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Structural Modification of Lipopolysaccharide Conferred by *mcr-1* in Gram-Negative ESKAPE Pathogens

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ABSTRACT *mcr-1* was initially reported as the first plasmid-mediated colistin resistance gene in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in China and has subsequently been identified worldwide in various species of the family *Enterobacteriaceae*. *mcr-1* encodes a phosphoethanolamine transferase, and its expression has been shown to generate phosphoethanolamine-modified bis-phosphorylated hexa-acylated lipid A in *E. coli*. Here, we investigated the effects of *mcr-1* on colistin susceptibility and on lipopolysaccharide structures in laboratory and clinical strains of the Gram-negative ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens, which are often treated clinically by colistin. The effects of *mcr-1* on colistin resistance were determined using MIC assays of laboratory and clinical strains of *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. Lipid A structural changes resulting from MCR-1 were analyzed by mass spectrometry. The introduction of *mcr-1* led to colistin resistance in *E. coli*, *K. pneumoniae*, and *A. baumannii* but only moderately reduced susceptibility in *P. aeruginosa*. Phosphoethanolamine modification of lipid A was observed consistently for all four species. These findings highlight the risk of colistin resistance as a consequence of *mcr-1* expression among ESKAPE pathogens, especially in *K. pneumoniae* and *A. baumannii*. Furthermore, the observation that lipid A structures were modified despite only modest increases in colistin MICs in some instances suggests more sophisticated surveillance methods may need to be developed to track the dissemination of *mcr-1* or plasmid-mediated phosphoethanolamine transferases in general.

KEYWORDS polymyxin resistance, lipid A, phosphoethanolamine, plasmid, plasmid-mediated resistance, polymyxins

Polymyxins, which include colistin (polymyxin E) and polymyxin B, are active against the majority of clinically relevant Gram-negative bacteria and are increasingly used as salvage therapy for infections caused by strains that have become resistant to all other commonly used antibacterial agents (1). Clinically, the key species against which colistin is most implemented due to extensive drug resistance include *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. These three ESKAPE pathogens, along with *Enterobacter* spp. and the Gram-positive organisms *Enterococcus faecium* and *Staphylococcus aureus*, are notable for their rapid drug resistance acquisition and nosocomial prevalence. Colistin is a polycationic peptide that acts by targeting the negative charges in bacterial lipopolysaccharide (LPS), the complex

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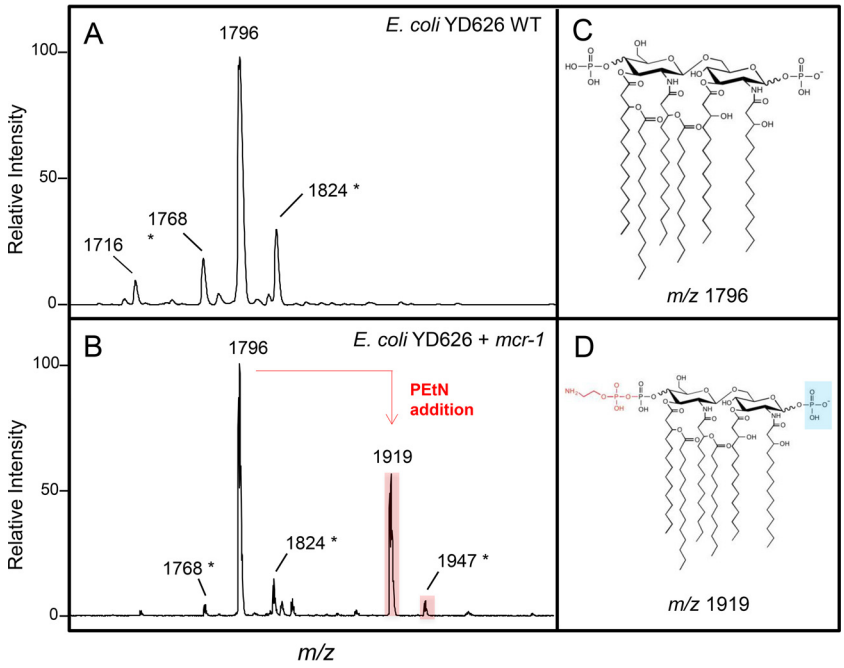

FIG 1 MS analysis of WT *E. coli* strain YD626 (A) and the strain expressing *mcr-1* (B). PEtN-modified species are represented in red (*m/z* 1,919 and 1,947). Proposed structures of WT *E. coli* lipid A (C) and PEtN-modified lipid A (D). Location of PEtN is suggestive; blue represents the lost phosphate moiety, the position of which is also suggestive. Asterisk (*) represents lipid A structure that varies in acyl chain length or hydroxylation status.

lipoglycan that comprises the majority of the Gram-negative outer membrane (1). Specifically, colistin attacks the highly electronegative phosphate groups associated with lipid A, the membrane-anchoring molecule of LPS, and its glycol core, resulting in the lysis and death of Gram-negative bacteria.

