CXC Chemokines Exhibit Bactericidal Activity against Multidrug-Resistant Gram-Negative Pathogens

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ABSTRACT The continued rise and spread of antimicrobial resistance among bacterial pathogens pose a serious challenge to global health. Countering antimicrobial-resistant pathogens requires a multifaceted effort that includes the discovery of novel therapeutic approaches. Here, we establish the capacity of the human CXC chemokines CXCL9 and CXCL10 to kill multidrug-resistant Gram-negative bacteria, including New Delhi metallo-beta-lactamase-1-producing Klebsiella pneumoniae and colistin-resistant members of the family Enterobacteriaceae that harbor the mobile colistin resistance protein MCR-1 and thus possess phosphoethanolamine-modified lipid A. Colistin-resistant K. pneumoniae isolates affected by genetic mutation of the PmrA/PmrB two-component system, a chromosomally encoded regulator of lipopolysaccharide modification, and containing 4-amino-4-deoxy-L-arabinose-modified lipid A were also found to be susceptible to chemokine-mediated antimicrobial activity. However, loss of PhoP/PhoQ autoregulatory control, caused by disruption of the gene encoding the negative regulator MgrB, limited the bactericidal effects of CXCL9 and CXCL10 in a variable, strain-specific manner. Cumulatively, these findings provide mechanistic insight into chemokine-mediated antimicrobial activity, highlight disparities amongst determinants of colistin resistance, and suggest that chemokine-mediated bactericidal effects merit additional investigation as a therapeutic avenue for treating infections caused by multidrug-resistant pathogens.

IMPORTANCE As bacterial pathogens become resistant to multiple antibiotics, the infections they cause become increasingly difficult to treat. Carbapenem antibiotics provide an essential clinical barrier against multidrug-resistant bacteria; however, the dissemination of bacterial enzymes capable of inactivating carbapenems threatens the utility of these important antibiotics. Compounding this concern is the global spread of bacteria invulnerable to colistin, a polymyxin antibiotic considered to be a last line of defense against carbapenem-resistant pathogens. As the effectiveness of existing antibiotics erodes, it is critical to develop innovative antimicrobial therapies. To this end, we demonstrate that the chemokines CXCL9 and CXCL10 kill the most concerning carbapenem- and colistin-resistant pathogens. Our findings provide a
unique and timely foundation for therapeutic strategies capable of countering antibiotic-resistant “superbugs.”

KEYWORDS Gram negative, antimicrobial resistance, carbapenem, chemokine, colistin

Antimicrobial-resistant bacterial pathogens are a serious threat to global health. Infections caused by multidrug-resistant (MDR) organisms have limited therapeutic options, resulting in frequent treatment failures and increased mortality rates (1). Carbapenem antibiotics have provided a clinical barrier against MDR Gram-negative pathogens; however, the continued emergence and spread of carbapenemases, β-lactamase enzymes capable of hydrolyzing and inactivating carbapenems, imperil the utility of these key antibiotics. Carbapenem-resistant Enterobacteriaceae (CRE), including carbapenemase-producing Klebsiella spp. and Escherichia coli, are resistant to nearly all classes of antibiotics. Consequently, CRE are listed by the U.S. Centers for Disease Control and Prevention as an immediate threat to public health that requires urgent and aggressive action (2).

The challenge posed by CRE has been countered by the use of colistin (CST), a decades-old polymyxin regarded as a last line of defense against highly drug-resistant Gram-negative pathogens. Unfortunately, extensive application of CST in animal husbandry has provided selective pressure for the development of CST resistance. Especially concerning is the dissemination of mcr-1, a plasmid-borne determinant of polymyxin resistance, among human pathogens (3). CST resistance arising from genetic alterations of chromosomal loci (e.g., mgrB and pmrB) has also been reported (4); however, unlike mcr-1, these altered genes are not transmissible between bacterial species. The acquisition of CST resistance by CRE highlights the emergence of extensively drug-resistant “superbugs” that are invulnerable to most, and in some cases all, available antibiotics.

