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A novel plasmid-encoded *mcr-4.3* gene in a colistin-resistant *Acinetobacter baumannii* clinical strain

Natacha Martins-Sorenson[1], Erik Snesrud[2], Danilo Elias Xavier[3], Luciana Camila Cacci[1], Anthony T. Iavarone[4], Patrick McGann[2], Lee W. Riley[5] and Beatriz Meurer Moreira [1]*

[1]Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; [2]Multidrug-Resistant Organism Repository and Surveillance Network, Walter Reed Army Institute of Research, Silver Spring, MD, USA; [3]Instituto Aggeu Magalhães/Fiocruz, Recife, Pernambuco, Brazil; [4]QB3/Chemistry Mass Spectrometry Facility, University of California, Berkeley, CA, USA; [5]Division of Infectious Disease and Vaccinology, School of Public Health, University of California, Berkeley, CA, USA

*Corresponding author. E-mail: bmeurer@micro.ufrj.br

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Objectives: To identify the molecular mechanism of colistin resistance in an MDR *Acinetobacter baumannii* clinical strain isolated in 2008 from a meningitis case in Brazil.

Methods: Long- and short-read WGS was used to identify colistin resistance genes in *A. baumannii* strain 597A with a colistin MIC of 64 mg/L. MS was used to analyse lipid A content. *mcr* was cloned into pET-26b (+) and transformed into *Escherichia coli* BL21(λDE3)pLysS for analysis.

Results: A novel plasmid (pAb-MCR4.3) harbouring *mcr-4.3* within a Tn*3*-like transposon was identified. The *A. baumannii* 597A lipid A MS spectra showed a main molecular ion peak at *m/z*=2034, which indicated the addition of phosphoethanolamine to the lipid A structure. *E. coli* BL21 transformed with pET-26b-mcr-4.3 gained colistin resistance with a colistin MIC of 8 mg/L.

Conclusions: Colistin resistance in *A. baumannii* 597A was correlated with the presence of a novel plasmid-encoded *mcr-4.3* gene.

Introduction

Over the past 15 years, carbapenem resistance in Gram-negative bacteria has resulted in greater reliance on colistin for treatment. Acquired polymyxin resistance is primarily mediated by chromosomal mutations that modify the bacterial outer membrane (OM).[1] However, plasmid-mediated polymyxin resistance (encoded by *mcr-1*) was first reported in China in 2015.[2] The *mcr* genes encode phosphoethanolamine transferases (MCR enzymes) that modify lipid A of LPS through the addition of phosphoethanolamine. Since the 2015 report, >40 countries have identified *mcr* genes in Enterobacteriaceae from environmental, animal or human origin.[3]

To date (June 2019), nine different *mcr*-like genes have been identified (*mcr-1* to *mcr-9*), which are frequently carried on transmissible plasmids in members of the Enterobacteriaceae.[3–5] Notably, *mcr-1* has been reported in *Acinetobacter lwoffii* from hospital surfaces in Italy and *mcr-4.3* has been recently identified in *Acinetobacter baumannii* isolated from pig faeces in China.[6,7] In Brazil, *mcr-1*- and *mcr-3.12*-harbouring *Escherichia coli* strains were obtained from clinical, animal and environmental samples.[8–10] The aim of this study was to investigate the mechanism of colistin resistance in an MDR *A. baumannii* recovered in Brazil.

Materials and methods

Bacterial isolates

In 2008, a colistin-resistant *A. baumannii* strain (termed 597A) with a colistin MIC of 64 mg/L was recovered from the CSF of a patient with meningitis in Brazil.[11] The strain was previously assigned to the international clonal complex CC113/79 based on the two MLST schemes, Oxford and Pasteur (https://pubmlst.org/abaumannii/).