The canonical lipid A structure found in Gram-negative bacteria consists of a β-1′,6-linked disaccharide glucosamine backbone that is hexa-acylated and phosphorylated at positions 1 and 4′ (Fig. 1C). Lipid A-modifying enzymes can introduce changes to the canonical lipid A structure by adding, removing, or altering different chemical moieties. These species can naturally develop resistance to colistin by modifying the structure of the lipid A component of LPS, reducing the overall negative charge of the lipid A moiety and therefore the binding of colistin (1). *K. pneumoniae* and *P. aeruginosa* accomplish this by the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A, a process that is governed by the *arnBCADTEF* operon (2). In contrast, *A. baumannii* uses phosphoethanolamine (PEtN) as the moiety to modify lipid A through a phosphoethanolamine transferase encoded by *pmrC* (3). In either of these pathways, the result is clinically relevant levels of colistin resistance (i.e., MICs of 4 μg/ml or higher).

Colistin resistance in patients usually occurs with exposure to this agent and was considered to be a strictly chromosomally mediated process (4–6). This paradigm changed when we reported the first plasmid-mediated colistin resistance mechanism in 2015 (7). Based on the observation that colistin resistance was transferable from some *Escherichia coli* strains of swine origin to *E. coli* laboratory strains, we identified *mcr-1*, a 1,626-bp gene with moderate sequence identity to known phosphoethanolamine transferase genes. The introduction of *mcr-1* to *E. coli* resulted in resistance to colistin by the addition of PEtN to lipid A. In addition to swine and poultry *E. coli* strains, the report also identified *mcr-1* in several colistin-resistant *E. coli* and *K. pneumoniae* strains of human origin (7). Subsequently, *mcr-1* has been identified in colistin-resistant strains of animal and human origins worldwide, indicating that this gene is widely disseminated. In addition to *E. coli* and *K. pneumoniae*, *mcr-1*-mediated colistin resistance has been found in *Salmonella enterica*, *Enterobacter cloacae*, and *Enterobacter aerogenes*, all

TABLE 1 MICs of *mcr-1*-positive strains used in the study

Strain	PCR for *mcr-1*	MIC (μg/ml)	Fold change
E. coli ATCC 25922	−	0.125	32
E. coli ATCC 25922(pMQ124-*mcr-1*)	+	4	
E. coli YD626	−	0.125	16
E. coli YD626(pMQ124-*mcr-1*)	+	2	
K. pneumoniae 2210291	−	0.125	32
K. pneumoniae 2210291(pMQ124-*mcr-1*)	+	4	
K. pneumoniae ATCC 13883	−	0.125	256
K. pneumoniae ATCC 13883(pMQ124-*mcr-1*)	+	32	
A. baumannii ATCC 17978	−	0.25	64
A. baumannii ATCC 17978(pMQ124XLAB1-*mcr-1*)	+	16	
A. baumannii SM1536	−	1	>128
A. baumannii SM1536(pMQ124XLAB1-*mcr-1*)	+	>128	
A. baumannii D773	−	0.25	>128
A. baumannii D773(pMQ124XLAB1-*mcr-1*)	+	>128	
P. aeruginosa TRPA179	−	2	4
P. aeruginosa TRPA179(pMQ124-*mcr-1*)	+	8	
P. aeruginosa 8542455	−	0.5	2
P. aeruginosa 8542455(pMQ124-*mcr-1*)	+	1	
P. aeruginosa ATCC 47085	−	0.5	4
P. aeruginosa ATCC 47085(pMQ124-*mcr-1*)	+	2	

of which belong to the family *Enterobacteriaceae* (8). A major concern from the epidemiological perspective is the potential for *mcr-1* to spread into health care-associated extensive drug-resistant (XDR) pathogens, including *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, which would lead to truly untreatable infections. The goal of this study was to investigate the impacts of *mcr-1* on colistin resistance and on lipopolysaccharide structure in these species.

RESULTS

***mcr-1* confers colistin resistance in *E. coli*, *K. pneumoniae*, and *A. baumannii* and confers reduced susceptibility in *P. aeruginosa*.** *mcr-1*-carrying recombinant plasmids were successfully introduced and maintained in the laboratory and clinical strains tested. In all species tested, colistin MICs were elevated upon the introduction of *mcr-1* (Table 1). Colistin MICs increased by 16- to 32-fold in *E. coli*, 32- to 256-fold in *K. pneumoniae*, 64- to >128-fold in *A. baumannii*, and 2- to 4-fold in *P. aeruginosa*.

