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MECHANISMS OF RESISTANCE

Identification of a Novel Plasmid Carrying *mcr-4.3* in an *Acinetobacter baumannii* Strain in China

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ABSTRACT Here, we identified *mcr-4.3* in *Acinetobacter baumannii*, which had not been previously observed to carry an *mcr* gene. The *mcr-4.3*-harboring *A. baumannii* strain AB18PR065 was isolated from pig feces from a slaughterhouse in Guangdong Province of China. The *mcr-4.3*-carrying pAB18PR065 is 25,602 bp in size and could not be transferred in conjugation, transformation, and electroporation experiments, as we did not find any conjugation-related genes therein. pAB18PR065 harbors two copies of type II toxin-antitoxin systems, which are functional in plasmid stabilization and maintenance. pAB18PR065 shares similarity only with one recently identified plasmid, pAb-MCR4.3 (35,502 bp), from a clinical *A. baumannii* strain. It is likely that the emergence of pAb-MCR4.3 was due to the insertion of an 11,386-bp, IS*Aba19*-based, composite transposon into pAB18PR065. These data indicate that *mcr-4.3* was captured by an *A. baumannii*-original plasmid via horizontal gene transfer.

KEYWORDS *Acinetobacter baumannii*, colistin resistance, *mcr-4.3*

Colistin is considered one of the last-resort treatments against human infections caused by multidrug-resistant Gram-negative bacteria (1). The first plasmid-mediated colistin resistance gene, *mcr-1* (1,626 bp), was identified on an IncI2 plasmid from *Escherichia coli* and *Klebsiella pneumoniae* in China (2). Since then, *mcr-1* has been proven to be disseminated ubiquitously among *Enterobacteriaceae* strains (3). While *mcr-1* remains the predominant plasmid-mediated colistin resistance gene, *mcr-2* (1,617 bp) (4), *mcr-3* (1,626 bp) (5), *mcr-4* (1,626 bp) (6), *mcr-5* (1,644 bp) (7), *mcr-6* (1,617 bp) (8), *mcr-7* (1,620 bp) (9), and *mcr-8* (1,698 bp) (10) have been identified in various species from humans and animals. There are 56 *mcr* variant sequences available in GenBank to date, including *mcr-1.1* to *mcr-1.15*, *mcr-2.1*, *mcr-2.2*, *mcr-3.1* to *mcr-3.24*, *mcr-4.1* to *mcr-4.6*, *mcr-5.1* to *mcr-5.3*, *mcr-6.1*, *mcr-7.1*, *mcr-8.1*, *mcr-8.2*, and *mcr-8.4* (11).

Acinetobacter baumannii, which is a member of the ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), is an emerging pathogen and a leading cause of nosocomial infections (12). As an opportunistic pathogen, *Acinetobacter* primarily attacks immunocompromised patients and causes infections such as ventilator-associated pneumonia, catheter-related bacteremia, wound and soft tissue infections, urinary tract infections, postsurgical endocarditis, and meningitis (13).

The *mcr-4* gene was first described to be located on an 8,749-bp ColE10 plasmid

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in pig-origin *Salmonella enterica* from Italy. Transformants with a *mcr-4*-carrying plasmid exhibited colistin MICs of 2 μg/ml, 8-fold higher than that of the untransformed recipient (6). *mcr-4.3* (originally named *mcr-4.2*) differs from *mcr-4* in two missense mutations, which lead to codon changes of V179G and V236F (11). *mcr-4.3* was first identified on a ColE10-type plasmid in 6 sequence type 54 (ST54) *Enterobacter cloacae* strains isolated from humans in Singapore in 2014 (14). This gene was also found on an 8,639-bp ColE plasmid in a clinical ST84 *E. cloacae* strain from China (15). Here, we identified a novel *A. baumannii*-original plasmid, pAB18PR065, carrying *mcr-4.3* in an *A. baumannii* strain recovered from pig feces in China. The transferability of pAB18PR065 was investigated using conjugation experiments, transformation, and electroporation. The birth and evolution of pAB18PR065, based on a comparative analysis of multiple plasmids, are also discussed.