WGS analysis

Short-read (550 bp) and long-read sequencing was performed with an Illumina MiSeq desktop sequencer (Illumina Inc., CA, USA) and a PacBio RS II (Pacific Biosciences, CA, USA), respectively. Newbler version 2.7 (454 Life Sciences, CT, USA) and HGAP 2.0 in the SMRT Analysis Portal were used to assemble Miseq and PacBio sequencing reads into *de novo* contigs. Antimicrobial resistance genes were annotated by ResFinder 2.1; ISs and the transposon were annotated after BLAST search. Plasmid maps were

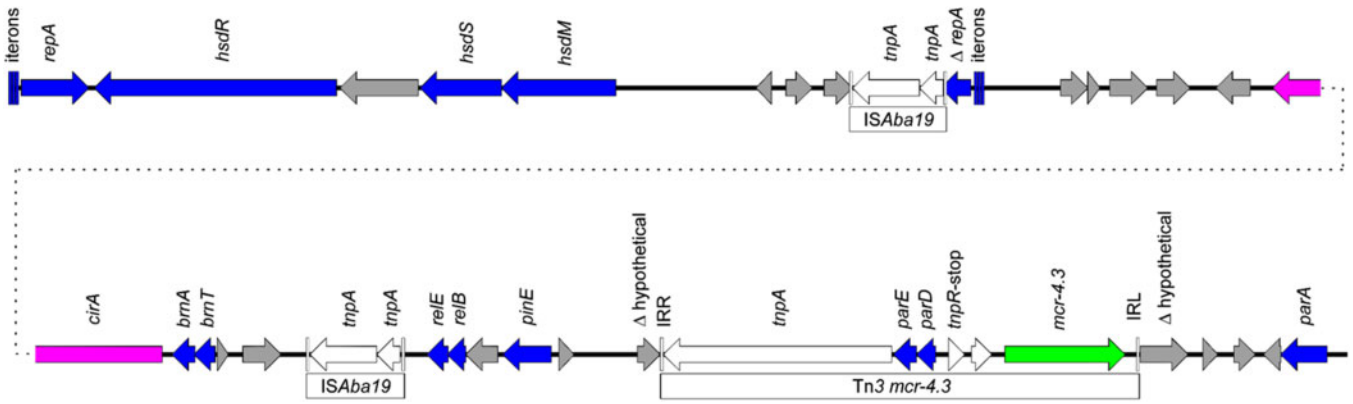

Figure 1. Schematic representation of the complete sequence of pAb-MCR4.3. Grey arrows indicate confirmed or putative ORFs and their orientations. Arrow size is proportional to predicted ORF length. The Tn*3*-like transposon carrying *mcr-4.3* is highlighted for emphasis, and *mcr-4.3* is represented by a green arrow. Transposon genes are indicated with white arrows. Blue arrows indicate genes involved in plasmid replication or maintenance. Pink arrows indicate genes involved in other metabolic functions (*cirA* from pAb-MCR4.3 is involved in iron acquisition and is a putative virulence factor). TSD, TTTCT; IRL, GGCTAAATTTGCCCAACGCGCCAAAAATGTAAAGTTAGC; and IRR, GGCTAATTTTGCCCAACGCGCCAAAAATGTAAAGTTAGC. Gene nomenclature is based on the closest BLAST match from the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

generated with Easyfig 2.1. Identification of plasmid genes, the replicon and the toxin–antitoxin system was based on comparison with sequences archived in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The plasmid replicon was characterized based on the PCR-based replicon system in *A. baumannii*.[12]

Cloning the mcr-4.3 gene into the expression vector pET-26b (+) and transformation of the E. coli BL21(λDE3)pLysS strain

The full coding sequence of *mcr-4.3* was amplified by PCR with forward eptA3-Fc (with an NdeI site) and reverse eptA3-Rp (with an XhoI site) primers (Table S1, available as Supplementary data at *JAC* Online). The purified *mcr-4.3* PCR product was inserted into the vector pET-26b (+) (Novagen, WI, USA) digested with the restriction enzymes NdeI and XhoI. Ligation was performed with the Rapid DNA ligation kit (Sigma, St Louis, MO, USA). The recombinant pET-26b-mcr-4.3 was transformed into the chemically competent *E. coli* DH5α strain (Invitrogen, CA, USA). Transformed cells were grown in LB agar supplemented with 30 mg/L kanamycin (Sigma) overnight at 37°C. The pET-26b-mcr-4.3 in DH5α was sequenced to confirm its integrity.