Lipid A is modified by the addition of phosphoethanolamine in all species examined. To investigate structural changes conferred by the phosphoethanolamine transferase activity of MCR-1, we isolated lipid A from wild-type (WT) and *mcr-1*-expressing strains and performed matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS). PEtN addition can be tracked by MS using the known mass for PEtN, which is determined by its structure (molecular weight [MW], 141; the addition to lipid A corresponds to a Δ*m/z* of 123 in negative-ion mode accounting for dehydration between PEtN and the lipid A phosphate moiety). All of the predicted lipid A structures were accessible to analysis in negative-ion mode, and therefore only negative-ion mode MS using the lipid matrix norharmane was performed. The resulting spectra were used to estimate the lipid A structures present in each strain, again based on their predicted structures and molecular weights.

***E. coli*.** *E. coli* expressing *mcr-1* contains lipid A with a PEtN addition (*m/z* 1,919), which is in agreement with the lipid A structures previously described by Liu et al. (Fig. 1; see also Fig. S1 in the supplemental material) (7). MS analysis also confirmed wild-type lipid A structures (*m/z* 1,796) in WT and plasmid-only strains *E. coli* YD626 and ATCC 25922 (Fig. 1; see also Fig. S1 and S3). This canonical structure is bis-phosphorylated and hexa-acylated, with acyl chains of 12 to 14 carbons in length (Fig. 1C). Mass differences of *m/z* 28 (*m/z* 1,768 and 1,824) were assigned to acyl chain heterogeneity, namely, in fatty-acid-chain length (a change of *m/z* 28 in negative-ion mode corresponds to a two-carbon change, for example, a change from a 12-carbon to

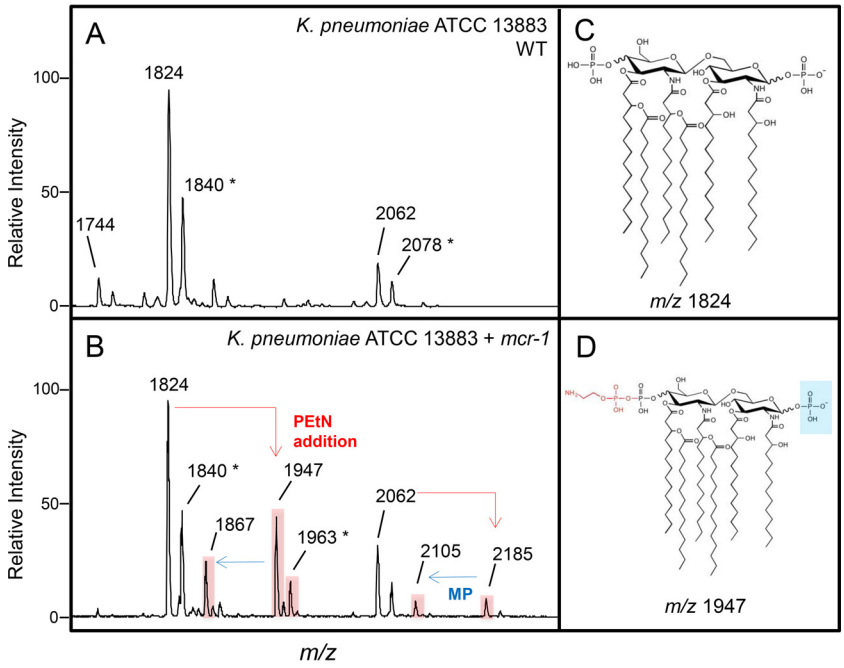

FIG 2 MS analysis of WT *K. pneumoniae* strain ATCC 13883 (A) and the strain expressing *mcr-1* (B). PEtN-modified species are represented in red (*m/z* 1,867, 1,947, 1,963, 2,105, and 2,185). Proposed structures of WT (C) and PEtN-modified (D) *K. pneumoniae* lipid A. Location of PEtN is suggestive; blue represents the lost phosphate moiety and monophosphate (MP) lipid A structures are designated with blue arrows. Asterisk (*) represents lipid A structure that varies in acyl chain length or hydroxylation status.

a 14-carbon acyl chain). Peak "clusters" differing by 28 *m/z* units were found across all samples and species and were therefore attributed to acyl chain heterogeneity independent of *mcr-1* activity. These differences are noted with asterisks in each figure. The peak at *m/z* 1,716 represents the canonical lipid A structure with a monophosphate group, annotated in blue (Fig. 1D). The loss of the phosphate moiety is suggested to be due to the nature of the extraction method, in which the labile phosphate bonds are susceptible to cleavage. Alternatively, it could represent a biosynthetic intermediate, as both the inner and the outer membranes are captured during the extraction process, and lipid A biosynthesis occurs across both membranes (9). A complete list of predicted lipid A structures for all strains can be found in Table S1 in the supplemental material.

