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MECHANISMS OF RESISTANCE

Chromosomally Encoded *mcr-5* in Colistin-Nonsusceptible *Pseudomonas aeruginosa*

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ABSTRACT Whole-genome sequencing (WGS) of historical *Pseudomonas aeruginosa* clinical isolates identified a chromosomal copy of *mcr-5* within a Tn*3*-like transposon in *P. aeruginosa* MRSN 12280. The isolate was nonsusceptible to colistin by broth microdilution, and genome analysis revealed no mutations known to confer colistin resistance. To the best of our knowledge, this is the first report of *mcr* in colistin-nonsusceptible *P. aeruginosa*.

KEYWORDS *Pseudomonas aeruginosa*, colistin, *mcr-5*

Pseudomonas aeruginosa is a leading cause of infection among immunocompromised patients and patients receiving treatment in intensive care units (ICUs) (1). Antimicrobial treatment of *P. aeruginosa* is challenging, due to the intrinsic resistance of this species to many antibiotics and its proclivity to develop resistance to other antibiotics via point mutations in intrinsic genes (2, 3). Furthermore, *P. aeruginosa* can readily acquire transmissible antibiotic resistance (AbR) genes, resulting in the emergence of successful multidrug-resistant or extensively drug-resistant strains (4). The emergence of such resistant strains has resulted in greater reliance on colistin (polymixin E) as a key antipseudomonal agent (5). Unfortunately, colistin resistance in *P. aeruginosa*, primarily due to mutations in regulatory two-component systems, has been extensively reported (reviewed in reference 6). However, colistin resistance mediated by the transferable colistin resistance gene *mcr* has not been described in this species to date. In this report, we describe the colistin-nonsusceptible *P. aeruginosa* strain MRSN 12280, carrying a chromosomal copy of *mcr-5*.

P. aeruginosa MRSN 12280 was sequenced as part of a larger effort to sequence all *P. aeruginosa* isolates in the Multidrug-resistant organism Repository and Surveillance Network (MRSN) repository ($n = 2{,}440$) (E. Snesrud, J. Stam, M. Hinkle, A. Jones, and P. McGann, unpublished data). The isolate was cultured from a sacral wound of a male patient in his mid-70s who was treated in the southwestern United States in 2012. There was no record of the patient ever receiving colistin. The MICs of colistin were determined using broth microdilution (BMD) with cation-adjusted Mueller-Hinton (CA-MH) medium, according to Clinical and Laboratory Standards Institute (CLSI) guidelines, and also with calcium-enhanced Mueller-Hinton (CE-MH) medium, as recommended by Gwozdzinski and colleagues for *Enterobacteriaceae* carrying *mcr* (7). *Escherichia coli* strain MRSN 388734 carrying *mcr-1* (8) and *P. aeruginosa* strain ATCC 27298 were used as the positive and negative controls, respectively. Colistin MICs were 4 μg/ml (intermediate) and 8 μg/ml (resistant) for *P. aeruginosa* MRSN 12280 in CA-MH medium and CE-MH medium, respectively. Notably, the colistin MICs for the control strains of *E. coli* MRSN 388734 and *P. aeruginosa* ATCC 27298 also increased from 8 to 16 μg/ml and from 0.25 to 1 μg/ml, respectively, in CE-MH medium, although the interpretations did not change.

Short-read and long-read whole-genome sequencing (WGS) analyses were performed with a NextSeq 550 system (Illumina, San Diego, CA) and a PacBio RS II

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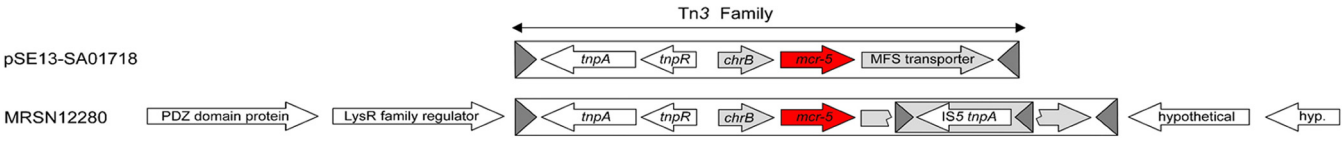

