanolamine to the lipid A moiety of the lipopolysaccharide (LPS) (the target of polymyxins) (3). Colistin resistance is therefore explained by a lack of interaction between polymyxins and a modified LPS structure. The progenitors of the different *mcr* genes have been shown to correspond to a variety of Gram-negative

bacterial species, namely, *Moraxella* spp. for *mcr-1* and *mcr-2*, *Aeromonas* spp. for *mcr-3*, and *Shewanella* spp. for *mcr-4* (6, 7, 12, 13). The process of acquisition of *mcr*-like genes from their progenitors to their respective plasmid supports remains poorly understood so far, with the exception of *mcr-1* that was shown to be, in some cases, associated with an IS*Apl1*-made functional composite transposon (2, 14, 15). Other *mcr*-like genes were often identified into structures comprising insertion sequences (IS), but the role of these IS in the acquisition of the resistance genes was not elucidated. The *mcr-5* gene was first described in a *Salmonella enterica* isolate, and *in silico* analysis identified an association with a Tn*3*-like transposon sharing 90% nucleotide identity with IS*Pa38* (8).

It is speculated that the use of colistin in veterinary medicine worldwide has contributed to the selection of colistin-resistant *Enterobacteriaceae* and thereby of MCR-producing isolates in animals (16). A recent prospective study performed in Portugal evidenced a strong link between the high use of MCR-producing *Escherichia coli* and *Klebsiella pneumoniae* strains and the use of colistin as a prophylactic treatment in pigs (17). Here, we aimed to characterize the genetic features of colistin resistance acquisition among colistin-resistant *E. coli* isolates recovered from pig farms in France.

RESULTS

Identification of colistin-resistant *E. coli* isolates. Among the 147 collected samples, 35 samples showed a positive culture for a colistin-resistant *E. coli* isolate. PCR experiments showed that out of 35 colistin-resistant *E. coli* isolates recovered, 15 isolates were positive for the *mcr-1* gene and 8 isolates were positive for the *mcr-5* genes. No *mcr*-like genes were identified among the other colistin-resistant isolates. They likely possessed mutations in chromosomally encoded proteins involved in lipid A modifications, as recently reported, and are under investigation (18). All MCR-positive isolates were identified from a single pig farm, being the only one where colistin is frequently given for treating piglet diarrhea. Pulsed-field gel electrophoresis (PFGE) analysis showed that all MCR-1 and all MCR-5 producers belonged to two distinct clones (data not shown). A single MCR-1-producing isolate (PS1) and a single MCR-5-producing isolate (PS8b) were retained for further characterization.

Both isolates had an MIC value for colistin of 8 μg/ml. Multilocus sequence typing results identified the PS1 and PS8b isolates as belonging to sequence type 5409 (ST5409) and ST5786, respectively. Both isolates belonged to the phylogroup B1, suggesting a commensal origin. Isolate PS1 showed resistance to penicillins, sulfamethoxazole-trimethoprim, chloramphenicol, and tetracycline, although isolate PS8b showed resistance only to narrow-spectrum penicillins and sulfamethoxazole-trimethoprim.

Plasmid analysis. Mating-out assays followed by plasmid analysis showed that the plasmid carrying the *mcr-1* gene in isolate PS1 (pPS1) was a conjugative IncHI2 plasmid. However, no *E. coli* transconjugant could be obtained using isolate PS8b as a donor, but electrotransformation experiments using a plasmid extract from isolate PS8b allowed us to obtain an *E. coli* transformant carrying a plasmid (pPS8b) on which the *mcr-5* gene was located. Plasmid pPS8b was nontypeable by PCR-based replicon typing (PBRT). Susceptibility testing showed that the *E. coli* transconjugant carrying plasmid pPS1 showed coresistance to sulfonamides and tetracycline, whereas no other resistance determinant was provided by plasmid pPS8b.

