Colocation of the Polymyxin Resistance Gene *mcr-1* and a Variant of *mcr-3* on a Plasmid in an *Escherichia coli* Isolate from a Chicken Farm

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ABSTRACT A colistin-resistant *Escherichia coli* isolate from a commercial poultry farm in China carried two colistin resistance genes, *mcr-1* and variant of *mcr-3*, in an IncP plasmid. The variant of the *mcr-3* gene, named *mcr-3.11*, encoded two amino acid substitutions compared with the *mcr-3* gene. A novel genetic structure, *ISKpn40-mcr-3-dgkA-ISKpn40*, might be the key element mediating the translocation of *mcr-3* through the formation of a circular form. The *mcr-1* and *mcr-3* genes, which are co-located on a plasmid, might pose a huge threat to public health.

KEYWORDS colistin, *mcr-3*, *mcr-1*, *Escherichia coli*, circular form

The emergence of multidrug resistance (MDR) in Gram-negative pathogens has become a challenging threat to public health in recent years. Colistin is considered one of the last lines of defense against multidrug-resistant Gram-negative bacteria.

The emergence of the plasmid-mediated mobile colistin resistance gene *mcr-1* was first identified on an IncI2 plasmid from *Escherichia coli* and *Klebsiella pneumoniae* in 2016 (1). Until now, five kinds of mobile colistin resistance genes, *mcr-1* to *mcr-5*, have been reported (1–5). The gene *mcr-3* was first characterized in the IncHI2-type plasmid from porcine *Escherichia coli*, followed by the characterization of 10 other *mcr-3* variants. Recently, the *mcr-3* gene has been proven to disseminate among *Enterobacteriaceae* (3, 6–8) and aeromonads (9, 10); aeromonads may act as a potential reservoir for *mcr-3*. Compared with MCR-1, the MCR-3 protein has only 32.5% amino acid identity, and the *mcr-3* gene in *Enterobacteriaceae* might have originated from *Aeromonas* species (3). In this study, we report the colocation of *mcr-1* and a variant of *mcr-3* on an IncP plasmid from an *E. coli* strain.

In the current study, *E. coli* strain ECSC102 was isolated from a chicken cloacal swab in Sichuan Province, China, in 2015. Species identification was performed using a BD Phoenix-100 system (Becton Dickinson, USA) and confirmed by 16S rRNA gene sequencing. The MICs of various antimicrobials were determined using the broth dilution method from the CLSI guidelines (11). Strain *E. coli* ECSC102 was resistant to ampicillin (MIC, >256 µg/ml), colistin (MIC, 8 µg/ml), cefotaxime (MIC, 16 µg/ml), ciprofloxacin (MIC, 32 µg/ml), ceftazidime (MIC, 64 µg/ml), trimethoprim-sulfamethoxazole (MIC, 64/1,216 µg/ml), streptomycin (MIC, 256 µg/ml), and tetracycline (MIC, 256 µg/ml), and it was susceptible to imipenem (MIC, 0.125 µg/ml), tigecycline (MIC, <0.125 µg/ml), aztreonam (MIC, 0.25 µg/ml), and amikacin (MIC, 0.5 µg/ml). PCR amplification (see Table S1 in the supplemental material) and Sanger sequencing indicated that this isolate cocarried the *mcr-1* and *mcr-3* genes.

Conjugation experiments were performed with *E. coli* ECSC102 as the donor and *E. coli* J53 (azide resistant [Azi^r]) as the recipient. Transconjugants were selected on Mueller-Hinton agar (MHA) plates (Oxoid) which contained 200 µg/ml sodium azide

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with 2 $\mu\text{g/ml}$ colistin. Positive transconjugants were identified by the detection of colistin resistance phenotypes and PCR screening for the presence of *mcr-1* and *mcr-3* (Table S1). The results showed that the transconjugants carried genes *mcr-1* and *mcr-3*, exhibiting an MIC of 8 $\mu\text{g/ml}$ for colistin, which was a 32-fold increase compared with that for the recipient *E. coli* J53 (Azi^r). The MIC of colistin for strain WCHECLL123 containing both *mcr-3.5* and *mcr-1* was 8 $\mu\text{g/ml}$, that for its transconjugant containing *mcr-3.5* was 4 $\mu\text{g/ml}$, and that for its transconjugant containing *mcr-1* was 4 $\mu\text{g/ml}$ (7). When *mcr-1* and *mcr-3* genes were simultaneously present in the *E. coli* strain, there was a higher colistin MIC value than for *mcr-1* or *mcr-3* alone in the strain.