The mounting human and economic burden of MDR bacteria, compounded by steep declines in the development of new antimicrobial agents (2), underscores the importance of developing novel therapeutic strategies. Previous work by our laboratory and others suggests antimicrobial chemokines as one possible avenue to counter the threat posed by antibiotic resistance (5, 6). Indeed, while best known for orchestrating immune cell recruitment to sites of infection, a subset of chemokines has been shown to exert antimicrobial activity against a range of microorganisms (7, 8). Here we report that the CXC chemokines CXCL9 and CXCL10 kill MDR bacterial pathogens, including CRE and CST-resistant clinical isolates.

RESULTS AND DISCUSSION

CXC chemokines exhibit bactericidal effects against CRE. The capacity of CXC chemokines to directly kill CRE was examined by using clinical isolates of MDR Klebsiella pneumoniae that possess either New Delhi metallo-β-lactamase-1 (NDM-1) or both NDM-1 and oxacillinase-48 (OXA-48) carbapenemases (Fig. 1A). NDM-1 presents a considerable challenge to antimicrobial therapy because of its high enzymatic and genetic stability (9, 10) and exceptional horizontal mobility among Gram-negative bacterial species (11). On the basis of prior investigations (12), CRE were treated with 48 μg/ml CXCL10. Viability determinations (CFU/ml) demonstrated that CXCL10 killed all of the MDR carbapenemase-producing organisms tested (Fig. 1B). Indeed, CXCL10-mediated killing of CRE was greater than that of the carbapenemase-negative control K. pneumoniae ATCC 43816. Human CXCL9 also efficiently killed CRE (Fig. 1C).

Chemokine-mediated antimicrobial activity against CRE was dependent on the chemokine concentration used (Fig. 1D). The half-maximal effective concentration (EC$_{50}$) of CXCL10 for isolate BL12125 was 9.6 ± 1.1 μg/ml (~1.1 μM). This value is comparable to the previously determined bactericidal capacity of human CXCL10 against Bacillus anthracis bacilli (EC$_{50}$, 0.5 μM) and E. coli (EC$_{50}$, 0.3 to 1.0 μM) and is also similar to those reported for the killing of K. pneumoniae by human (EC$_{50}$, 0.3 μM) and

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murine (EC50, 1.6 μM) CCL28 (5, 12, 13). Our findings demonstrate that CXCL9 and CXCL10 kill carbapenem-resistant bacterial isolates that also maintain broad co-resistance against additional classes of antibiotics. Given the increasingly limited options available to treat infections caused by MDR bacterial pathogens, this novel observation highlights the potential utility of chemokine-mediated antimicrobial activity as a foundation for developing innovative therapeutic strategies to counter a range of antibiotic-resistant pathogens.

**CXC chemokines mediate antimicrobial activity against mcr-1-harboring bacteria.** CST is a cationic lipopeptide that targets Gram-negative bacteria through electrostatic interactions with phosphate groups on the lipid A component of lipopolysaccharide (14). While the genetic basis of CST resistance is diverse, the principal mechanisms are analogous; chemical modification of lipid A phosphates mask their negative charge, thereby decreasing affinity between CST and the bacterial cell envelope (4). Most concerning among determinants of CST resistance is mcr-1, a horizontally transmissible, plasmid-borne gene that encodes a phosphoethanolamine (pEtN) transferase that catalyzes the addition of pEtN onto phosphate moieties of lipid A (3). MCR-1 has been shown to reduce CST susceptibility in a number of important human pathogens, including *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (15).