RESULTS AND DISCUSSION

Identification of the *mcr-4.3*-harboring *A. baumannii* strain. Among the samples collected, we identified 84 *mcr*-harboring isolates from pigs ($n = 63$), patients ($n = 10$), and healthy individuals ($n = 11$). These isolates included *E. coli* (*mcr-1*, $n = 75$; *mcr-3*, $n = 1$; *mcr-1* and *mcr-3*, $n = 3$), *Shewanella* spp. (*mcr-4.3*, $n = 4$), and *A. baumannii* (*mcr-4.3*, $n = 1$) (see Table S1 in the supplemental material). The *mcr-4.3*-harboring *Shewanella* sp. strains did not show resistance to colistin, and *mcr-4.3* was found to be located on ~140- to 220 kb plasmids (Fig. S1). However, *A. baumannii* strain AB18PR065 exhibited resistance to both colistin and polymyxin B, with MICs of 8 μg/ml, while remaining susceptible to almost all other antimicrobial agents tested (Table S2). This strain was recovered from pig feces in a slaughterhouse in Guangdong province on 25 May 2018. Sequence typing of AB18PR065 determined that alleles of the seven housekeeping genes were *cpn60*-48, *gdhB*-49, *gltA*-51, *gpi*-25, *gyrB*-90, *recA*-11, and *rpoD*-4 in the Oxford scheme (newly assigned to ST1929) and *cpn60*-3, *fusA*-3, *gltA*-16, *pyrG*-1, *recA*-13, *rplB*-1, and *rpoB*-15 in the Pasteur scheme (newly assigned to ST1303), both of which indicated a new ST (Table S3). Except for *mcr-4.3*, this strain harbors the naturally occurring $bla_{\text{OXA-51-like}}$ ($bla_{\text{OXA-430}}$ with additional H7Q and S90G mutations) and $bla_{\text{ADC-like}}$ ($bla_{\text{ADC-184}}$ with an additional P113S mutation) genes in *A. baumannii* (16). The IS*Aba1* element can provide a promoter to upregulate the expression of bla_{ADC} or bla_{OXA}, leading to resistance to cephalosporins or carbapenems in *A. baumannii* (17, 18). No IS*Aba1* element was found upstream of the $bla_{\text{OXA-51-like}}$ and $bla_{\text{ADC-like}}$ genes in AB18PR065. A retrospective screening of *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* using a strain collection of 90 colistin-resistant *A. baumannii* clinical isolates identified in 2012 to 2015 by our group did not find a *mcr*-carrying strain.

A. baumannii is considered a serious threat to human health because it causes serious infections that are associated with high morbidity and mortality rates. The challenge in treating *Acinetobacter* infections is primarily related to its high intrinsic tolerance to most antibiotics. Compromise of antibiotics to treat *A. baumannii* infections is due to the exceptionally low permeability to antibiotics, constitutively expressed efflux pumps, resistance genes harbored on genetic islands, and high genetic plasticity of *Acinetobacter* (19, 20). Polymyxins have remained effective against *Acinetobacter* infections (13). Available approaches use novel combinations of drugs, such as daptomycin-colistin-teicoplanin and intensified meropenem-polymyxin B (21). The presence of *mcr-4.3* and its location on a plasmid would facilitate the spread of *mcr-4.3* among *Acinetobacter* strains.

***mcr-4.3* was found on pAB18PR065 without a flanking mobile element.** S1-nuclease digestion–pulsed-field gel electrophoresis (S1-PFGE) and Southern blotting hybridization revealed that *mcr-4.3* was located on an ~25-kb plasmid within AB18PR065 (Fig. 1). Using whole-genome sequencing (WGS) data, we found a scaffold of 25,602 bp in the genome assembly that carries *mcr-4.3*. BLASTn was used to analyze the close plasmid sequences. Unexpectedly, this contig showed a high level of similarity (94% coverage, 99% identity) only to a 35,502-bp *mcr-4.3*-carrying plasmid (pAbMCR4.3 [GenBank accession no. CP033872]) identified in *A. baumannii* (Fig. S2). pAb-

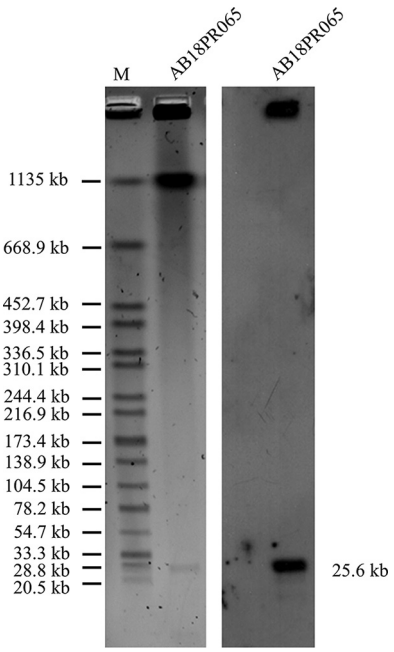

FIG 1 S1-PFGE and Southern hybridization with the *mcr-4.3* probe. (Left) S1-PFGE map of the AB18PR065 strain. (Right) Southern blotting hybridization using the *mcr-4.3* probe. M, *Salmonella* H9812 (New England BioLabs, Beverly, MA).