FIG 1 Alignment of Tn*3*-family transposons carrying *mcr-5*. The Tn*3*-like transposon carrying *mcr-5* in plasmid pSE13-SA01718 from *Salmonella enterica* (10) is compared with the Tn*3*-like transposon carrying *mcr-5* in *P. aeruginosa* MRSN 12280. Transposons are enclosed in rectangles, with the IRs depicted as shaded arrowheads. Arrows represent coding sequences (red arrows, *mcr-5*; white arrows, genes associated with DNA mobility; gray arrows, other genes) and indicate the direction of transcription.

sequencer (Pacific Biosciences, Menlo Park, CA), respectively, as described previously (9). *In silico* multilocus sequence typing (MLST) assigned *P. aeruginosa* MRSN 12280 to a novel sequence type (ST) that is a single-locus variant of ST-235, the most prevalent clone associated with multidrug-resistant *P. aeruginosa* epidemics worldwide (10). An analysis of the WGS data detected five AbR genes that are commonly found in *P. aeruginosa*, namely, *aph(3′)-IIb*, bla_{OXA-50}, bla_{PAO}, *catB7*, and *fosA*, and the recently described colistin resistance gene *mcr-5* (11). The *mcr-5* gene was first reported in 2017 in a cluster of colistin-nonsusceptible *Salmonella enterica* strains, and the protein shares sequence identity of just 36.11% with Mcr-1 (11). Borowiak and colleagues reported that the gene was part of a Tn*3*-family transposon that was found primarily on small, multicopy, ColE-type plasmids; however, one isolate (*S. enterica* 12-02546-2) had the gene present in a single copy on the chromosome and demonstrated a colistin MIC of 4 mg/liter (11). In *P. aeruginosa* MRSN 12280, a single copy of *mcr-5* was present on the chromosome, where it was inserted into a noncoding region between genes encoding a LysR-family regulator and a hypothetical protein of unknown function (Fig. 1). The *mcr-5* gene was embedded within an 8,522-bp Tn*3*-family transposon with 38-bp inverted repeats (IRs), and it generated a 5-bp target site duplication (TSD) (TCCAT) upon insertion. Notably, an IS*5* insertion sequence has been inserted into a gene encoding a putative major facilitator superfamily (MFS) protein, directly downstream of *mcr-5* (Fig. 1).

To better understand the genetic environment surrounding *mcr-5*, a search for the same transposon sequence was conducted at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed May 2018). Eleven different submissions from five species were found to harbor the sequence (Table 1). Nine were harbored on Tn*3* transposons, and six of those were >99% identical to the Tn*3* in *P. aeruginosa* MRSN 12280 but without the IS*5* insertion (Fig. 2A). The remaining three Tn*3* structures carried identical passenger genes, including *mcr-5*, but had different *tnpA* and *tnpR* genes. These structures appear to be the products of resolvase-mediated recombination at the *res* site between two different Tn*3* transposons (Fig. 2B), like

TABLE 1 GenBank submissions with *mcr-5*

GenBank accession no.	Species	Strain name	Structure[a]	TSD[b]
CP028162	*Pseudomonas aeruginosa*	MRSN 12280	Tn*3*; chromosome	TCCAT
KY807921	*Salmonella enterica*	13-SA01718	Tn*3*; plasmid	ATGTA
CP010516	*Cupriavidus gilardii*	CR3	Tn*3*; chromosome	AACTC/ATAAT
NBAR00000000.1	*Salmonella enterica*	12-02546-2	Tn*3*; chromosome	ATAAA
BESA01000094	*Escherichia coli*	PV1176	Tn*3*; plasmid	ATTAT
BENI01000099	*Escherichia coli*	10138	Tn*3*; plasmid	AATAA
BBQK01000039	*Pseudomonas aeruginosa*	KF702	Partial Tn*3*; chromosome	Unknown
BEPM01000040	*Escherichia coli*	10875	Tn*3*; plasmid	AAACT/AAATA
BDIH01000107	*Escherichia coli*	NIID080884	Tn*3*; plasmid	TACGC/NGTGA
CP028567	*Aeromonas hydrophila*	WCHAH045096	Tn*3*; plasmid	Unknown
MG800820	*Aeromonas hydrophila*	pI064-2	MIC; plasmid	AAACG
OEUH00000000.1	*Salmonella enterica*	STY142	MIC; plasmid	AAATA

[a]Location and structure of the *mcr-5*-carrying region. Tn*3* indicates that *mcr-5* is located with a Tn*3*-like transposon. MIC indicates that *mcr-5* is located within a putative mobile insertion cassette.