Sequencing of plasmid pPS8b (GenBank accession number MH674200.1) revealed a size of 6,268 bp and a sequence identical to that of the recently identified *mcr-5*-bearing plasmid pEC0674 (GenBank accession number MF684783.1) (19). By analyzing the sequence of plasmid pPS8b, we found that *mcr-5* was part of a 3,810-bp-long inserted cassette. This cassette also contained a chromate resistance gene, *chrB,* and the inverted repeat left (IRL) of the Tn*3*-like structure, as previously identified in the original study that identified the *mcr-5* gene (8) (Fig. 1). Noteworthy, a 5-bp-long direct repeat (AATTA) was identified at each extremity of the cassette, suggesting that this cassette had been acquired through a transposition mechanism, even though no transposase gene was identified on plasmid pPS8b. A 148-bp-long sequence sharing

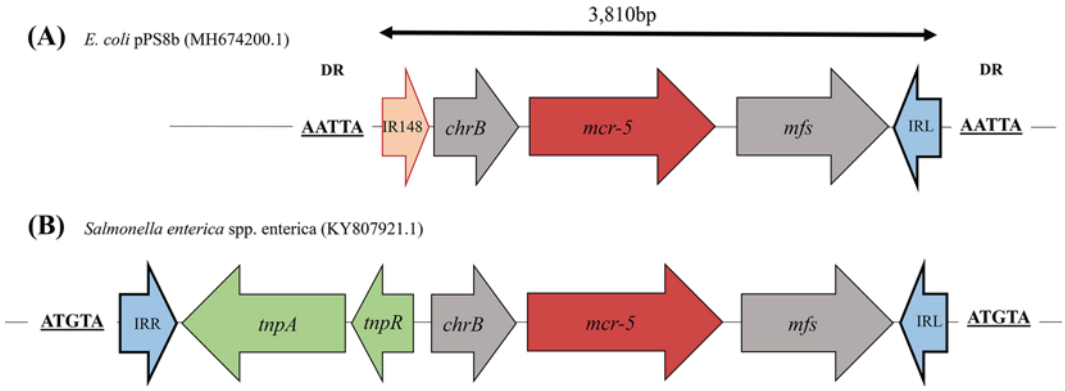

FIG 1 (A and B) Schematic representation of the *mcr-5* cassette inserted into the pPS8b plasmid (A) and comparison with a similar structure previously described (B). The direct repeats (DRs) being signature of transposition events are in bold and underlined. IRL corresponds to a Tn*3*-like left inverted-repeat sequence.

85% identity with the IRL of a Tn*3*-like transposon was identified at the 5′ extremity of the *mcr-5* gene (Fig. 2).

In silico analysis showed that this 148-bp-long IR-like structure was actually present in similar genetic contexts. Indeed, this exact same sequence, later named IR148, was identified in the near vicinity of Tn*3*-like transposons that were lacking their cognate transposase gene. Hence, IR148, together with the original IR of a truncated Tn*3*-like structure, likely corresponded to the boundaries of the whole cassette, being bracketed by direct repeats (Fig. 1). This suggested that IR148 could be involved in the mobilization of the *mcr-5* cassette that likely occurred through a nonautonomous transposition mechanism. Those features resemble those described as miniature inverted-repeat transposable elements (MITEs), being reported as mobile elements possessing left and right IRs and lacking a transposase gene. Some studies even confirmed that they may be mobilized by the in *trans* activity of specific transposases recognizing those IRs (20).

Mobilization of the *mcr-5* cassette by the Tn*As1* transposase. By using the BLAST tool of the ISfinder webpage (https://isfinder.biotoul.fr/blast.php) (21), we found that the sequence of IR148 shared the highest nucleotide identity with the IRL of the Tn*As1* transposase (90%). In addition, the IR right (IRR) of the Tn*As1* shared 80% nucleotide identity with the Tn*3*-like IRL of the *mcr-5* transposon. Hence, we speculated that the Tn*As1* transposase might be able to mobilize the *mcr-5*-containing structure in *trans* and therefore performed corresponding *in vitro* transposition experiments.