***K. pneumoniae*.** *K. pneumoniae*, also a member of the *Enterobacteriaceae* family, has likewise been reported to have *mcr-1*-mediated colistin resistance (7, 10, 11). MIC analysis of *K. pneumoniae* strains ATCC 13883 and 2210291 containing the *mcr-1* plasmid showed increases in colistin resistance by 256- and 32-fold, respectively, suggesting the presence of PEtN-modified lipid A species in these isolates. Wild-type *K. pneumoniae* lipid A contains a 14-carbon acyl chain at the 3 position of the 3′ chain, compared with a 12-carbon acyl chain in the canonical *E. coli* lipid A structure (Fig. 2C). As expected, MS analysis revealed lipid A structures with PEtN modification in *mcr-1*-expressing strains ATCC 13883 and 2210291 (Fig. 2; see also Fig. S1). *mcr-1*-expressing ATCC 13883 showed PEtN modifications on mono- and bis-phosphorylated species (*m/z* 1,867 and *m/z* 1,947 and 1,963, respectively). Interestingly, only the monophosphorylated lipid A structures were observed to have PEtN additions (*m/z* 1,867 and 1,883) in the 2210291 strain when *mcr-1* was present. The loss of the phosphate moiety could be due to the nature of the extraction method, as previously discussed. The observed *m/z* variation of 28 units is attributed to acyl chain length heterogeneity, as described previously and annotated with an asterisk. Also of note is *m/z* 2,062, which represents lipid A modified with palmitate (MW, 238), a 16-carbon acyl chain. The outer membrane lipid A biosynthetic enzyme, PagP, is responsible for the transfer of palmi-

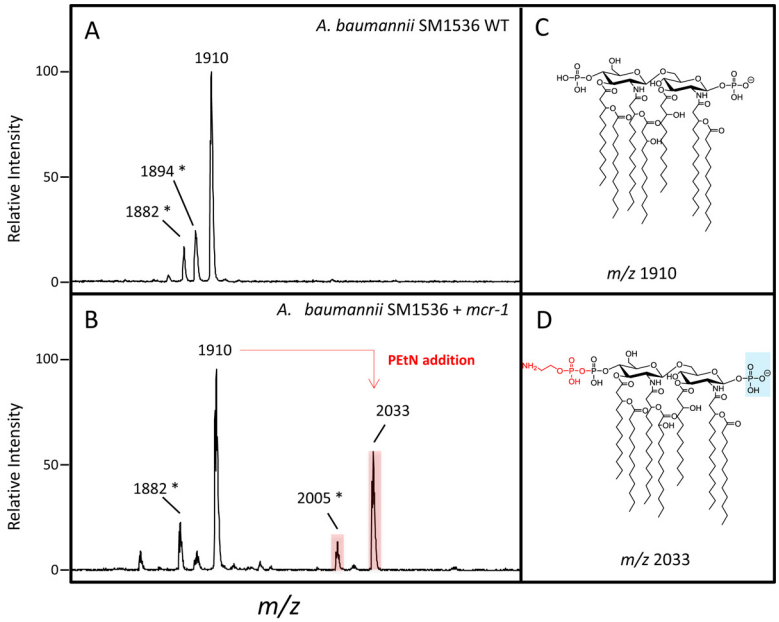

FIG 3 MS analysis of WT *A. baumannii* strain SM1536 (A) and the strain expressing *mcr-1* (B). PEtN-modified species are represented in red (*m/z* 2,005 and 2,033). Proposed structures of WT (C) and PEtN-modified (D) *A. baumannii* lipid A. Location of PEtN is suggestive; blue represents the lost phosphate moiety. Asterisk (*) represents lipid A structure that varies in acyl chain length or hydroxylation status.

tate from outer membrane phospholipids to lipid A (12). This palmitate-containing hepta-acylated lipid A species was also observed to be modified with PEtN in strain ATCC 13883 expressing *mcr-1* (*m/z* 2,185 and 2105) (Fig. 2B). Palmitate-modified lipid A was observed in both WT and plasmid-only strains of *K. pneumoniae* and is therefore not thought to be influenced by the presence or activity of *mcr-1*.