The *mcr-3* gene in this strain is a novel variant (designated *mcr-3.11*) encoding MCR-3 proteins with two amino acid substitutions (G373V and T468Q) compared with the original *mcr-3* on plasmid pWJ1 (GenBank accession no. [KY924928](#)). Conserved domains of MCR-3.11 were predicted using CD-Search (12) in NCBI against the Pfam database. MCR-3.11 can be divided into two domains, a domain assigned with phosphoethanolamine transferase (sulfatase) (amino acids [aa] 233 to 518, pfam00884), and a domain predicted to be a transmembrane module (aa 56 to 206, pfam08019), which is the same as MCR-3 (Fig. S3). Phylogenetic analysis revealed that MCR-3.11 has close genetic distance from MCR-3 and MCR-3-like sequences (Fig. S3). Notably, MCR-3.11 originated from *Aeromonas* species. To assess the colistin resistance phenotype conferred by the *mcr-3.11* gene, the corresponding 2,219-bp region (Table S1) derived from the plasmid DNA of the *E. coli* ECSC102 strain was cloned into a pUC57 vector and transformed into the colistin-susceptible strain *E. coli* DH5 α (TaKaRa). The MIC of colistin for *E. coli* transformants containing *mcr-3.11* was 4 $\mu\text{g/ml}$, which was 16-fold higher than that of *E. coli* DH5 α alone (0.25 $\mu\text{g/ml}$). The MIC against *E. coli* W3110 carrying a cloned vector, pUC19, of the original *mcr-3* gene was 4 $\mu\text{g/ml}$ (3). These results revealed that the amino acid substitutions of MCR-3.11 have not altered its activity against colistin compared with the origin MCR-3.

The total genomic DNA from the transconjugant that carried genes *mcr-1* and *mcr-3* was extracted and sequenced on the Illumina MiSeq platform (Majorbio, Shanghai, China) using a 400-bp paired-end TruSeq library with a 2×300 run. The paired-end reads were assembled with SOAPdenovo version 2.04 and GapCloser version 1.12. The gaps between different contigs were closed by PCR and Sanger sequencing; the primers used to close the gaps are listed in Table S1. Gene prediction and annotation were performed using the Rapid Annotations using Subsystems Technology (RAST) server (<http://rast.nmpdr.org/>) and the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identification of antibiotic resistance genes was performed using ResFinder 2.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>). Plasmid replicon types were determined by the PlasmidFinder tool (<http://genomicepidemiology.org/>). Sequence comparative analysis and generation of plasmids were performed with Easyfig (13) and BRIG (14).

The variant of *mcr-3* and the *mcr-1* gene were collocated in a conjugative 60.7-kb IncP plasmid, designated pECSC102. It had a length of 60,744 bp, with 47.2% G+C content (Fig. S2). Plasmid pECSC102 aligned very well to two *mcr*-carrying IncP plasmids, pMCR3_WCHEC-LL123 (GenBank accession no. [MF489760](#)), found in an *E. coli* isolate, and pMCR_1511 (GenBank accession no. [KX377410](#)), found in a *K. pneumoniae* isolate (Fig. 1). All three of these strains were isolated in Sichuan Province in Southwest China; however, pMCR3_WCHEC-LL12 was recovered from an abdominal abscess of a hospital patient (7), pMCR_1511 was recovered from the sewage of the hospital (15), and pECSC102 in this study was isolated from the cloacal swab from a chicken. The apparent spread of the same IncP plasmid with one or two *mcr* genes between different species and a patient, the hospital environment, and animal production is worrying. Sequence typing for strain ECSC102, performed as previously described (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>), revealed that it belonged to sequence type 167 (ST167).