The BL21(λDE3)pLysS strain (Novagen) was used for the expression of the *mcr-4.3* gene after induction with 1 mM IPTG. The colistin MIC was determined by broth dilution in a constitutively expressed construct using the recommendations of CLSI. The pET-26b-mcr-4.3 was purified from the *E. coli* DH5α recombinant and transformed into the chemically competent BL21(λDE3)pLysS strain. The *E. coli* strain harbouring pET-26b-mcr-4.3 was grown aerobically at 37°C in LB broth (Merck, Darmstadt, Germany) with 30 mg/L kanamycin.

Isolation and characterization of lipid A using MS

A. baumannii 597A, *E. coli* BL21(λDE3)pLysS and the recombinant pET-26b-mcr-4.3 BL21(λDE3)pLysS were grown with shaking at 37°C in 250 mL of LB broth to an OD_{600} of 0.05 to 0.8–1.0. Cells were harvested and washed with 1× PBS. Isolation consisted of solvent extractions with chloroform, methanol and water, followed by mild acid hydrolysis.[13] For *A. baumannii*, boiling time was increased to 1 h after mild acid hydrolysis. Dried samples were stored at −20°C until MS analysis.

Lipid extracts were analysed in the negative ion mode with a Synapt G2-Si High Definition Mass Spectrometer equipped with a nanoelectrospray ionization source (Waters, Milford, MA, USA). MS data acquisition and analysis were performed with MassLynx software (version 4.1, Waters).

Results and discussion

mcr-4.3 gene identified in colistin-resistant A. baumannii 597A

We identified the phosphoethanolamine transferase-encoding gene *mcr-4.3* in the *A. baumannii* 597A clinical strain. The gene was located on a novel plasmid, pAb-MCR4.3, and embedded within a Tn*3*-family transposon (6504 bp) harbouring a resolvase and a Tn*3*-family transposase (Figure 1). The *mcr-4.3* gene (1626 bp), previously annotated as *mcr-4.2*, shows 100% nucleotide identity to a putative sulphatase present in the chromosome of *Shewanella frigidimarina* NCIMB 400 (CP000447) (*mcr-4*).[14] In China, the *mcr-4.3* allele has been recently identified in *A. baumannii* recovered from pig faeces (accession number MK016505.1) and in a clinical *Enterobacter cloacae* (NG_057461) isolate.[7,15] Five additional *mcr-4* alleles from *Salmonella enterica* and Shiga toxin-producing *E. coli* have been reported in swine farms in Italy and Spain.[16,17]

Another *mcr-4.3* sequence was found in the NCBI database in *Acinetobacter nosocomialis* (accession number MG948623.2) without related scientific publications.

pAb-MCR4.3 is 35 502 bp in length and harbours two putative type II toxin–antitoxin systems (*relBE* and *parDE*); however, the *parDE* locus was found on the Tn*3 mcr4.3* transposon. A GR22 replicase gene of the Rep-3 superfamily is found on pAb-MCR4.3 based on 93% sequence identity of the replicase *repB* to repAci22. Interestingly, the replicase from GR22 was encoded by a plasmid detected in a carbapenem-resistant *A. baumannii* assigned to the MLST CC104 isolated in Argentina.[18] However, none of the CC104 *A. baumannii* isolates was colistin resistant or carried *mcr*.

