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MECHANISMS OF RESISTANCE

Coidentification of *mcr-4.3* and bla_{NDM-1} in a Clinical *Enterobacter cloacae* Isolate from China

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ABSTRACT We describe the first report of a clinical colistin-resistant ST84 *Enterobacter cloacae* isolate coharboring *mcr-4.3* (previously named *mcr-4.2*) and bla_{NDM-1} from a patient in China. The bla_{NDM-1}-harboring IncX3 plasmid and the novel *mcr-4.3*-harboring ColE plasmid were completely sequenced. Although this isolate showed a high level of resistance to colistin, *mcr-4.3* plasmid transformation, gene subcloning, susceptibility testing, and lipid A matrix-assisted laser desorption ionization mass spectrometry analysis indicated that *mcr-4.3* itself does not confer resistance to colistin.

KEYWORDS colistin, *mcr*-4, NDM-1, plasmid, carbapenem resistant

Colistin, a cationic antimicrobial peptide, is one of the last-resort antibiotics used to treat infections caused by multidrug-resistant Gram-negative organisms (1, 2). The major mechanism for resistance to colistin is associated with chromosomal gene-mediated lipopolysaccharide (LPS) modification, e.g., transfer of the phosphoethanolamine (PEA) moiety to the suggestive 4′-phosphate position of LPS-bound lipid A (3); however, in 2016, the first report was published describing a plasmid carrying a gene (*mcr-1*) responsible for colistin resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates recovered from animals and patients in China (4). Since that report, >10 distinct alleles of *mcr-1* have been identified in *E. coli*, *Klebsiella*, and *Salmonella* isolates (5, 6). In 2016, a second colistin resistance gene, *mcr-2*, was discovered in porcine and bovine *E. coli* isolates in Belgium (7), and the list now includes *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7*, *and mcr-8* isolated from *Enterobacteriaceae* (8–13).

Similar to the various alleles found in *mcr-1*, different alleles of *mcr-4* have been reported. Recently, a new variant of the *mcr-4* gene, *mcr-4.2*, was identified in two *Salmonella* isolates of human origin collected in 2013 in Italy (14). Compared to the prototype *mcr-4.1* gene (GenBank accession no. MF543359), the *mcr-4.2* gene (GenBank accession no. MG581979) contains a mutation at position 331, resulting in amino acid transition (Q→R) (Table 1) (14). A second study, published around the same time, described another *mcr-4* gene variant that was also named *mcr-4.2* (but later renamed *mcr-4.3*; see below). This mutant was described in six *Enterobacter cloacae* clinical isolates from Singapore that coharbored bla_{KPC-2} (15). Compared to the prototype *mcr-4.1* gene, this *mcr-4.2* gene (GenBank accession no. MG026621) contains two missense mutations at positions 179 (V→G) and 236 (V→F) (Table 1). In a recent proposal to streamline the nomenclature of *mcr* genes, this *mcr-4.2* has been renamed *mcr-4.3* (31). In addition, *mcr-4.4* and *mcr-4.5* gene variants were identified from *E. coli*

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TABLE 1 Variants of *mcr-4* gene

mcr-4 variant	Mutation					Accession no.	Reference
	P110L	V179G	H205N	V236F	Q331R		
mcr-4.1	–	–	–	–	–	MF543359	9
mcr-4.2	–	–	–	–	+	MG822663	14
mcr-4.3[a]	–	+	–	+	–	MG026621	15
mcr-4.4	–	–	+	–	+	MG822665	17
mcr-4.5	+	–	–	–	+	MG822664	17
mcr-4.6[b]	–	–	–	+	–	MH423812	16

[a]Originally named *mcr-4.2*.
[b]Originally named *mcr-4.3*.

isolates obtained from the feces of pigs in Spain. The *mcr-4.4* gene contains missense mutations at positions 205 (H→N) and 331 (Q→R), whereas the *mcr-4.5* gene harbors mutations at positions 110 (P→L) and 331 (Q→R) (17). Moreover, an *mcr-4.6* gene (originally named *mcr-4.3*) was identified from *Salmonella enterica* serovar Kedougou strain 151570, and this mutant contains a missense mutation at position 236 (V→F) (Table 1) (16, 31). Here, we report the identification of another example of the *mcr-4.3* gene, and in this instance, it is uniquely found with bla_{NDM-1} in an *Enterobacter* strain recovered from a patient in China.