MCR4.3 was found in an ST233 (Oxford scheme) or ST79 (Pasteur scheme) *A. baumannii* strain that had been collected from the cerebrospinal fluid of a patient in Brazil in 2008; the plasmid sequence had been released in GenBank by 26 November 2018. We used the pAb-MCR4.3 sequence as the reference to design primers to close the plasmid. The complete sequence of the 25,602-bp plasmid pAB18PR065 was found to encode 31 open reading frames (ORFs).

mcr-4.3 was found downstream of two ORFs that encode putative recombinase on pAB18PR065, whereas no mobile element was found adjacent to *mcr-4.3*. We identified a Tn*3* element (3,087 bp) upstream of *mcr-4.3* at a 1,528-bp distance. However, this transposon is more likely to mediate the location of type II toxin-antitoxin (TA) systems of *parE* and *phd*, since a 4,460-bp sequence including Tn*3*, *parE*, *phd*, and the two ORFs encoding recombinases has been found in various plasmids (*Klebsiella oxytoca*, pKOR-b08d [GenBank accession no. CP026279]; *E. cloacae*, pENT-2c5 [GenBank accession no. CP017992]; *E. coli*, pECO-dc1b [GenBank accession no. CP026207]). Of note, this sequence combined with the *mcr-4.3* gene was found to be homologous to the genomic sequence of *Shewanella frigidimarina* NCIMB 400 (GenBank accession no. CP000447), and *mcr-4.3* showed 100% nucleotide identity to the sequence in the genome. These data supported the conclusion that *mcr-4* and its alleles originated from *Shewanella frigidimarina* (6), while the identification of *mcr-4.3* in *A. baumannii* resonated with the indication of horizontal gene transfer of *mcr-4.3*, according to the other two reports (14, 15).

pAB18PR065 is a novel nonconjugative plasmid and carries plasmid stabilization elements. pAB18PR065 encodes a replication initiation protein, RepB, that differs from the known Inc types. Phylogenetic analysis using replicon sequences revealed that *repB* within pAB18PR065 did not link with other plasmid types (Fig. S3). Conjugation experiments using AB18PR065 failed to transfer the plasmid into an *E. coli* recipient. Indeed, we did not find any conjugation-related ORFs on this plasmid. However, either heat shock transformation or electroporation using multiple recipient strains failed to yield pAB18PR065-harboring transformants. We could not determine whether transfer of pAB18PR065 could confer resistance to colistin. According to the other two studies, *mcr-4.3* alone did not confer phenotypic resistance to colistin (14) and showed a lack of lipid A modification function (15). Recently, a study on the action and mechanism of MCR-4