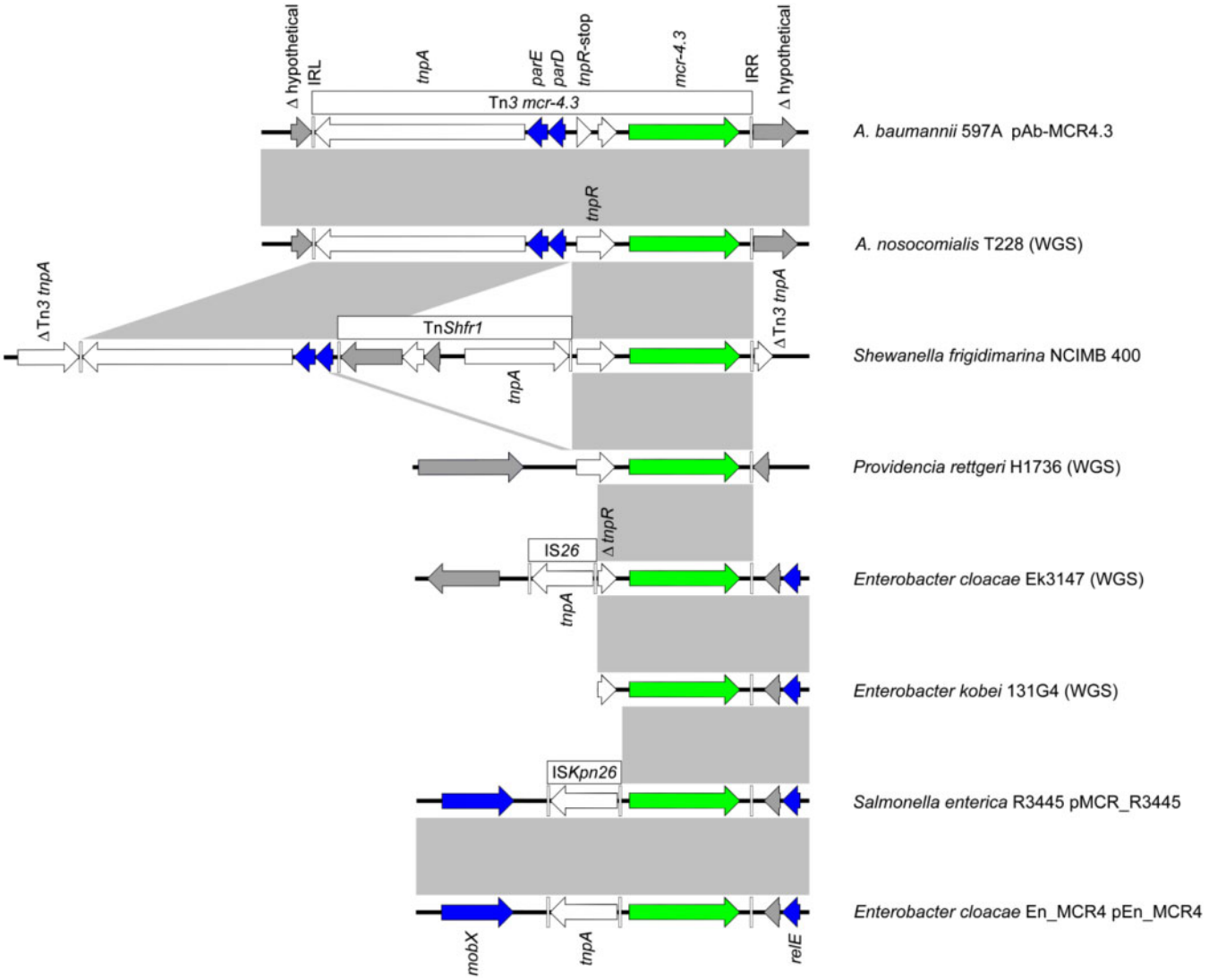

Figure 2. Alignment of *mcr-4.3*. Comparison of the Tn*3*-family structure carrying *mcr-4.3* in pAb-MCR4.3 from *A. baumannii* 597A and seven other sequences obtained from the NCBI. Subsequent genetic events in and around the Tn*3*-family structure are evidenced, including deletions, insertions and rearrangements. Arrow size is proportional to predicted ORF length and the colour scheme is the same as that used in Figure 1. The grey-shaded rectangles indicate areas of identity >95% between each sequence. WGS results of *A. baumannii* 597A and pAb-MCR4.3 were deposited in NCBI GenBank with accession numbers CP033869 and CP033872, respectively. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The Tn*3*-family transposon harbouring *mcr-4.3* is characterized by inverted repeats and a 5 bp target site duplication (TSD) (TTTCT). Similar TSDs were found in the *mcr-4.3* region from the *S. frigidimarina* chromosome NCIMB 400 (accession number CP000447). *mcr-4* variants are found closely related to the Tn*3*-family transposons or truncated sequences (CP000447, MF543359, MH061380, CVLT01000089, JRUA01000041, QKNF01000006 and QMCU01000033). Other *mcr* genes including *mcr-1* and *mcr-2* are found in composite transposons, while *mcr-3.5* and *mcr-5* are embedded within Tn*3*-family and Tn*21*-subfamily transposons, respectively.[19–23] Subsequent genetic events have resulted in gene loss or gain due to the movement of other mobile genetic elements including IS*26* and IS*Kpn26* (Figure 2).

Cloning and lipid A structure experiments confirmed the function of the mcr-4 gene

As a phosphoethanolamine transferase, MCR catalyses the attachment of phosphoethanolamine to lipid A of LPS. *A. baumannii* 597A lipid A MS spectra had the main ion peak at *m/z*=2034 consisting of the addition of phosphoethanolamine (+124 Da) to the heptacylated bisphosphorylated form of lipid A (*m/z*=1910) (Figure S1).

We used the expression vector pET-26b (+) to assess the function of the *mcr-4.3* gene. The colistin MIC for the recipient *E. coli* strain increased from 0.0125 to 8 mg/L, indicating that the gene conferred phenotypic resistance. Furthermore, the mass spectrum of *E. coli* BL21(λDE3)pLysS carrying the plasmid exhibited an ion