To define the capacity of CXCL9 and CXCL10 to kill mcr-1+ CST-resistant bacteria, three *E. coli* isolates were used, FI-4531, an *mcr-1*+ clinical isolate; J53, a laboratory reference strain; and J53 Δmcr-1, a J53 transconjugant carrying the *mcr-1*-containing plasmid from FI-4531. The CST resistance of *mcr-1*+ isolates was confirmed using a modified Etest (Fig. 2A). To verify the presence or absence of pEtN-modified lipid A in the outer membrane of the above bacterial strains (Fig. 2B; see Fig. S1 in the supplement).
mental material), lipid extracts from each were prepared and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Fig. 2C). The canonical, unmodified bis-phosphorylated hexa-acylated base peak was observed in all spectra. Major ions at m/z 1,919 and 1,839, corresponding to pEtN-modified lipid A and pEtN-modified lipid A lacking the second phosphate moiety, were detected only in spectra generated from mcr-1⁺ isolates. E. coli isolates were treated with 4 or 12 µg/ml CXCL10 (D) or CXCL9 (E); K. pneumoniae strains were treated with 48 µg/ml CXCL10 (F). Different chemokine concentrations were assayed on the basis of the inherent susceptibilities of these organisms. Survival was measured by CFU determination (limit of detection, 500 CFU/ml; n.d., none detected). Data are expressed as percentages of the respective untreated-control value and represent the mean ± the standard error of the mean (n = 3). ns, not significant.

FIG 2 Chemokine-mediated antimicrobial activity against mcr-1⁺ bacteria. (A) CST susceptibility testing of E. coli by modified Etest. CST concentrations are in µg/ml. Images are representative of two or three separate tests. Printed CO indicates a CST test strip. (B) Molecular structures and mass-to-charge (m/z) ratios of signature ions identified in E. coli mass spectra. (C) Lipid extracts were analyzed by MALDI-TOF mass spectrometry. The unmodified base peak (m/z 1,796) was observed in all spectra. Major ions at m/z 1,919 and 1,839, corresponding to pEtN-modified lipid A and pEtN-modified lipid A lacking the second phosphate moiety, were detected only in spectra generated from mcr-1⁺ isolates. E. coli isolates were treated with 4 or 12 µg/ml CXCL10 (D) or CXCL9 (E); K. pneumoniae strains were treated with 48 µg/ml CXCL10 (F). Different chemokine concentrations were assayed on the basis of the inherent susceptibilities of these organisms. Survival was measured by CFU determination (limit of detection, 500 CFU/ml; n.d., none detected). Data are expressed as percentages of the respective untreated-control value and represent the mean ± the standard error of the mean (n = 3). ns, not significant.
against the E. coli strains examined (Fig. 2E). Furthermore, introduction of a mcr-1-carrying recombinant plasmid into CST-susceptible K. pneumoniae ATCC 13883 did not promote resistance to chemokine-mediated antimicrobial activity (Fig. 2F), despite conferring CST resistance (MIC, 32 μg/ml) (15). These data demonstrate that CXCL9 and CXCL10 are able to effectively kill mcr-1-mediated CST-resistant pathogens that maintain pEtN-modified lipid A in their outer membrane.

Disparate chromosomal determinants of CST resistance differentially impact the bactericidal activity of CXC chemokines. Similar to pEtN, the modification of lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N) reduces the net charge of the bacterial cell envelope, thereby promoting CST resistance. The PmrA/PmrB two-component regulatory system governs the expression of chromosomal loci that encode the enzymes responsible for L-Ara4N synthesis and incorporation into lipid A (16). Discrete, nonsynonymous mutations in pmrA or pmrB are associated with the constitutive activation of this signaling pathway and CST resistance (4). Particular mutations in PhoP/PhoQ, an upstream activator of the response regulator PmrA (17), and genetic disruption of MgrB, a negative regulator of PhoP/PhoQ signaling (18), also result in the activation of PmrA and CST-resistant phenotypes (4, 19). While not horizontally transmissible, these determinants have been identified in a number of human pathogens and present an emergent clinical challenge (19).

To test the effects of chromosomal determinants of CST resistance on chemokine-mediated antimicrobial activity, we examined MDR, mcr-1 negative, CST-resistant K. pneumoniae clinical isolates (Fig. 3A). Bacteria were treated with 48 μg/ml CXCL10, and survival was measured by CFU determination (Fig. 3B). Curiously, CXCL10 exhibited strain-specific bactericidal activity against the isolates examined, i.e., three susceptible