A 75-year-old man was injured in a traffic accident in March 2013 and presented to a tertiary hospital in eastern China. The patient was a local farmer with no travel history for >2 years. He was diagnosed with bronchiectasis with severe aspiration pneumonia, and sputum samples grew *E. cloacae*. Susceptibility testing by Vitek 2 Compact (bioMérieux) showed that the *E. cloacae* isolate (named En_MCR4) exhibited resistance to multiple antimicrobial agents, including ceftazidime (MIC, ≥64 μg/ml), ceftriaxone (MIC, ≥64 μg/ml), cefepime (MIC, ≥64 μg/ml), imipenem (MIC, ≥16 μg/ml), meropenem (MIC, ≥16 μg/ml), aztreonam (MIC, >32 μg/ml), ciprofloxacin (MIC, >2 μg/ml), gentamicin (MIC, >8 μg/ml), tobramycin (MIC, >8 μg/ml), piperacillin-tazobactam (MIC, >64/4 μg/ml), and nitrofurantoin (MIC, ≥512 μg/ml). Additional broth microdilution testing showed that it also had high-level resistance to colistin (MIC, >256 μg/ml) (18).

To genotype the resistance mechanisms underlying the strain's multidrug resistance phenotype, whole-genome sequencing was performed using an Illumina NextSeq platform with 150-bp paired-end reads. *In silico* multilocus sequence typing (MLST) analysis revealed that En_MCR4 belongs to ST84 (allele profile, 60-1-61-1-36-22-1) (19), and the mining of acquired resistance genes showed that En_MCR4 harbors 10 antimicrobial resistance genes encoding resistance to β-lactams (bla_{NDM-1}, $bla_{CTX-M-9}$, and *ampC*), aminoglycosides [*aadA2* and *ant(2″)-Ia*], colistin (*mcr-4.3*), fluoroquinolones (*qnrA*), phenicol (*catA1*), sulfonamide (*sul1*), and trimethoprim (*dfrA16*) (20).

Sequencing and characterization of the bla_{NDM-1}- and *mcr-4.3*-harboring plasmids were achieved by first segregating the plasmids into an *E. coli* host. Conjugation experiments were performed using the *E. coli* J53Az^r strain as a recipient as described previously (21). The transconjugants were selected on lysogeny broth (LB) agar plates with 100 μg/ml sodium azide in combination with 2 μg/ml imipenem or colistin. Multiple attempts to transfer *mcr-4.3* plasmid failed; however, the bla_{NDM-1}-bearing plasmid was successfully transferred to *E. coli* J53 by conjugation. Plasmid DNA was isolated from En_MCR-4, electroporated into *E. coli* DH10B (Invitrogen) as described previously (21), and selected on LB agar plates with 0.5, 1, and 2 μg/ml colistin. Growth was observed only on plates with colistin 0.5 μg/ml, and the transformants were screened for the presence of *mcr-4.3* by PCR using primers described previously (9). Plasmid DNA from bla_{NDM-1}-harboring *E. coli* J53 transconjugants and from *mcr-4.3*-harboring *E. coli* DH10B transformant was extracted using the Qiagen Plasmid Midi kit (Qiagen, Valencia, CA) and sequenced using an Illumina NextSeq system as described previously (22).

The bla_{NDM-1}-harboring plasmid pEn_NDM is 45,739 bp in size and has an average