***A. baumannii*.** *A. baumannii* strains ATCC 17978, SM1536, and D773 transformed with *mcr-1* had increased colistin resistance. ATCC 17978 had a 64-fold increase, while strains SM1536 and D773 had increases of greater than 128-fold (Table 1). We predicted PEtN additions on lipid A would be observed in these strains due to their colistin resistance. The wild-type lipid A structure was confirmed in both untransformed and plasmid-only *A. baumannii* strains (Fig. 3A; see also Fig. S2A and S4A and B). This structure is hepta-acylated, with acyl chains ranging from 12 to 14 carbons in length (*m/z* 1,910) (Fig. 3C). In strains SM1536 and ATCC 17978, PEtN-modified bis-phosphorylated hepta-acylated lipid A species were observed (*m/z* 2,033) (Fig. 3; see also Fig. S2). Monophosphorylated PEtN-modified lipid A species (*m/z* 1,953) were observed in strain ATCC 17978 (Fig. S2). Furthermore, the monophosphorylated non-PEtN-modified lipid A species (*m/z* 1,830) was seen in the *mcr-1*-expressing strain of ATCC 17978 but not in the wild-type strain. Mass differences of *m/z* 28 and *m/z* 16 were observed in both WT and *mcr-1*-expressing *A. baumannii* strains, including plasmid-only control strains. As previously described, differences of *m/z* 28 can be attributed to heterogeneity in acyl chain length. Differences of *m/z* 16 correspond to changes in the hydroxylation status of the acyl chains, a common alteration in Gram-negative bacteria (13). As these acyl chain variations were observed in WT and plasmid-only control strains, they were not attributed to the presence of *mcr-1*. PEtN-modified lipid A was also observed in strain D773 (data not shown).

***P. aeruginosa*.** Only moderate changes in colistin susceptibility were observed in *mcr-1*-expressing *P. aeruginosa* strains 8542455, TRPA179, and ATCC 47085 (2- or 4-fold increase), yet PEtN-modified lipid A was still readily detected. Notably, all *P. aeruginosa* strains with *mcr-1* showed PEtN-modified bis- and monophosphorylated lipid A species (*m/z* 1,569 and 1,489, respectively) (Fig. 4; see also Fig. S2) (data not shown for ATCC

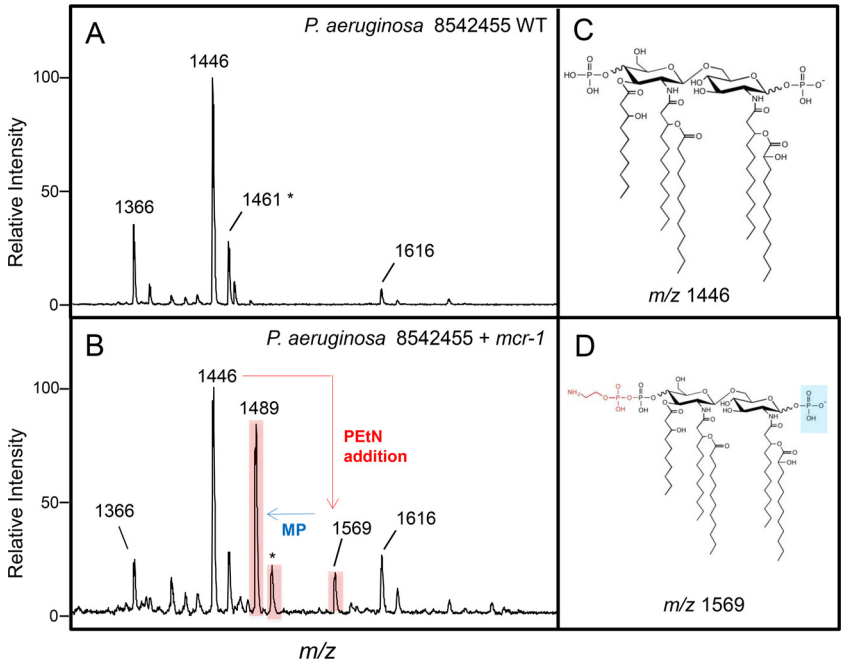

FIG 4 MS analysis of WT *P. aeruginosa* strain 8542455 (A) and the strain expressing *mcr-1* (B). PEtN-modified species are represented in red (*m/z* 1,568 and 1,488). Proposed structures of WT (C) and PEtN-modified (D) *P. aeruginosa* lipid A. Location of PEtN is suggestive; blue represents the lost phosphate moiety and monophosphate (MP) lipid A structure is designated with a blue arrow. Asterisk (*) represents lipid A structure that varies in acyl chain length or hydroxylation status.

