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
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

![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.](http://aac.asm.org/)

FIG 1
of which belong to the family Enterobacteriaceae (8). A major concern from the epidemiological perspective is the potential for mcr-1 to spread into healthcare-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 Δm/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 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PCR for mcr-1</th>
<th>MIC (µg/ml)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>-</td>
<td>0.125</td>
<td>32</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922(pMQ124-mcr-1)</td>
<td>+</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> YD626</td>
<td>-</td>
<td>0.125</td>
<td>16</td>
</tr>
<tr>
<td><em>E. coli</em> YD626(pMQ124-mcr-1)</td>
<td>+</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 2210291</td>
<td>-</td>
<td>0.125</td>
<td>32</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 2210291(pMQ124-mcr-1)</td>
<td>+</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 13883</td>
<td>-</td>
<td>0.125</td>
<td>256</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 13883(pMQ124-mcr-1)</td>
<td>+</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em> ATCC 17978</td>
<td>-</td>
<td>0.25</td>
<td>64</td>
</tr>
<tr>
<td><em>A. baumannii</em> ATCC 17978(pMQ124XLAB1-mcr-1)</td>
<td>+</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em> SM1536</td>
<td>-</td>
<td>1</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> TRPA179</td>
<td>-</td>
<td>2</td>
<td>4</td>
</tr>
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<td>+</td>
<td>8</td>
<td></td>
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<tr>
<td><em>P. aeruginosa</em> BS42455</td>
<td>-</td>
<td>0.5</td>
<td>2</td>
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<tr>
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<td>+</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 47085</td>
<td>-</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 47085(pMQ124-mcr-1)</td>
<td>+</td>
<td>2</td>
<td></td>
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LPS Modification by mcr-1 in ESKAPE Pathogens

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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-
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 \textit{mcr}-1 (m/z 2,185 and 2105) (Fig. 2B). Palmitate-modified lipid A was observed in both WT and plasmid-only strains of \textit{K. pneumoniae} and is therefore not thought to be influenced by the presence or activity of \textit{mcr}-1.

\textit{A. baumannii}. \textit{A. baumannii} strains ATCC 17978, SM1536, and D773 transformed with \textit{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 \textit{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 \textit{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 \textit{mcr}-1-expressing \textit{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 \textit{mcr}-1. PEtN-modified lipid A was also observed in strain D773 (data not shown).

\textit{P. aeruginosa}. Only moderate changes in colistin susceptibility were observed in \textit{mcr}-1-expressing \textit{P. aeruginosa} strains 8542455, TRPA179, and ATCC 47085 (2- or 4-fold increase), yet PEtN-modified lipid A was still readily detected. Notably, all \textit{P. aeruginosa} strains with \textit{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...
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.
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 repilcon 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′-GCTCTAGATACCGGATAAACAACCCC-3′) were used to amplify an approximately 2-kb fragment containing mcr-1 and its native promoter from pHN5HP4S 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′-TCCAAAAATGCCCTACAGACC-3′) and mcr-1-R (5′-GCCACCCAGCCAGT 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 × 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 to endotoxin-free water (1:1 [vol/vol]), snap-frozen on dry ice, and lyophilized overnight. The resultant material was then resuspended in 1 ml of a mixture of chloroform, methanol, and water (3:1:0.25 [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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