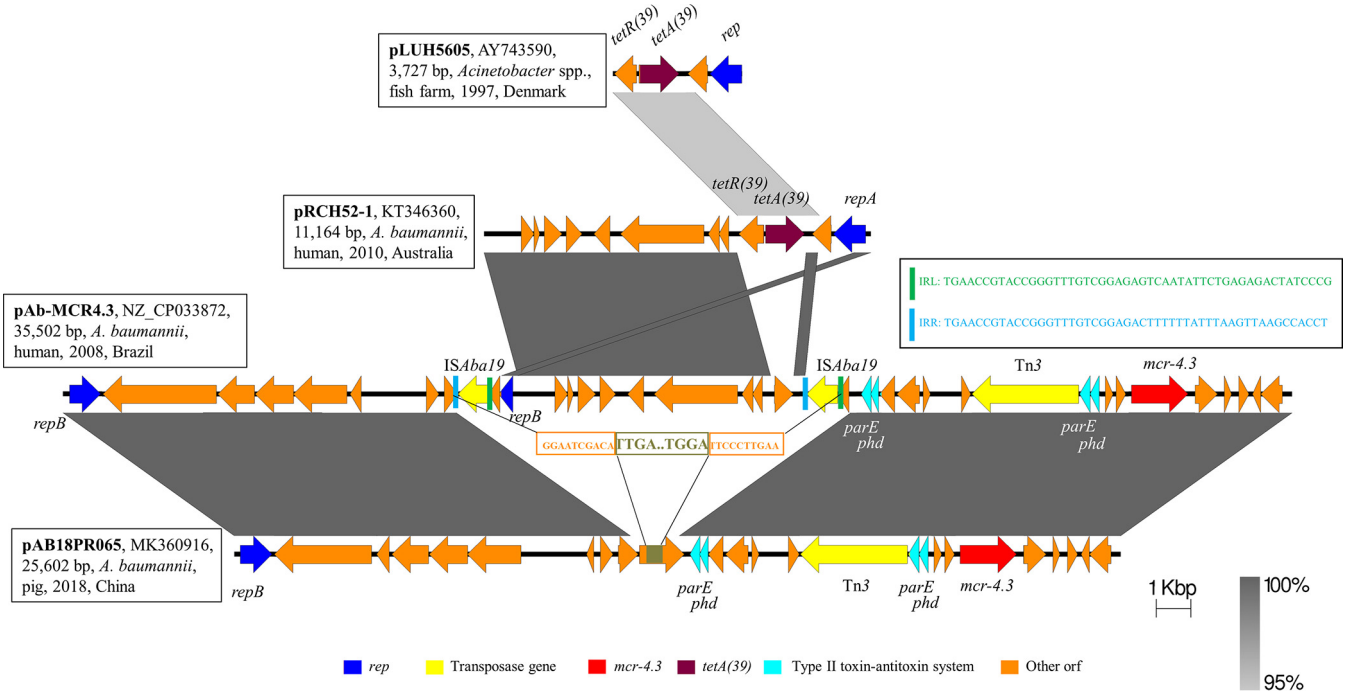

FIG 2 Schematic presentation of the major structural features of pAB18PR065, in comparison with the reference plasmids pLUH5605, pRCH52-1, and pAb-MCR4.3. Areas shaded gray indicate homologous regions of ≥95% nucleotide sequence identity in the plasmid scaffold regions. ORFs are portrayed by arrows to indicate the direction of transcription and are colored based on their predicted gene functions. The figure is drawn to scale.

showed that revertants of *mcr-4.3* (either V179G or V236F) led to its expression and conferred colistin resistance of 8 μg/ml, together with the acquired function of lipid A modification (22).

Although pAB18PR065 is nonconjugative, it employs type II TA systems as a post-segregational killing operation to ensure plasmid stabilization and maintenance. Type II TA systems are generally composed of two genes, encoding a labile antitoxin and a stable toxin (23). These systems are thought to move from one genome to another by horizontal gene transfer, since TA loci were found in integrons in *Vibrio cholerae* and closely linked *attC* sites, which are regarded as the recombination sites of gene cassettes (24). pAB18PR065 has two TA gene families (*parDE* and *phd/doc*), and each has two copies. These systems preferentially guarantee the growth of pAB18PR065-carrying daughter cells in a bacterial population via killing of newborn bacteria that did not inherit a plasmid copy at cell division (25).

pAB18PR065 involvement in recombination in *A. baumannii*. Given the fact that pAB18PR065 matched only pAb-MCR4.3, we analyzed their genetic contexts to understand the structural evolution (Fig. 2). The main difference between them was that pAB18PR065 lacked an 11,386-bp sequence that existed in pAb-MCR4.3, at the positions from 11,367 bp to 22,751 bp. Of note, this ~11-kb sequence was flanked by two copies of IS*Aba19* in the same direction, and the sequences shared 99% nucleotide identity when IS*Aba19*, with a unique 11,164-bp plasmid (pRCH52-1 [GenBank accession no. KT346360]) identified in clinical *A. baumannii* isolates recovered prior to 2010 in Australia (26), was excluded. IS*Aba19* is an *A. baumannii*-original insertion sequence with a length of 1,309 bp, which was found inserted into the bla_{OXA-78} gene in clinical *A. baumannii* isolates (27). Additionally, pRCH52-1 harbors a *tetA*(39)-*tetR*(39) unit (1,895 bp); the original study showed that *tetA*(39) was located on a plasmid from *A. baumannii* isolated in 1997, but only a 3,727-bp fragment was sequenced (28, 29). With all of this information, we hypothesized that the emergence of the *mcr-4.3*-carrying plasmids involved recombination in *A. baumannii* (Fig. 2). The process may be summarized as follows. The *tetA*(39)-*tetR*(39) unit was first inserted into a plasmid, causing

the production of pRCH52-1. This unit was then transferred to other environments, which led to the appearance of a pRCH52-1-like plasmid without this unit. The transfer of two copies of intact IS*Aba19* constituted a composite transposon to translocate the pRCH52-1-like plasmid into a DNA polymerase-encoding ORF on pAB18PR065, leading to the formation of pAb-MCR4.3.