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**FIG 3** Effects of chromosomal determinants of CST resistance on the bactericidal activity of CXC chemokines. (A) MLSTs, carbapenemase genes, and antimicrobial susceptibilities of CST-resistant K. pneumoniae isolates are shown. MICs [μg/ml] were interpreted as resistant (red), intermediate (yellow), or susceptible (green) on the basis of established breakpoints. For definitions of abbreviations, see the legend to Fig. 1. Bacteria were treated with 48 μg/ml CXCL10 (B) or CXCL9 (C), and survival was measured by CFU determination. Data are expressed as percentages of the respective untreated-control value and represent the mean ± the standard error of the mean (n = 3). **, P < 0.01; ***; P < 0.001; ns, not significant (compared to ATCC 43816 [panel B] or the untreated control [panel C]). (D) Visualization of mgrB amplicons generated by PCR from the isolates indicated. Amplicon size of intact K. pneumoniae mgrB, 235 bp. Markers indicate 1,000 and 200 bp.
isolates (<25% survival; BA2664, BL8800, and BA2880), three intermediate isolates (25 to 75% survival; MS84, BA3783, and BU19801), and one unaffected isolate (BL849). The intermediate phenotype displayed by isolate MS84 was also observed upon treatment with CXCL9 (Fig. 3C). MALDI-TOF analysis demonstrated that all of the bacterial isolates examined possessed similar relative amounts of l-Ara4N-modified lipid A in their outer membrane (Fig. S2); thus, the reduction in anionic surface charge afforded by l-Ara4N modification of lipid A does not appear to explain the differences in susceptibility to chemokine-mediated antimicrobial activity. This observation is consistent with our finding that pEtN modification of lipid A also does not reduce susceptibility to CXCL9 or CXCL10. Taken together, these results support the notion that CXCL9 and CXCL10 kill bacterial pathogens via a mechanism distinct, at least in part, from those reliant upon electrostatic interaction as proposed for a number of cationic antimicrobial peptides, including CST.

We next examined the possibility that disparate chromosomal determinants conferring CST resistance account for the noted variability in CXCL10 susceptibility. To identify the specific mutations/disruptions that underlie CST resistance in the above clinical isolates, five loci (mgrB, pmrA, pmrB, phoP, and phoQ) previously reported to be associated with CST-resistant phenotypes were examined by amplicon sequencing and analysis of whole-genome sequence data (4, 20). All of our CST-resistant, CXCL10-susceptible K. pneumoniae isolates (BA2664, BL8800, and BA2880) possessed a nonsynonymous pmrB point mutation resulting in a Thr157Pro amino acid substitution. This particular substitution has been proposed to impact dimerization and phosphotransferase by PmrB, leading to constitutive activation of PmrA and, consequently, CST resistance (21). In contrast, CST-resistant organisms that exhibited partial or complete resistance to CXCL10 were found to possess mgrB loci disrupted by insertion sequences (MS84, IS3-like element; BA3783, IS1-like element; BU19801, ISKpn25 element) or to be missing the mgrB gene entirely (BL849) (Fig. 3D). Functional inactivation of mgrB eliminates the negative feedback loop governing PhoP/PhoQ signaling, resulting in both direct and indirect (via PmrD-dependent activation of PmrA) transcriptional activation of the biosynthetic machinery necessary for l-Ara4N modification of lipid A in K. pneumoniae (4, 22). No isolates were found to have genetic aberrations previously associated with CST resistance in the pmrA, phoP, or phoQ loci.

That impairment of PhoP/PhoQ autoregulatory control curtails the antimicrobial activity of CXCL10, but direct disruption of PmrA/PmrB does not, reasonably suggests that disparate chromosomal determinants of CST resistance inequitably affect CXCL10 susceptibility. Indeed, while constitutive activation of PmrA, either directly or indirectly, would be expected to result in the incorporation of l-Ara4N-modified lipid A into the bacterial outer membrane, disruption of PhoP/PhoQ autoregulation would also be expected to influence the expression and/or activity of a number of additional enzymes and regulatory systems central to defensive alteration of the bacterial outer membrane (23). These findings highlight the underappreciated prospect that distinct chromosomal determinants of CST resistance differentially impact outer membrane physiology and thus variably affect microbial traits beyond CST resistance.