47085). The wild-type lipid A structure is penta-acylated (Fig. 4C) but is synthesized in a mechanism that involves hexa- and hepta-acylated lipid A intermediates (14). This hexa-acylated intermediate (*m/z* 1,616) is observed in all strains of *P. aeruginosa* (including WT and plasmid-only control strains) (Fig. 4A; see also Fig. S2C and S4C and D) and is not attributed to the presence of *mcr-1*. Drug-resistant strains of *P. aeruginosa* have been reported to have 4-amino-4-deoxy-L-arabinose (L-Ara4N)-modified lipid A structures, which mediate resistance to cationic antibiotics such as polymyxins in a way similar to that by PEtN (2). This type of lipid A modification was not observed in any of the *P. aeruginosa* strains tested, reinforcing the idea that *mcr-1* specifically encodes PEtN lipid A modification and not general resistance modifications.

DISCUSSION

Colistin is currently one of the last lines of defense against XDR Gram-negative bacteria, and resistance to colistin (or polymyxin B, which differs from colistin by a single amino acid and demonstrates comparable activity) in these organisms can result in pandrug resistance (PDR) (15). Colistin resistance in XDR bacteria mostly develops upon the treatment of infection with this agent and is an imminent threat in intensive care settings. For example, over 40% of *K. pneumoniae* strains producing *K. pneumoniae* carbapenemase (KPC)-type carbapenemase in Italian hospitals are reportedly resistant to colistin (16). However, resistance to colistin outside this context had largely been overlooked until the first plasmid-mediated colistin resistance gene, *mcr-1*, was reported from animals and humans in China in the fall of 2015 (7). *mcr-1* encodes a phosphoethanolamine transferase and its expression leads to the addition of the phosphoethanolamine moiety to lipid A. Since this first report, over 150 articles and letters have been published on *mcr-1*. It is now clear that it has existed undetected for decades, has a worldwide distribution, is found more commonly in food animals than in humans (especially in *E. coli*), and its prevalence is on the rise where data are available. Furthermore, a second plasmid-mediated colistin resistance gene, *mcr-2*, which shares 76.7% nucleotide identity with *mcr-1*, was recently reported from Belgium

(17). Fortunately, the majority, if not all, of *mcr-1*-carrying strains are still susceptible to other commonly used antimicrobial agents, and mortality due to the failure of colistin treatment for infections caused by *mcr-1*-expressing strains has not been reported.

Our objectives for this study were to determine the impacts of *mcr-1* expression on the level of colistin resistance and on the lipid A structure in three ESKAPE species against which colistin is utilized clinically the most, namely, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. Of those tested, both laboratory and clinical strains from all four species were observed to have *mcr-1*-mediated increases in colistin resistance, as determined by MIC, and PEtN-modified lipid A. This supports the previous findings in *E. coli* that *mcr-1* mediates its effects through lipid A modification, namely, by masking the negatively charged phosphates, thereby reducing the affinity of cationic substances such as colistin for the bacterial membrane. It also indicates that the native promoter of *mcr-1* found in *E. coli* (18) is functional across the four species tested. *K. pneumoniae* and *A. baumannii* likewise showed correlative changes from colistin susceptibility to colistin resistance at a clinically relevant range (16 to 32 μg/ml) and lipid A structural modification. This suggests *K. pneumoniae* and *A. baumannii* would be amenable to *mcr-1* plasmid-mediated colistin resistance. Interestingly, we observed only modest changes in *P. aeruginosa* susceptibility to colistin despite the presence of PEtN-modified lipid A. This finding suggests that the acquisition of *mcr-1* alone may not confer clinically relevant colistin resistance in *P. aeruginosa*, at least with expression from the native promoter of *mcr-1*. However, it is still possible that the modest rise in MIC may negatively impact the activity of colistin against *mcr-1*-expressing *P. aeruginosa* considering the suboptimal pharmacokinetics of this agent (19). Another caveat of this modest resistance observed in *P. aeruginosa* is that traditional susceptibility-based surveillance assays intended to detect the presence of *mcr-1* may not prove effective for all bacterial species. Furthermore, the recently reported emergence of *mcr-2*, which only has moderate sequence identity to *mcr-1*, indicates that PCR-based surveillance methods may likewise have limitations (17). Given the prevalence of these pathogens as threats to human health and the risk *mcr-1*-expressing strains pose to effectively treating bacterial infection, the development of effective surveillance techniques is crucial.