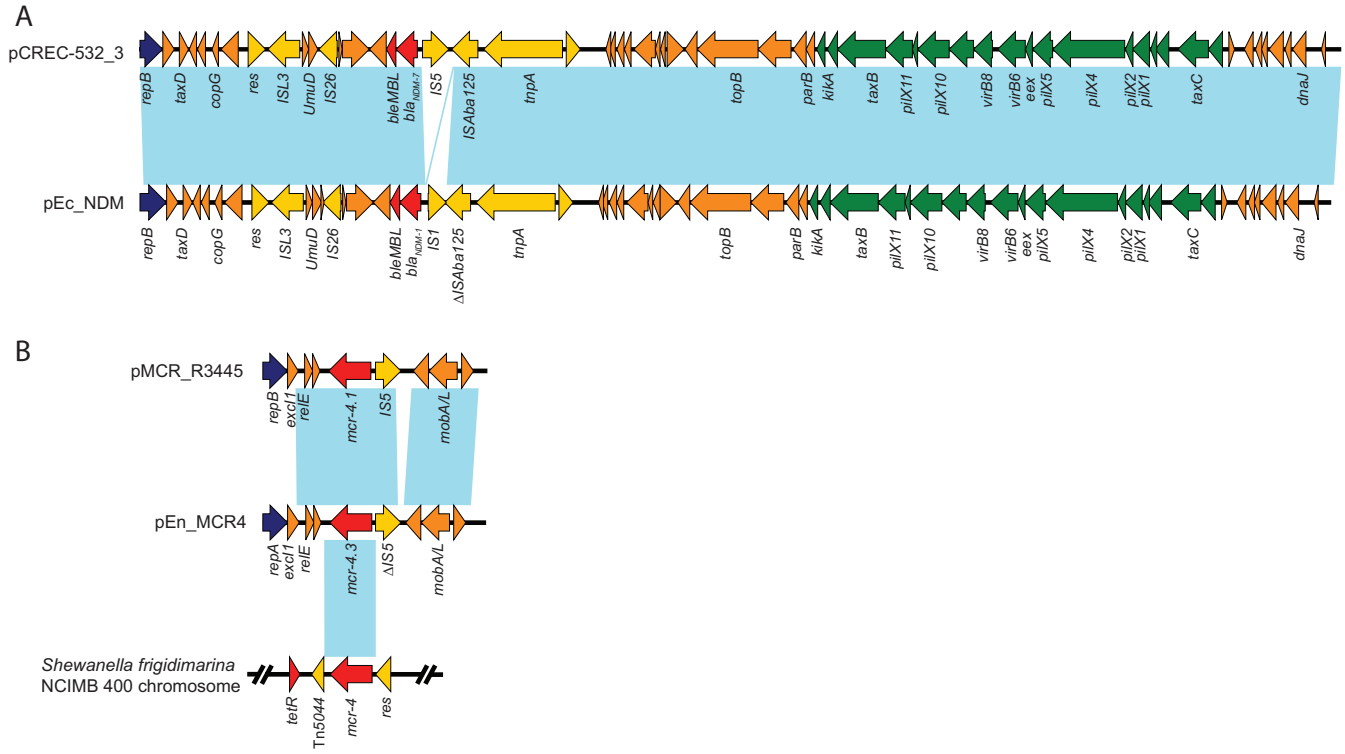

FIG 1 The plasmid structures. (A) Comparison of IncX3 plasmids pCREC-532_3 (CP024833) and pEn_NDM (MH061381). (B) Comparison of *mcr-4*-harboring plasmids pMCR_R3445 (MF543359), pEn_MCR4 (MH061380), and *S. frigidimarina* NCIMB 400 chromosome (CP000447). Colored arrows indicate ORFs. Dark blue, yellow, green, red, and orange arrows represent replication genes, mobile elements, plasmid transfer genes, the resistance gene, and plasmid backbone genes, respectively. Blue shading denotes regions of shared homology among different plasmids or chromosome sequences.

G+C content of 46.6%. The plasmid contains 61 predicted open reading frames (ORFs) and belongs to the IncX3 incompatibility group (Fig. 1A). A BLAST search of the plasmid sequences against the GenBank database showed that pEn_NDM is highly similar to several bla_{NDM}-harboring IncX3 plasmids, such as the bla_{NDM-7}-harboring plasmid pCREC-532_3 from *E. coli* (GenBank accession no. CP024833), bla_{NDM-4}-harboring plasmids pJEG027 from *K. pneumoniae* (23) and pM216_X3 from *E. coli* (24), and bla_{NDM-5}-harboring plasmid pNDM5_IncX3 from *K. pneumoniae* (25). Plasmid pEn_NDM harbored an intact set of conjugative transfer genes to facilitate the transfer of plasmids among different members of *Enterobacteriaceae*, which is consistent with its ability to conjugate into *E. coli* J53Az^r as described above (Fig. 1A).