**Restoration of PhoP/PhoQ autoregulatory control abolishes protection from CXCL10.** To substantiate that mgrB disruption and the resultant loss of PhoP/PhoQ autoregulation account for reduced CXCL10 susceptibility, we performed genetic complementation and susceptibility determination. For these experiments, native K. pneumoniae mgrB was introduced into isolate MS84. This MDR isolate was chosen for mgrB complementation since it demonstrated reduced CXCL10 susceptibility yet was still susceptible to TET and thus amenable to screening selection.

Complementation of isolate MS84 with pACYC::mgrB restored CST susceptibility (Fig. 4A), elicited the loss of l-Ara4N-modified lipid A (Fig. 4B and C), and markedly increased susceptibility to CXCL10 (Fig. 4D). In contrast, the empty-vector control was equivalent to the MS84 parent strain in all of the regards examined. These data were validated by using an additional set of three K. pneumoniae isolates, KKBO1, KKBO4, and KKBO4 mgrB. KKBO1 and KKBO4 are sequentially collected, K. pneumoniae carbapen-
emase (KPC)-producing clinical isolates that are CST susceptible or resistant, respectively. The CST resistance of KKBO4 has previously been shown to be caused by insertional inactivation of \textit{mgrB} \textsuperscript{(24)}. Genetic complementation with pACYC::\textit{mgrB} reestablished CST susceptibility in KKBO4 (Fig. 4E) and eliminated L-Ara4N-modified lipid A (Fig. S2). Determination of chemokine susceptibility demonstrated that only \textit{K. pneumoniae} isolates harboring intact \textit{mgrB} alleles were fully sensitive to the bactericidal effects of CXCL10 (Fig. 4F). These observations demonstrate that diminished CXCL10 susceptibility is associated with the functional inactivation of \textit{mgrB}.

A pair of additional observations can be made from the lipid A mass spectra presented in this work. In \textit{K. pneumoniae}, the PhoP/PhoQ-activated loci \textit{lpxO} and \textit{pagP} encode lipid A-modifying enzymes responsible for the hydroxylation of 2′-linked

**FIG 4** Effect of \textit{mgrB} complementation on resistance phenotypes. (A) CST susceptibility testing of \textit{K. pneumoniae} strains by modified E test. CST concentrations are in \textmu g/ml. Images are representative of three separate tests. (B) Molecular structures and \textit{m/z} values of selected ions identified in \textit{K. pneumoniae} mass spectra. (C) MALDI-TOF analysis demonstrated the canonical \textit{K. pneumoniae} lipid A base peak \( (\textit{m/z} 1,824) \). Major ions at 1,855 \( (\textit{m/z} 1,824 + \text{L-Ara4N}) \), 1,971 \( (\textit{m/z} 1,840 + \text{L-Ara4N}) \), and 1,891 \( (\textit{m/z} 1,971 - \text{PO}_3^-) \) were observed in mass spectra from strains harboring disrupted \textit{mgrB}. Additional ions observed at \textit{m/z} 1,840, 2,063, and 2,079 are indicative of lipid A hydroxylation, palmitoylation, or both, respectively. (D) Bacteria were treated with 48 \textmu g/ml CXCL10. Survival was measured by CFU counting (limit of detection, 500 CFU/ml; n.d., none detected). Data are expressed as percentages of the respective untreated-control value and represent the mean ± the standard error of the mean \((n = 3)\). (E) CST susceptibility testing by modified E test. Images are representative of two or three separate tests. (F) Bacteria were treated with 48 \textmu g/ml CXCL10. Survival was measured by CFU counting (limit of detection, 500 CFU/ml; n.d., none detected). Data are expressed as percentages of the respective untreated control and represent the mean ± the standard error of the mean \((n = 3)\).
myristate (LpxO) and the addition of palmitate (C16:0) onto the hydroxyl group of hydroxymyristate (PagP) (25). The reinforcement of hydrogen bond networks by hydroxylated lipid A and the strengthening of hydrophobic interactions by palmitoylated lipid A are proposed to fortify outer membrane barrier function and impede penetration by antimicrobial peptides. Although hydroxylation and palmitoylation of lipid A have each been reported to promote bacterial resistance to antimicrobial peptides (26, 27), the presence of these PhoP/PhoQ-regulated modifications did not appear to account for CXCL10 resistance in isolates harboring disruptions in mgrB. This conclusion is evidenced by the detection of 2-hydroxylated lipid A (12/12 isolates) and palmitoylated lipid A (6/12 isolates) in both CXCL10-susceptible and -resistant K. pneumoniae strains (Fig. 4; Fig. S2).