Initial attempts to mobilize the *mcr-5* cassette by using the transposase identified in the original Tn*3* transposon found in association with the *mcr-5* gene (8) were unsuccessful, despite the high level of nucleotide identity between its IRs and those of the *mcr-5* cassette. Therefore, transposition experiments were done using the *E. coli* RZ211 recombinant strain containing the pOX38 conjugative plasmid, together with the pACYC-*mcr-5* and the pBAD-Tn*As1.1* recombinant plasmids. That recombinant strain was therefore used as the host of putative transposition events, speculating that the *mcr*-5 cassette could be mobilized onto plasmid pOX38 by transposition through the in *trans* activity of the Tn*As1.1* transposase. In order to evidence such a putative transposition event, mating-out assays were performed using the azide-resistant J53 strain as a recipient, and 10 *E. coli* transconjugants were analyzed. Susceptibility testing

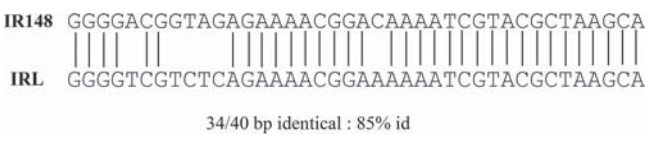

FIG 2 Alignment of the 40 first nucleotides of the IR148 with the original IR identified on the *mcr-5* cassette.