Plasmid pEn_MCR4 is 8,639 bp in size with a G+C content of 45.3%. It harbors eight predicted ORFs and belongs to the ColE incompatibility group (Fig. 1B). A BLAST search of all plasmid sequences against the GenBank database showed the highest 75% query coverage and 99% identity to the *mcr-4.1* prototype plasmid pMCR_R3445 (GenBank accession no. MF543359) from *Salmonella* species (9). Unlike pMCR_R3445, plasmid pEn_MCR4 carries a different replication gene, *repA*, identical to plasmid pPSP-b98 (CP009870) from the *Pantoea* species (26). Interestingly, the *mcr-4.3* gene in plasmid pEn_MCR4 showed 100% nucleotide identity with the genome of *Shewanella frigidimarina* NCIMB 400 (CP000447) and encoded for a putative member of phosphoethanolamine transferases (9). pEn_MCR4, pMCR_R3445, and NCIMB 400 contain conserved 59-bp *mcr-4* upstream sequences, encompassing the predicted −35 (TTATTT) and −10 (AGCTAGTAT) promoter regions. In addition, the *mcr-4.3* gene was identical to the *mcr-4*-like gene located on a 7.7-kb contig and found in six bla_{KPC-2}-harboring *E. cloacae* isolates from Singapore (previously also named *mcr-4.2*) (Table 1) (15). Only an ~7.7-kb *mcr-4.3*-harboring contig was obtained in that study; however, because the contig sequence is currently not available for comparison, it is not clear whether *mcr-4.3* was carried by the same ColE plasmids as pEn_MCR4. Moreover, all six *mcr-4.3*-

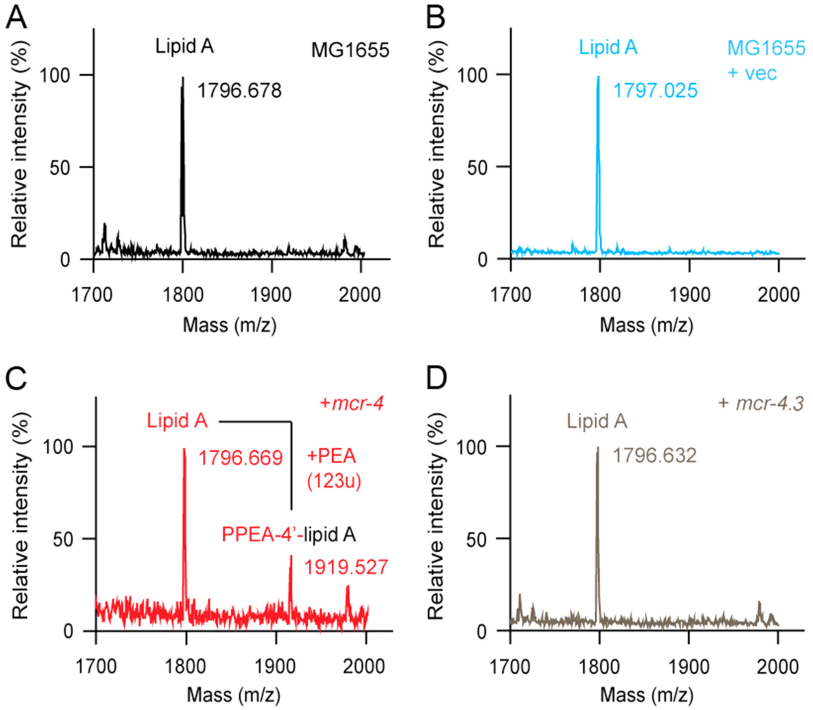

FIG 2 MS evidence that no addition of PEA to lipid A occurs in the *mcr-4.3 E. coli* MG1655 constructs. MALDI-TOF MS profile of LPS-lipid A from MG1655 alone (A) or with the empty vector pBAD24 (B). (C) MALDI-TOF MS profile of LPS-lipid A from MG1655 expressing the wild-type *mcr-4.1*. Two distinct MS spectra of LPS-lipid A are present. The unmodified lipid A appears at *m/z* 1,796.669, while the PEA-4′-lipid A (lipid A with addition of PEA) is present at *m/z* 1,919.527. (D) The presence of *mcr-4.3* in *E. coli* MG1655 fails to modify the LPS-lipid A species.

harboring *E. cloacae* isolates from Singapore belonged to ST54 (allele profile, 41-3-54-37-3-15-17), which is distinct from the ST84 found in our study, suggesting the horizontal transfer of *mcr-4.3* into different *E. cloacae* strains.