[b]Nucleotide sequence of the TSD generated upon insertion. Different TSDs formed during resolvase-mediated recombination of different Tn*3* transposons are shown separated by a forward slash. Note that the N in the TSD from *E. coli* NIID080884 reflects the annotation in GenBank for this submission. Unknown indicates that subsequent genetic events have removed the TSD from one or both ends.

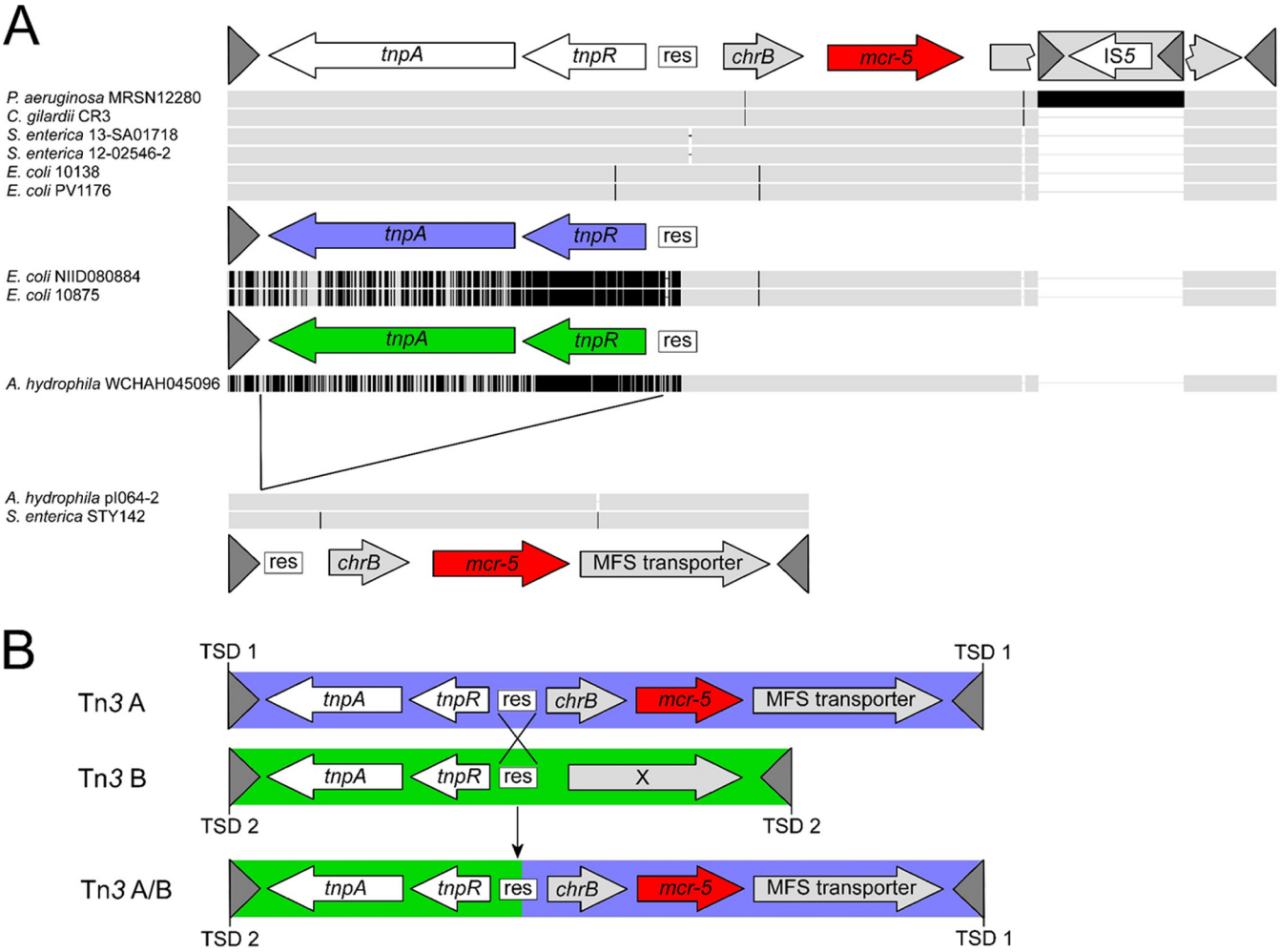

FIG 2 Genetic environment of *mcr-5*. (A) Alignment of all 11 structures carrying *mcr-5*, as of this writing (May 2018). Arrows represent coding sequences (red arrows, *mcr-5*; gray arrows, other genes) and indicate the direction of transcription. Different colors are used to represent the different *tnpA* and *tnpR* genes, with the corresponding *res* sites displayed as white rectangles. IRs are depicted as shaded arrowheads flanking the structures. Areas of >99% homology between sequences are represented by light gray rectangles, with nonhomologous regions represented by black rectangles. Deletions are denoted with a center dot. The homologous *mcr-5* region that constitutes the mobile insertion cassette structure in *A. hydrophilia* pI064-2 and *S. enterica* STY142 is delineated with two black lines. (B) Schematic representation of possible resolvase-mediated recombination of two different Tn*3* transposons, flanked by different TSDs, to form a new mosaic Tn*3* structure. Labeling is consistent with that in panel A.

that observed in the formation of Tn*2* (12). Finally, the remaining two sequences were present on putative mobile insertion cassettes (13). The mobile insertion cassettes were bounded by imperfect Tn*3*-like IRs and flanked by 5-bp TSDs (Table 1). However, additional experimental evidence would be required to confirm that these structures are mobile and were not formed by the loss of the *tnpA* and *tnpR* genes.

TSDs were identified in 9 of the 11 structures, with subsequent genetic events removing one of the TSD pairs in *Aeromonas hydrophilia* WCHAH045096 and *P. aeruginosa* KF702. All 9 structures generated a 5-bp TSD (Table 1). Furthermore, the 5′ and 3′ TSDs flanking the Tn*3* in *E. coli* 10875 and *E. coli* NIID080884 were different, consistent with the hypothesis that these structures were formed by resolvase-mediated recombination of two different Tn*3* transposons flanked by different TSDs (Fig. 2B). In addition, the TSDs flanking the Tn*3* from *Cupriavidus gilardii* CR3 are different, suggesting that this structure might have been created by resolvase-mediated recombination between two identical Tn*3* transposons.