Complete sequence analysis of plasmid pECSC102 revealed that *mcr-1* was located on a composite transposon, Tn6330 (16), with direct repeats of IS*ApI1* flanking *mcr-1*. Gene *mcr-3* was located on an IS*Kpn40*-*mcr-3*-*dgkA*-IS*Kpn40* fragment, with direct

FIG 1 Comparison of the genetic configuration of pECSC102 with other plasmids. The open reading frames are illustrated by arrows pointing in the directions of their respective orientation. Important genes are indicated above and below the plasmid sequence. Resistance genes are in red, mobile genetic elements are in green, and other genes are in orange.

repeats of ISKpn40 flanking *mcr-3*. A truncated form of *mcr-3* was located on pMCR_1511, and the mechanism for the truncation of *mcr-3* was unknown. The genetic configuration comparison between pECSC102 and pMCR3_LL123 showed that the *mcr-1* gene was absent from pMCR3_LL123, and the genetic contexts of *mcr-3* are not that similar in these two plasmids, as pMCR3_LL123 lacks ISKpn40 (Fig. 1). The identical backbone suggested that pECSC102, pMCR3_LL123, and pMCR_1511 were derived from a common ancestor.

ISKpn40 has a size of 1,213 bp and is flanked by 12-bp inverted repeats (TGTAATG ACCCA), which belong to the IS3 family; ISKpn40 was first found in an *Escherichia coli* strain isolated from a swine feedlot (3). IS911 and IS150 are members of the large IS3 family and proved to be able to form a circular intermediate (17, 18). Since direct repeats of insertion sequences (IS) may mediate the dissemination of genes via the formation of a circular form (16, 19), inverse PCR assays (Table S1) were performed to amplify the putative circular form that harbored the *mcr-3* gene. One circular form of 3,535 bp, *mcr-3*-*dgkA*-ISKpn40, was confirmed by an inverse PCR assay. After aligning with the pECSC102 sequence, we found that this circular form was derived from the ISKpn40-*mcr-3*-*dgkA*-ISKpn40 element, which was 100% homologous to this region.

FIG 2 The genetic context of *mcr-3* on pECSC102 compared with three plasmids and the formation of a *mcr-3* circular intermediate. (a) The genetic context of *mcr-3* on pECSC102 compared with pMCR_1511, pWJ1, and pMCR3_WCHEC_LL123. Regions of >99% nucleotide sequence identity are marked with light-gray shading. The number 1 represents a truncated (41-bp remnant) ISKpn40, and number 2 represents 12-bp AT-rich (ATTTTACAAA) nucleotides. The $\Delta mcr-3$ represents a truncated (645-bp remnant) *mcr-3*. (b) Formation of a circular intermediate by the *mcr-3* gene; reverse primers dgkA-F and MCR-R were designed to detect the circular form.

BLASTN (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis showed that the 3,535-bp circular form exhibited 99% identity to the corresponding region of pWJ1 (GenBank accession no. KY924928.1). A truncated (41-bp remnant) *ISKpn40* was found upstream of *mcr-3* in pWJ1, which presumably suggests directly repeated upstream and downstream *ISKpn40* copies at first and subsequently loss of the upstream IS copy during the mobilization of *mcr-3* into the pWJ1. Compared with the *mcr-3.11*-containing segment, a 12-bp AT-rich (ATTTTACAAA) deletion was found upstream of *mcr-3* in pWJ1. The 12-bp nucleotides exist in pMCR3_WCHEC-LL123, which has IS26 downstream of *mcr-3.5* instead of *ISKpn40* (Fig. 2). These findings suggest that the association of the resistance gene *mcr-3* with *ISKpn40* might have facilitated its translocation.

Here, we report for the first time the cooccurrence of *mcr-1* and *mcr-3.11* on a single plasmid in an *E. coli* strain from a commercial poultry farm in China. The *mcr-3* circular intermediate originating from *ISKpn40-mcr-3-dgkA-ISKpn40* may be the key element responsible for mediating the translocation of the *mcr-3* gene into various plasmids. The coexistence of the *mcr-1* and *mcr-3* genes in *E. coli* isolates may pose a huge threat to public health and warrant further investigation.

Accession number(s). The complete nucleotide sequence of the plasmid pECSC102 characterized in this study was submitted to the GenBank database and assigned accession number [MG552133](https://doi.org/10.1128/MG552133).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00501-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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