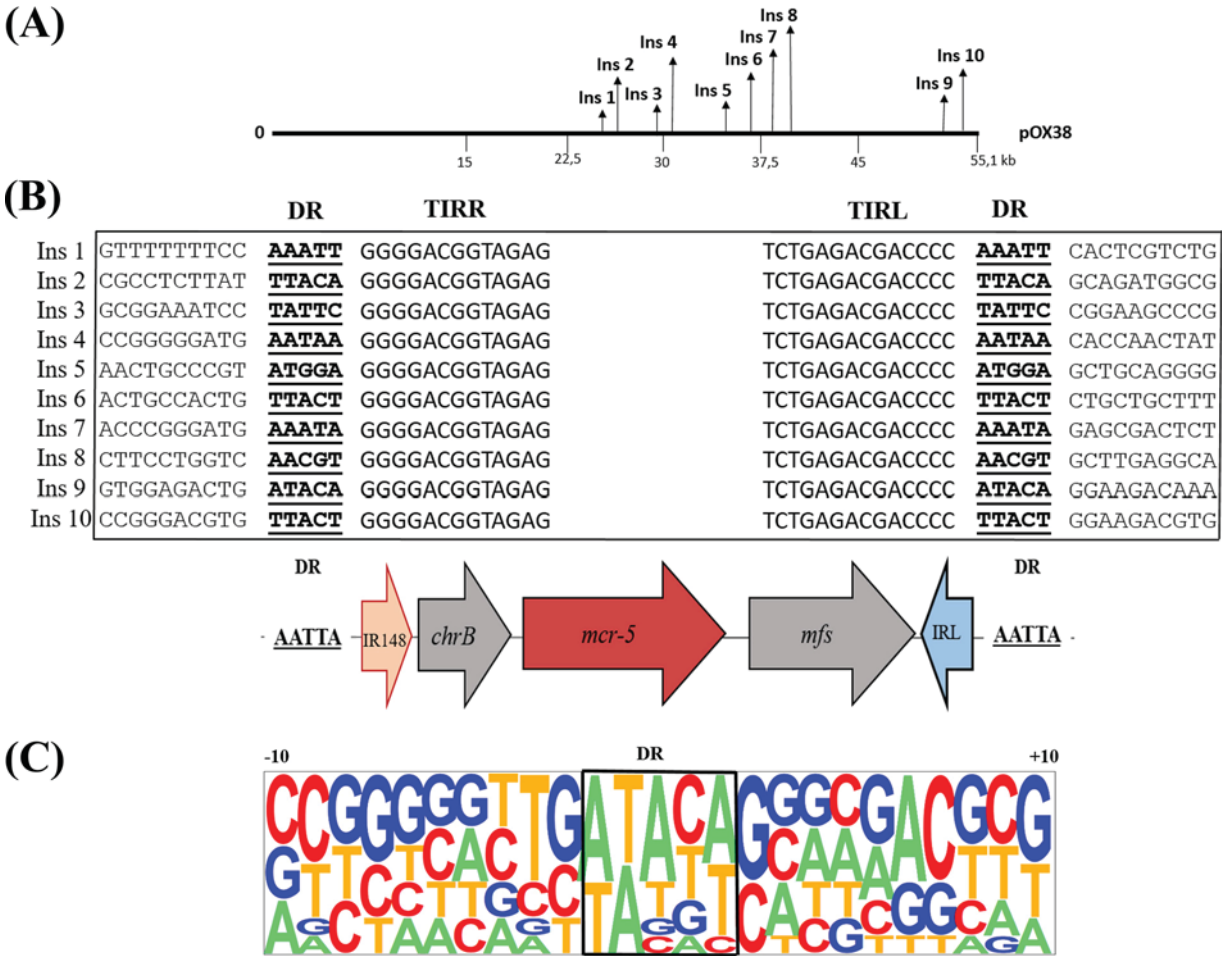

FIG 3 Target site analysis of the *mcr-5* cassette. (A) Schematic representation of the positions of the integration sites of the MITE-like structure onto plasmid pOX38. (B) Sequence alignment of 10 transposition events identified onto plasmid pOX38. The IR148 (IR-like) and inverted repeat left (IRL) sequences of this MITE structure are boxed. The 5-bp duplicated direct repeat (DR) sequences are highlighted in bold and underlined. (C) Pictogram showing the relative frequencies of A, T, C, and G nucleotides at the target site and their vicinities 10 bp downstream and upstream.

confirmed that those transconjugants were resistant to gentamicin, sodium azide, and colistin but remained susceptible to chloramphenicol and ampicillin, which was in agreement with a transfer of plasmid pOX38 carrying the *mcr-5* cassette. PCR experiments confirmed that the *mcr-5* cassette had indeed transposed onto the pOX38 conjugative plasmid.

Sequence analysis identified 10 different insertion sites of this transposed structure that were systematically bracketed by a 5-bp direct repeat (DR) sequence (Fig. 3A and B). Analysis of the GC content of the close genetic environment of the insertion site revealed an AT-rich sequence whose consensus was found to be A/T, A/T, A/T, B, A/T (Fig. 3C). The genetic environment surrounding the different target sites did not display a particular feature.

DISCUSSION

We first identified here MCR-5-producing *E. coli* isolates in France. Sequence analysis of plasmid pPS8b revealed a particular genetic structure displaying an *mcr-5* cassette surrounded by two imperfect inverted-repeat sequences but with no transposase-encoding gene, suggesting that the *mcr-5* cassette could correspond to a nonautonomous mobile genetic structure, similarly to MITEs. MITEs are defined as small genetic elements bracketed by two IRs that can be recognized by a transposase acting in *trans* (21). Such transposase activity can therefore mobilize the MITE element by recognizing the two IR sequences. In the case that such a mobilized genetic element actually