Further investigation is required to clarify the molecular basis of PhoP/PhoQ-dependent protection from the bactericidal effects of CXCL9 and CXCL10 and to determine whether this limitation can be overcome. Of interest, K. pneumoniae isolate BU19801 demonstrated mgrB disruption yet was only marginally resistant to CXCL10 (Fig. 3). This phenotype is distinct from the pronounced CXCL10 resistance exhibited by other clinical isolates harboring interrupted mgrB and may provide an opportunity to elucidate the molecular basis of protection from CXCL10.

While it is unknown whether antimicrobial chemokines act through a common mechanism, they are generally considered to target bacteria via electrostatic interactions between positively charged topological “patches” present on the chemokine and negatively charged constituents of the bacterial cell envelope, notably, the lipid A component of lipopolysaccharide in Gram-negative bacteria (7, 28). This basic mechanism is analogous to those proposed for a number of cationic antimicrobial peptides, including CST, and is in agreement with many experimental results (6, 29). However, pEtN or l-Ara4N modification of lipid A phosphate moieties and consequent reduction of the bacterial surface charge were not found to specifically diminish the bactericidal effects of CXCL9 and CXCL10. These findings suggest that factors other than charge-based interactions may be critical for the bactericidal action of these CXC chemokines. This notion is supported by previous findings that CXCL10 may interact directly with the conserved bacterial component FtsX, which displays homology with CXCR3, the cognate cellular receptor of CXCL10 (30, 31). Elucidation of the underlying principles of chemokine-mediated antimicrobial activity will inform the development of innovative strategies for treating infections caused by antimicrobial-resistant pathogens.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial isolates used in this study and their derivatives are summarized in Table 1. MDR K. pneumoniae clinical isolates BL849 (CFSAN044563), BU19801 (CFSAN044564), MS84 (CFSAN044565), BL71215 (CFSAN044566), BL12456 (CFSAN044568), BA3783 (CFSAN044569), BL13802 (CFSAN044570), BA2664 (CFSAN044571), BL8800 (CFSAN044572), and BA2880 (CFSAN044573) were obtained in Pakistan (20). K. pneumoniae ATCC 43816 was used as a species-matched control for phenotypic assays. Sequence typing of the above isolates was performed and assignments were made in accordance with the Institut Pasteur K. pneumoniae multilocus sequence typing scheme (http://bigdb.pasteur.fr/). K. pneumoniae KKBO1 and KKBO4 (24), and E. coli FI-4531 were obtained in Italy (32). E. coli J53 and K. pneumoniae ATCC 13883 were used as host backgrounds for mcr-1-containing plasmids. E. coli DH5α was used as an intermediate in molecular cloning. Positive control strains for antimicrobial susceptibility testing included Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 29212, E. coli ATCC 25922, and P. aeruginosa ATCC 27853. Unless otherwise noted, bacteria were cultured in Luria-Bertani (LB) medium at 37°C with continuous shaking. E. coli FI-4531 and J53 mcr-1 were grown in the presence of 1 µg/ml CST freshly prepared. K. pneumoniae MS84 derivatives and KKBO4 mcrB, each harboring pACYC constructs, were grown in the presence of 25 µg/ml TET. K. pneumoniae strains derived from ATCC 13883 and containing pBCS3 constructs were grown in the presence of 50 µg/ml chloramphenicol.