Colistin resistance in *P. aeruginosa* has been attributed primarily to mutations in up to five different two-component regulatory systems (PhoPQ, PmrAB, ParR/S, ColR/S, and CprR/S) (reviewed in reference 6) but, to the best of our knowledge, Mcr-mediated

colistin resistance has not been described in *P. aeruginosa* to date. Because mutations in the two-component regulatory systems could potentially contribute to colistin resistance in *P. aeruginosa* MRSN 12280, we examined the amino acid sequences of PmrA, PmrB, PmrE, PhoP, PhoQ, ParR, ParS, ColR, ColS, MigA, LpxC, CprR, and CprS for nonsynonymous mutations (6, 14–16). In comparison to *P. aeruginosa* PAO1, *P. aeruginosa* MRSN 12280 had nonsynonymous mutations in PhoQ (Y85F), PmrA (L71R), PmrB (S2P, A4T, G68S, Y345H, and G362S), ParR (L153R and S170N), and ParS (H398R). When the sequences were compared to the genomes of 100 ST-235 strains deposited in GenBank, however, the same mutations were present in all strains. Furthermore, a review of the literature indicated that all of the mutations in PhoQ, PmrA, and PmrB were observed previously in colistin-sensitive strains (15, 16). Finally, an analysis of 1,135 *parS* genes from the NCBI revealed that 1,117 sequences have an arginine at position 398, indicating that the ParS protein from *P. aeruginosa* PAO1 is a poor representative of ParS in *P. aeruginosa*. Although the evidence presented here is not definitive, additional experiments to clone and to express the *mcr-5* transposon in *P. aeruginosa* are under way to confirm these findings.

We report the first identification of *mcr-5* in a colistin-nonsusceptible strain of *P. aeruginosa* that was closely related to the globally distributed epidemic clone ST-235 (10). The gene was chromosomally encoded and embedded within a Tn*3*-family transposon that appears to be an important vehicle for *mcr-5* transmission. Notably, the transposon appears to have a tendency to undergo resolvase-mediated recombination with other Tn*3*-like transposons, bestowing greater mobility on *mcr-5*.