The *mcr-4.3*-harboring *E. coli* DH10B transformant was subjected to susceptibility testing for colistin using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (27). The transformant showed a colistin MIC of 0.5 μg/ml, which is only 2-fold higher than that of native *E. coli* DH10B cells (MIC, 0.25 μg/ml). Consistent with this finding, the entire coding sequence of *mcr-4.3* cloned with its native promoter into plasmid pET-28a (+) in *E. coli* DH10B cells also had a colistin MIC of 0.5 μg/ml. Our results are in agreement with those of Teo et al. (15), which show that the *mcr-4.3* gene does not confer resistance to colistin. In contrast, in the original *mcr-4.1* study, plasmid pMCR_R3445 was transformed into DH5α *E. coli* and had an MIC of 2 μg/ml for colistin, which is an 8-fold increase compared with the MIC of the DH5α *E. coli* host (MIC, 0.25 μg/ml) (9).

We therefore hypothesize that two missense mutations at positions 179 (V→G) and 236 (V→F) might cause a decrease in the colistin MIC in *mcr-4.3*-harboring strains. Subsequently, to investigate structural changes in lipid A, we applied matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) to an array of *E. coli* strains with and without *mcr-4* genes (Fig. 2). Briefly, the entire coding sequence of the *mcr-4.3* gene from En_MCR4 and *mcr-4.1* (generated by site-directed mutagenesis from *mcr-4.3*) was cloned into an arabinose-inducible plasmid pBAD24 and expressed into the *E. coli* MG1655 strain. LPS-lipid A was extracted and purified as described previously (28), and the structure of lipid A was analyzed by MALDI–time of flight mass (TOF) MS (Bruker, ultrafleXtreme) in negative-ion mode (28). As expected, a single MS spectrum of lipid A (*m/z*, ~1,796.6) appears in colistin-susceptible *E. coli* MG1655 (Fig. 2A) and strain MG1655 carrying the empty vector pBAD24 (Fig. 2B). In contrast, the MS peak of PEA-4′-lipid A, a chemically decorated lipid A with addition of PEA, is detected in strain

MG1655 harboring *mcr-4.1* (Fig. 2C). Interestingly, the presence of *mcr-4.3* in *E. coli* MG1655 fails to modify the LPS-lipid A (Fig. 2D). Collectively, we present comprehensive evidence that *mcr-4.3* is a variant of *mcr-4* without a lipid A modification function and, consequently, does not confer resistance to colistin.

Our studies indicate that the presence of *mcr-4.3* cannot explain the high colistin resistance level (>256 μg/ml) observed in En_MCR4. It is known that chromosomes encoding resistance mechanisms, such as mutations in the PmrAB or PhoPQ two-component regulatory system and MgrB inactivation, are usually associated with high-level colistin resistance (29). Consequently, we mined the *mgrB*, *phoP*, *phoQ*, *pmrA*, and *pmrB* gene variations in En_MCR4 and compared them with 80 colistin-susceptible strains (all MICs, <1 μg/ml) from our previous *Enterobacter* spp. genomic study (30). No insertion, deletion, or stop codons were identified in these genes in En_MCR4; however, a number of unique (found only in En_MCR4 but not in the susceptible strains) missense mutations, such as I10V in MgrB; R2K, I4L, L5M, R69Q, I102V, and L168P in PhoQ; G21S, N72D, and Q143C in PmrA; and K91Q, T173S, N233P, L276R, and G331A in PmrB, were found. We suspect that some of these missense mutations may contribute to the colistin resistance in En_MCR4, and we are currently investigating the correlation of colistin resistance and these mutations.

In summary, this study describes the first report of an *mcr-4.3*-positive bacterial isolate coharboring bla_{NDM-1} of human origin from China. We completely characterized the bla_{NDM-1}-harboring IncX3 plasmid and the novel *mcr-4.3*-harboring ColE plasmid from a clinical ST84 *Enterobacter* isolate. Although this isolate showed high-level resistance to colistin, *mcr-4.3* does not appear to contribute to this resistant phenotype, suggesting that the two amino acid substitutions (i.e., V179G and V236F) in *mcr-4.3* significantly alter the *mcr-4* function. Further studies comparing additional amino acid substitutions in *mcr-4* (Table 1) are necessary to understand the correlation of enzyme changes and colistin resistance and the molecular evolution of *mcr-4*.

Accession number(s). The complete nucleotide plasmid sequences were deposited in GenBank as accession numbers MH061380 and MH061381. The raw whole-genome sequencing data from this study were submitted to the NCBI Sequence Read Archive (SRA) under accession no. SRR6833776.

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