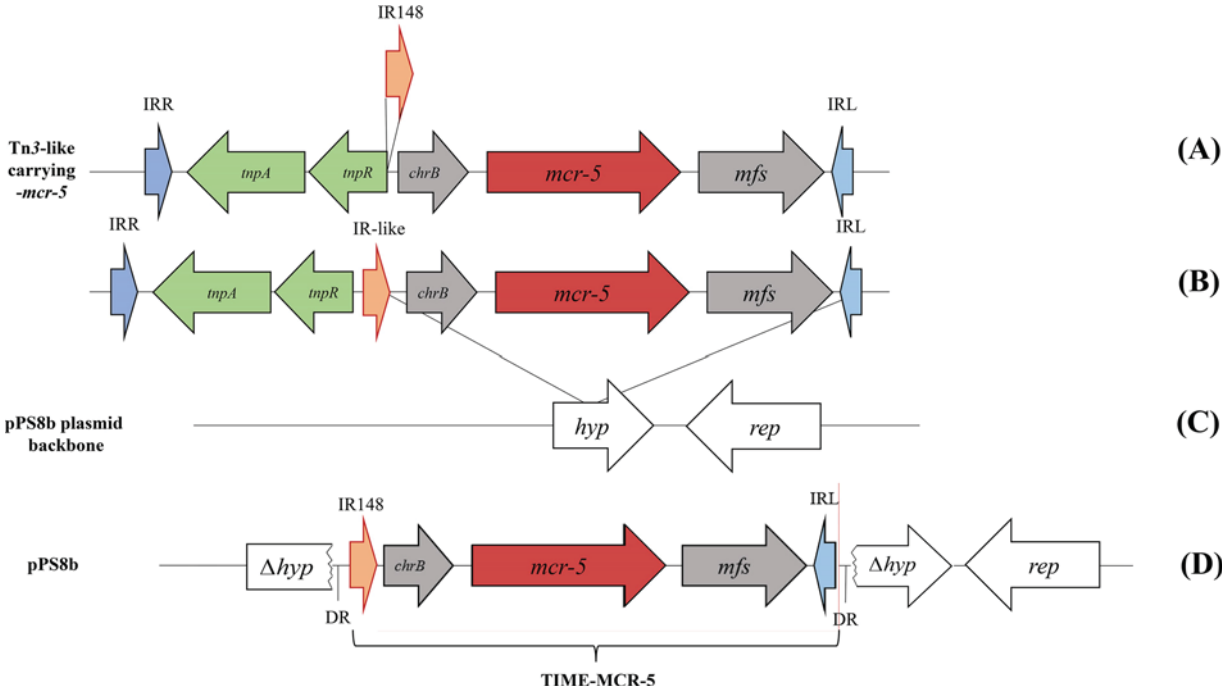

FIG 4 Proposed model of the chronology of acquisition of the *mcr*-5 cassette onto the pPS8b plasmid backbone.

contains an antibiotic resistance gene, such a MITE therefore constitutes a genetic structure being an original source of acquired resistance. This has been shown for instance with the GES-5 carbapenemase-encoding gene (22) or with the QnrS2 quinolone resistance-encoding gene (23). Here, we showed that the Tn*As1* transposase was able to recognize the extremities of this *mcr-5* cassette leading to its mobilization through a transposition mechanism. The Tn*As1* transposase belongs to the Tn*3* family, and its inverted-repeat sequences shared high nucleotide identities with the IR148 and IRL of the *mcr-5* cassette. *In silico* analysis showed that Tn*As1* is not found in *Aeromonas* spp. A particular class of MITEs whose mobilization is mediated by Tn*3* elements but which does not carry any putative open reading frame, accordingly designated TIME (standing for Tn*3*-derived inverted-repeat miniature element), has been previously reported (24). Here, we may consider our structure bearing the *mcr-5* cassette to be part of this class of transposable elements, and we therefore named it TIME–MCR-5.

This finding constitutes the first evidence of a MITE-mediated mobilization of an *mcr*-like gene. In addition, the *mcr-5*-bearing cassette was identified on a small nonconjugative plasmid that cannot be transferred by conjugation; however, it can be mobilized by another plasmid, therefore prompting it to be horizontally transferred and to disseminate among different strains.

Noteworthy, *in silico* analysis revealed that this same *mcr-5* cassette was identified in *E. coli* and *Aeromonas* sp. isolates, respectively (19, 25), suggesting that it constituted an effective transposable element. According to our experimental data, the chronology of the genetic events might be the following: the IR148 sequence was inserted into the original Tn*3* transposon carrying the *mcr*-5 gene (Fig. 4A and B). Then, the IR-like structure and the IRL of Tn*3* were recognized by transposase activity provided in *trans* that was responsible for the excision of the new cassette and of its insertion into the pPS8b plasmid backbone sequence (Fig. 4C), accordingly generating DRs (Fig. 4D). Further experiments will be necessary in order to investigate the mechanism responsible for the initial step corresponding to insertion of IR148 into transposon Tn*3*. Interestingly, *in silico* investigations showed that IR148 sequences have been found in similar genetic structures, likely resulting from a similar MITE-like mobilization mechanism (Fig. 5). This IR-like structure was found to be associated with other IRs, all sharing