peak at *m/z*=1920, indicating the addition of phosphoethanolamine to lipid A (*m/z*=1796; Figure S1). Our findings contradict the lack of functionality of the *mcr-4.3* gene previously observed in *E. cloacae* strains carrying the gene, where no phosphoethanolamine addition to lipid A or increased colistin MIC was reported.[13–20] In our additional analysis, multiple zinc-binding sites (E240, T278, H377, D452 and H453) were conserved in MCR-4 variants as compared with MCR-1 and MCR-2 that could support the enzymatic function of the protein.[24]

Mutations in genes related to colistin resistance and other antimicrobial resistance genes

In *Acinetobacter* species, colistin resistance has primarily been mediated by lipid A modification with the addition of phosphoethanolamine due to mutations in the *pmrCAB* operon.[1] However, no mutations in the *pmrCAB* operon were identified in *A. baumannii* strain 597A. An additional *pmrC*-related gene, defined as a phosphoethanolamine transferase-encoding gene (*eptA-1*), was found in the chromosome of the 597A strain with 50.5% nucleotide identity to the *mcr-4.3* gene. The phosphoethanolamine transferase EptA and MCR catalytic domains are highly conserved in *A. baumannii*; however, their different functionality related to the mechanism of colistin resistance is unclear.[24] No mutations that have been implicated in *A. baumannii* colistin resistance were found in *lpxCAD* (lipid A biosynthesis pathway proteins), *naxD* (deacetylase), *lpsB* (glycosyltransferase), *lolA* (OM lipoprotein), *lolB* (OM lipoprotein), *pldA* (OM phospholipase A) or *ttg2E* (putative toluene tolerance protein).[25] However, we cannot exclude that additional secondary unknown mechanisms of colistin resistance could be associated with the high-level colistin resistance in *A. baumannii* 597A.

Multiple antimicrobial resistance-encoding genes, including resistance to β-lactams (bla_{OXA-23} and bla_{TEM-1A}), aminoglycosides [*strB*, *strA*, *aph(3')-VIa* and *aadA1*], sulphonamides (*sul2*) and trimethoprim (*dfrA1*), were identified in *A. baumannii* 597A. The antimicrobial resistance genotype correlated with the phenotype.

Conclusions

We provide evidence that colistin resistance in *A. baumannii* 597A strain CC113/79 is mediated by *mcr-4.3*. The gene was carried on a novel plasmid within a Tn*3*-family transposon.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online.

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