Accession number(s). The complete and closed genome of *Pseudomonas aeruginosa* MRSN 12280 has been deposited in GenBank under accession no. CP028162.

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Material has been reviewed by the Walter Reed Army Institute of Research, and there is no objection to its presentation. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

REFERENCES

1. Kerr KG, Snelling AM. 2009. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. J Hosp Infect 73:338–344. https://doi.org/10.1016/j.jhin.2009.04.020.
2. Livermore DM. 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis 34:634–640. https://doi.org/10.1086/338782.
3. Poole K. 2011. *Pseudomonas aeruginosa*: resistance to the max. Front Microbiol 2:65. https://doi.org/10.3389/fmicb.2011.00065.
4. Oliver A, Mulet X, Lopez-Causape C, Juan C. 2015. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updat 21-22:41–59. https://doi.org/10.1016/j.drup.2015.08.002.
5. Montero M, Horcajada JP, Sorli L, Alvarez-Lerma F, Grau S, Riu M, Sala M, Knobel H. 2009. Effectiveness and safety of colistin for the treatment of multidrug-resistant *Pseudomonas aeruginosa* infections. Infection 37:461–465. https://doi.org/10.1007/s15010-009-8342-x.
6. Olaitan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643. https://doi.org/10.3389/fmicb.2014.00643.
7. Gwozdzinski K, Azarderakhsh S, Imirzalioglu C, Falgenhauer L, Chakraborty T. 2018. An improved medium for colistin susceptibility testing. J Clin Microbiol 56:e01950-17. https://doi.org/10.1128/JCM.01950-17.
8. McGann P, Snesrud E, Maybank R, Corey B, Ong AC, Clifford R, Hinkle M, Whitman T, Lesho E, Schaecher KE. 2016. *Escherichia coli* harboring *mcr-1* and bla_{CTX-M} on a novel IncF plasmid: first report of *mcr-1* in the United States. Antimicrob Agents Chemother 60:4420–4421. https://doi.org/10.1128/AAC.01103-16.
9. Snesrud E, Ong AC, Corey B, Kwak YI, Clifford R, Gleeson T, Wood S, Whitman TJ, Lesho EP, Hinkle M, McGann P. 2017. Analysis of serial isolates of *mcr-1*-positive *Escherichia coli* reveals a highly active IS*Apl1* transposon. Antimicrob Agents Chemother 61:e00056-17. https://doi.org/10.1128/AAC.00056-17.
10. Treepong P, Kos VN, Guyeux C, Blanc DS, Bertrand X, Valot B, Hocquet D. 2018. Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. Clin Microbiol Infect 24:258–266. https://doi.org/10.1016/j.cmi.2017.06.018.
11. Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. J Antimicrob Chemother 72:3317–3324. https://doi.org/10.1093/jac/dkx327.
12. Partridge SR, Hall RM. 2005. Evolution of transposons containing bla_{TEM} genes. Antimicrob Agents Chemother 49:1267–1268. https://doi.org/10.1128/AAC.49.3.1267-1268.2005.
13. Chen Y, Braathen P, Leonard C, Mahillon J. 1999. MIC231, a naturally

occurring mobile insertion cassette from *Bacillus cereus*. Mol Microbiol 32:657–668. https://doi.org/10.1046/j.1365-2958.1999.01388.x.
14. Dosselmann B, Willmann M, Steglich M, Bunk B, Nubel U, Peter S, Neher RA. 2017. Rapid and consistent evolution of colistin resistance in extensively drug-resistant *Pseudomonas aeruginosa* during Morbidostat culture. Antimicrob Agents Chemother 61:e00043-17. https://doi.org/10.1128/AAC.00043-17.
15. Lee JY, Chung ES, Na IY, Kim H, Shin D, Ko KS. 2014. Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. J Antimicrob Chemother 69:2966–2971. https://doi.org/10.1093/jac/dku238.
16. Lee JY, Ko KS. 2014. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. Diagn Microbiol Infect Dis 78:271–276. https://doi.org/10.1016/j.diagmicrobio.2013.11.027.
17. Jolley KA, Maiden MCJ. 2010. BIGSdb: scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11:595. https://doi.org/10.1186/1471-2105-11-595.