In conclusion, our data demonstrate that *mcr-1* is capable of conferring variable levels of resistance to colistin in ESKAPE Gram-negative pathogens and consistently results in phosphoethanolamine-modified lipid A in medically pervasive bacterial strains. This highlights the threat that *mcr-1* may disseminate into already difficult-to-manage XDR organisms, in particular, *A. baumannii*.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* TOP10 was used as the recipient strain for the cloning of *mcr-1* into various vectors. *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 17978, and *P. aeruginosa* ATCC 47085 were the laboratory strains used as the recipients of the recombinant plasmids. In addition, the following carbapenem-resistant clinical strains were used as the recipients: *E. coli* YD626, *K. pneumoniae* 2210291, *A. baumannii* SM1536, *A. baumannii* D773, *P. aeruginosa* TRPA179, and *P. aeruginosa* 8542455. All the clinical strains except *A. baumannii* D773 were clinical strains previously identified at the University of Pittsburgh Medical Center. *A. baumannii* D733 was a kind gift from Carl Urban at the New York Hospital Presbyterian/Queens.

Cloning vector pBCSK was used for the initial cloning of *mcr-1*. Shuttle vectors pMQ124 (20) and pMQ124XLAB1 (a derivative of pMQ124 possessing the replicon of *Acinetobacter* plasmid pWH1266) were used to introduce *mcr-1* to *P. aeruginosa* and *A. baumannii*, respectively.

Cloning of *mcr-1* and MIC measurements. Primers *mcr-1*-EcoRI (5′-CGAATTCCGAAGCACCAAGACA TCAA-3′) and *mcr-1*-XbaI (5′-GCTCTAGAATACGGCATAACAAACCCC-3′) were used to amplify an approximately 2-kb fragment containing *mcr-1* and its native promoter from pHNSHP45 and ligated to pBCSK. Electrocompetent *E. coli* TOP10 was transformed with either this construct, pBCSK-*mcr-1*, or pBCSK vector control and selected on a lysogeny broth (LB) agar plate containing chloramphenicol at 30 μg/ml. The sequence of this 2-kb fragment was confirmed by Sanger sequencing and further ligated to pMQ124 and pMQ124XLAB1. The pMQ124-*mcr-1* construct was used to transform *E. coli*, *K. pneumoniae*, and *P. aeruginosa* strains, whereas the pMQ124XLAB1-*mcr-1* construct was used to transform *A. baumannii* strains, all by electroporation. All strains were transformed with empty vectors as controls. Cells were plated on LB agar plates containing gentamicin at 50 μg/ml. The introduction of *mcr-1* was confirmed by PCR using primers *mcr-1*-F (5′-TCCAAAATGCCCTACAGACC-3′) and *mcr-1*-R (5′-GCCACCACAGGCAGT AAAAT-3′) in all instances.

Colistin sulfate was purchased from Sigma-Aldrich (St. Louis, MO). MICs of colistin were determined by the recommended ISO-standard broth microdilution method stipulated by the joint Clinical and Laboratory Standards Institute-EUCAST Polymyxin Breakpoints Working Group. *E. coli* ATCC 25922 was used as the control strain.

Lipid A structural analysis. Lipid A was extracted from cell pellets using an ammonium hydroxide-isobutyric acid-based procedure (21). Briefly, approximately 5 ml of cell culture was pelleted and resuspended in 400 μl of 70% isobutyric acid and 1 M ammonium hydroxide (5:3 [vol/vol]). Samples were incubated for 1 h at 100°C and centrifuged at 2,000 $\times$ *g* for 15 min. Supernatants were collected, added to endotoxin-free water (1:1 [vol/vol]), snap-frozen on dry ice, and lyophilized overnight. The resultant material was washed twice with 1 ml methanol, and lipid A was extracted using 100 μl of a mixture of chloroform, methanol, and water (3:1:0.25 [vol/vol/vol]). Once extracted, 1 μl of the concentrate was spotted on a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) plate followed by 1 μl of 10 mg/ml norharmane matrix in chloroform-methanol (2:1 [vol/vol]) (Sigma-Aldrich, St. Louis, MO) and then was air dried. All samples were analyzed on a Bruker Microflex mass spectrometer (Bruker Daltonics, Billerica, MA) in the negative-ion mode with reflectron mode. An electrospray tuning mix (Agilent, Palo Alto, CA) was used for mass calibration. Spectral data were analyzed with Bruker Daltonics FlexAnalysis software. The resulting spectra were used to estimate the lipid A structures present in each strain based on their predicted structures and molecular weights. Structural diversity of lipid A within a single bacterial membrane is well-described (22, 23).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00580-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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REFERENCES

1. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev 30:557–596. https://doi.org/10.1128/CMR.00064-16.
2. Moskowitz SM, Ernst RK, Miller SI. 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J Bacteriol 186:575–579. https://doi.org/10.1128/JB.186.2.575-579.2004.
3. Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. Antimicrob Agents Chemother 55:3370–3379. https://doi.org/10.1128/AAC.00079-11.
4. Pelletier MR, Casella LG, Jones JW, Adams MD, Zurawski DV, Hazlett KR, Doi Y, Ernst RK. 2013. Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. Antimicrob Agents Chemother 57:4831–4840. https://doi.org/10.1128/AAC.00865-13.
5. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, Vatopoulos A, Rossolini GM, COLGRIT Study Group. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob Agents Chemother 58:5696–5703. https://doi.org/10.1128/AAC.03110-14.
6. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Hoiby N, Moskowitz SM. 2011. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 55:5761–5769. https://doi.org/10.1128/AAC.05391-11.
7. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168. https://doi.org/10.1016/S1473-3099(15)00424-7.
8. Schwarz S, Johnson AP. 2016. Transferable resistance to colistin: a new but old threat. J Antimicrob Chemother 71:2066–2070. https://doi.org/10.1093/jac/dkw274.
9. Whitfield C, Trent MS. 2014. Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem 83:99–128. https://doi.org/10.1146/annurev-biochem-060713-035600.
10. Gu DX, Huang YL, Ma J H, Zhou HW, Fang Y, Cai JC, Hu YY, Zhang R. 2016. Detection of colistin resistance gene *mcr-1* in hypervirulent *Klebsiella pneumoniae* and *Escherichia coli* isolates from an infant with diarrhea in China. Antimicrob Agents Chemother 60:5099–5100. https://doi.org/10.1128/AAC.00476-16.
11. Rolain JM, Kempf M, Leangapichart T, Chabou S, Olaitan AO, Le Page S, Morand S, Raoult D. 2016. Plasmid-mediated *mcr-1* gene in colistin-resistant clinical isolates of *Klebsiella pneumoniae* in France and Laos. Antimicrob Agents Chemother 60:6994–6995. https://doi.org/10.1128/AAC.00960-16.
12. Bishop RE, Gibbons HS, Guina T, Trent MS, Miller SI, Raetz CR. 2000. Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria. EMBO J 19:5071–5080. https://doi.org/10.1093/emboj/19.19.5071.
13. MacArthur I, Jones JW, Goodlett DR, Ernst RK, Preston A. 2011. Role of *pagL* and *lpxO* in *Bordetella bronchiseptica* lipid A biosynthesis. J Bacteriol 193:4726–4735. https://doi.org/10.1128/JB.01502-10.
14. Ernst RK, Adams KN, Moskowitz SM, Kraig GM, Kawasaki K, Stead CM, Trent MS, Miller SI. 2006. The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. J Bacteriol 188:191–201. https://doi.org/10.1128/JB.188.1.191-201.2006.
15. Paterson DL, Harris PN. 2016. Colistin resistance: a major breach in our last line of defence. Lancet Infect Dis 16:132–133. https://doi.org/10.1016/S1473-3099(15)00463-6.
16. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Kleb-*

siella pneumoniae: a rapidly evolving problem in Italy, November 2013 to April 2014. Euro Surveill 19:pii=20939. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20939.
17. Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. Euro Surveill 21:pii=30280. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=22525.
18. Poirel L, Kieffer N, Brink A, Coetze J, Jayol A, Nordmann P. 2016. Genetic features of MCR-1-producing colistin-resistant *Escherichia coli* isolates in South Africa. Antimicrob Agents Chemother 60:4394–4397. https://doi.org/10.1128/AAC.00444-16.
19. Bergen PJ, Landersdorfer CB, Lee HJ, Li J, Nation RL. 2012. 'Old' antibiotics for emerging multidrug-resistant bacteria. Curr Opin Infect Dis 25:626–633. https://doi.org/10.1097/QCO.0b013e328358afe5.
20. Shanks RM, Kadouri DE, MacEachran DP, O'Toole GA. 2009. New yeast recombineering tools for bacteria. Plasmid 62:88–97. https://doi.org/10.1016/j.plasmid.2009.05.002.
21. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res 46:1773–1778. https://doi.org/10.1194/jlr.D500014-JLR200.
22. Dixon DR, Darveau RP. 2005. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. J Dent Res 84:584–595. https://doi.org/10.1177/154405910508400702.
23. Shaffer SA, Harvey MD, Goodlett DR, Ernst RK. 2007. Structural heterogeneity and environmentally regulated remodeling of *Francisella tularensis* subspecies *novicida* lipid A characterized by tandem mass spectrometry. J Am Soc Mass Spectrom 18:1080–1092. https://doi.org/10.1016/j.jasms.2007.03.008.