Conclusion. We identified the colistin resistance gene *mcr-4.3* on a novel nonconjugative plasmid, pAB18PR065, in *A. baumannii* from pig feces in China. There was no mobile element adjacent to *mcr-4.3* on pAB18PR065, which suggests that *mcr-4.3* might have been acquired in the *A. baumannii* community via horizontal gene transfer.

MATERIALS AND METHODS

Bacterial strains. To detect *mcr*-carrying isolates in humans and animals, 185 pig fecal samples, 320 rectal swabs from healthy persons, and 170 rectal swabs from patients were collected from a pig slaughterhouse and hospitals in Guangdong Province, China, with one sample per individual, in April to June 2018. Samples were cultured by adding 3 ml of nutrient broth and incubated for 18 to 24 h at 37°C. Subsequently, the total DNA was extracted and screened for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* using multiplex PCR, as described previously (30). Sanger sequencing using the positive amplicons was used to determine the *mcr* variants. Isolation of *mcr*-harboring strains from the PCR-positive samples was performed by inoculating the culture cells on LB agar containing colistin at 2 μg/ml, and different morphological colonies were selected to screen the *mcr* genes. Preliminary species identification was achieved by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) and 16S rRNA sequencing, and the identification of *A. baumannii* species was confirmed by WGS.

Antimicrobial susceptibility testing. MICs were determined for colistin, polymyxin B, tigecycline, ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, cefepime, gentamicin, amikacin, ertapenem, imipenem, meropenem, fosfomycin, and ciprofloxacin for all *mcr*-carrying isolates, using the agar dilution method except for colistin, for which used the broth dilution method was used, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (31). The results were interpreted according to CLSI instructions (31), and colistin resistance was defined according to EUCAST clinical breakpoints (32).

S1-PFGE and Southern blotting. The plasmid and/or chromosomal locations of *mcr-4.3* were determined by S1-PFGE (33), followed by Southern blotting hybridizations. Southern blotting hybridizations of plasmid DNA were performed with a digoxigenin-labeled *mcr-4.3* probe, according to the manufacturer's instructions (Roche Diagnostics, Germany).

Plasmid conjugation, transformation, and electroporation. Conjugation experiments were performed to test the transferability of the *mcr-4.3*-harboring plasmid, using streptomycin-resistant *E. coli* C600 as the recipient strain. Briefly, cultured cells of *mcr-4.3*-carrying isolates and *E. coli* C600 were mixed (at a ratio of 1:9) and subjected to overnight incubation (34). The mixture was then spread on LB agar plates containing sodium streptomycin (2,000 μg/ml) plus colistin (2 μg/ml) to select transconjugants, which were checked by PCR and Sanger sequencing. When the *mcr-4.3*-harboring plasmid could not be transferred by conjugation, we tried transformation and electroporation to harvest the transformants. Plasmid DNA was isolated from donor strains and transformed into *E. coli* DH5α cells or electroporated into *E. coli* DH10B, *E. coli* MG1655, *E. coli* C600, and *A. baumannii* ATCC 19606 cells, and transformants were selected on LB agar plates with 0.5, 1, and 2 μg/ml colistin.

Whole-genome sequencing. The genomic DNA of the *mcr-4.3*-carrying isolates was extracted using the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany). DNA libraries were constructed with 350-bp paired-end fragments and sequenced using an Illumina HiSeq 2000 platform. Raw reads were assembled using SPAdes 3.10 (35). *In silico* multilocus sequence typing (MLST) was performed using MLST 1.8 (https://cge.cbs.dtu.dk/services/MLST). Antimicrobial resistance genes (ARGs) were identified by submitting the sequence to the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/ResFinder). The virulence factors of *A. baumannii* were identified using the VFDB database (36). Plasmid assembly was performed using plasmidSPAdes (37) and was confirmed by a PCR-based closure, using the primers listed in Table S4 in the supplemental material. Plasmid replicon types were detected using PlasmidFinder 1.3 (38). Insertion sequence elements were confirmed by searching in ISFinder (https://isfinder.biotoul.fr).

Accession number(s). Genome assemblies of newly sequenced *A. baumannii* strain AB18PR065 (GenBank accession no. RZNI00000000) and the sequence of pAB18PR065 (GenBank accession no. MK360916) have been deposited in the NCBI database under BioProject no. PRJNA512224.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00133-19.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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