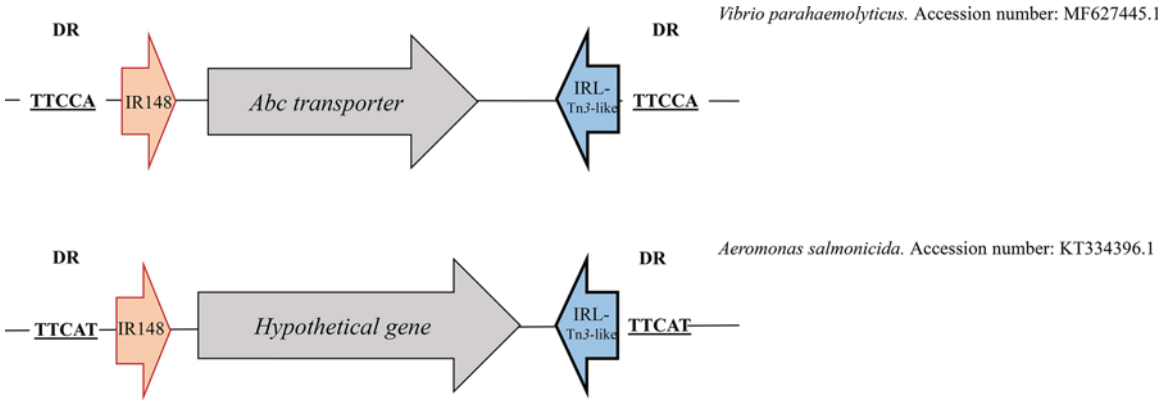

FIG 5 Examples of genetic contexts in which the IR-like structure in different genetic contexts forming a MITE-like structure in the genomes of *V. parahaemolyticus* and *A. salmonicida*. A 5-nucleotide (nt) direct repeat sequence was identified *in silico* in every MITE-like structure. The direct repeats (DRs) being signature of transposition events are in bold and underlined.

high identity with Tn*3*-like IRs. This suggests that the IR148 element may be responsible for the mobilization of numerous genes and represent an efficient mobilization unit.

This is the first study identifying a MITE-like element at the origin of acquisition of a colistin resistance determinant. Our study highlights the diversity of mobile elements associated with resistance determinants and the remarkable genetic engineering of bacterial genomes for adaptive resistance toward antimicrobial agents.

MATERIALS AND METHODS

Design of the study. A collection of 147 rectal samples recovered from pigs and from 4 different farms were collected in the Bourgogne region in France in 2016. All samples were cultured overnight in Luria-Bertani (LB) broth supplemented with colistin (1 μg/ml). After 16 h of incubation, 10 μl of culture was inoculated onto Superpolymyxin agar plates (ELITech, Signes, France) and was incubated at 37°C for another 16 h. Colistin resistance of the recovered isolates was confirmed by the broth microdilution method using cation-adjusted Mueller-Hinton (MH) broth (Bio-Rad, Cressier, Switzerland), as recommended by the CLSI (26).

The presence of *mcr*-like genes (*mcr-1* to *-8*) was investigated by PCR analysis using specific primers, and all positive signals were sequenced using the Sanger method (Microsynth, Balgach, Switzerland) (Table 1). Antimicrobial susceptibility testing was performed according to the standard disk diffusion method with MH agar plates (http://www.eucast.org/).

Clonality evaluation was performed by pulsed-field gel electrophoresis (PFGE). Briefly, total DNA of all MCR producers was digested by using the XbaI enzyme (New England BioLabs, Ipswich, MA, USA). The generated fragments were separated by PFGE using a CHEF-DR III system (Bio-Rad, Cressier, Switzerland).

TABLE 1 Oligonucleotides used in this work

Primer	Direction	Sequence (5′ to 3′)	Reference
mcr-1	Forward	ATGCCAGTTTCTTTCGCGTG	29
	Reverse	TCGGCAAATTGCGCTTTTGGC	
mcr-2	Forward	AAGGCTGACACCCCATGTCAT	29
	Reverse	GATGGCGGTCTATCCTGTAT	
mcr-3	Forward	ACCAGTAAATCTGGTGGCGT	29
	Reverse	AGGACAACCTCGTCATAGCA	
mcr-4	Forward	TTGCAGACGCCCATGGAATA	29
	Reverse	GCCCGCATGAGCTAGTATCGT	
mcr-5	Forward	GGACGCGACTCCCTAACTTC	29
	Reverse	ACAACCAGTACGAGAGCACG	
mcr-7	Forward	GGTGAATTTGTTGCTGGTGC	This study
	Reverse	GGCACTGGCTGAAAATATCG	
mcr-8	Forward	TCGGCAACATAGCACTTTGG	This study
	Reverse	TGTGTTTGTTCATTGGGGGC	

Multilocus sequence type was obtained using the Center for Genomic Epidemiology server (MLST 1.8). Phylogroup determination was performed using the PCR-based Clermont method (27).

Plasmid analysis. Plasmid carrying the *mcr-5* gene was extracted using the Zyppy miniprep kit (Zymo Research, Irvine, CA, USA) and was sequenced by the Sanger method (Microsynth, Balgach, Switzerland) using a PCR walking method. PBRT was used to determine the incompatibility group of the plasmids carrying *mcr* genes (28).

Conjugation experiments were performed using the azide-resistant *E. coli* J53 recipient strain. Both donor and recipient strains were cultured in exponential phase and then mixed on solid LB agar using filters at a 1:10 donor/recipient ratio. After 5 h of incubation, filters were resuspended in 0.85% NaCl, and the bacterial mixture was plated onto agar plates supplemented with colistin (1 μg/ml) and sodium azide (100 μg/ml).

Plasmid constructs for the transposition assay. Two different recombinant plasmids were obtained for the transposition experiments. pACYC-*mcr-5* was obtained by cloning the *mcr-5* cassette (containing the *mcr-5* gene bracketed by the two identified IRs) into the tetracycline resistance gene of the low-copy-number cloning vector pACYC184 carrying both tetracycline and chloramphenicol resistance genes. The pBAD-Tn*As1.1* plasmid was obtained by cloning the resolvase and transposase genes of transposon Tn*As1* into the L-arabinose-inducible $pBAD_b$ plasmid carrying an ampicillin resistance determinant. The pBAD-Tn*3*-like plasmid was obtained by cloning the resolvase and transposase of the original Tn*3* transposon containing the *mcr-5* cassette in the $pBAD_b$ plasmid (14).

Transposition experiments. Both pACYC-*mcr*-5 and pBAD-*TnAs1*.1 were transformed into the *E. coli* RZ211 strain carrying the conjugative pOX38 plasmid used as a DNA receptor for transposition events and carrying the gentamicin resistance determinant. The RZ211 *E. coli* strain containing the three plasmids was incubated overnight at 37°C in LB broth supplemented in L-arabinose (0.1%), ampicillin (100 μg/ml), gentamicin (8 μg/ml), chloramphenicol (25 μg/ml), and colistin (1 μg/ml). After mating-out assays with the J53 strain, the putative transposants were selected on LB agar supplemented with azide (100 μg/ml), gentamicin (8 μg/ml), and colistin (1 μg/ml). Each colony obtained was checked for chloramphenicol and ampicillin susceptibility in order to eliminate strains in which whole-plasmid integration events might have occurred.

Determination of the location of the transposition events was performed by PCR, followed by sequencing using one outward primer located at the 5′ extremity of the *mcr-5* cassette and a second primer further designed after sequencing of this 5′ extremity and targeting the adjacent sequences on plasmid pOX38.

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