Antimicrobial susceptibility testing and carbapenemase gene detection. Antimicrobial resistance was first established with the Vitek 2 system (BioMérieux, Marcy l’Etoile, France). broth microdilution, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (33), was subsequently used to determine the MICs of antibiotics representing the main families of antimicrobial agents, i.e., ampicillin, amoxicillin-clavulanic acid, AMP-sulbactam, piperacillin-tazobactam, cefazolin, cefoxitin, cefotaxime, ceftriaxone, aztreonam, ceftazidime, ertapenem, doripenem, imipenem, meropenem, CST, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, TET, TGC, and trimethoprim-sulfamethoxazole. Antimicrobial susceptibilities were interpreted on the basis of 2016 CLSI breakpoints (34),
then resuspended in 200 μl of membrane lipids. Water, frozen on dry ice, and lyophilized overnight. The resulting dry pellets contained whole-cell extracts remove cell debris, the supernatants were transferred to fresh tubes, combined in a 1:1 ratio of distilled incubated at 100°C for 30 to 45 min. After centrifugation (2,000 relative centrifugal force for 15 min) to K. pneumoniae of BL12125 MDR, NDM-1 *, CST * 20 BL12456 MDR, NDM-1 *, CST * 20 BL13802 MDR, NDM-1 *, OXA-48 *, CST * 20 ATCC 13883 Reference/recipient, CST * ATCC 13883 + vector pBCSK, CST * 15 ATCC 13883 mcr-1 pBCSK::mcr-1, CST * 15 MS84 MDR, NDM-1 *, CST * (mgrB disruption) 20 MS84 + vector pACYC, CST * 20 MS84 mgrB pACYC::mgrB, CST * This study BL849 MDR, NDM-1 *, CST * (mgrB disruption) 20 BA3783 MDR, NDM-1 *, OXA-48 *, CST * (mgrB disruption) 20 BA2664 MDR, OXA-48 *, CST * (pmrB Thr157Pro) 20 BU19801 MDR, NDM-1 *, CST * (mgrB disruption) 20 BL8800 MDR, OXA-48 *, CST * (pmrB Thr157Pro) 20 BA2880 MDR, OXA-48 *, CST * (pmrB Thr157Pro) 20 KKB01 MDR, KPC *, CST * 24 KKB04 MDR, KPC *, CST * (mgrB disruption) 24 KKB04 mgrB pACYC::mgrB, CST * 24 *ATCC, American Type Culture Collection, Manassas, VA, USA.

except those for CST and TGC, which are not defined by the CLSI. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for CST and TGC (35). The presence of genes encoding the NDM-1 and OXA-48 carbapenemases was determined by PCR amplification (36). CST Etest. While CST resistance was established by broth microdilution, the maintenance of resistant phenotypes was confirmed with a modified Etest. Standardized inocula were prepared by diluting overnight cultures 1:35 (vol/vol) in sterile LB broth and incubating them at 37°C with shaking. When an optical density at 600 nm (OD600) of 0.6 was reached, a 1.5-ml volume was removed and centrifuged at 16,000 relative centrifugal force for 1 min. The supernatant was discarded, and 7 μl of wet pellet was transferred into 5 ml of 0.9% sterile saline in a 15-ml glass tube. The suspensions were adjusted to a bacterial concentration equivalent to a 1.0 McFarland turbidity standard and inoculated onto LB agar with sterile polyester-tipped swabs. Plates were allowed to dry for 10 min prior to the application of CST Etest strips (BioMérieux). Sample plates were imaged after 20 h of incubation at 37°C. K. pneumoniae Etest results not presented here are included in Fig. S3.

Chemokine treatment. Recombinant human CXCL9 and CXCL10 were purchased from PeproTech (Rocky Hill, NJ) and reconstituted at 1 mg/ml in distilled water containing 0.3% human serum albumin. Overnight bacterial cultures were used to inculcate sterile LB medium at an OD600 of 0.1 to 0.2. Subcultures were incubated at 37°C with continuous shaking until an OD600 of 0.6 was attained. Bacteria were then diluted to a concentration of 3 × 10^6 CFU/ml in 10 mM potassium phosphate buffer (pH 7.4) supplemented with 1% trypticase soy broth and treated with the chemokine indicated or an equal volume of 0.3% human serum albumin (untreated control) in the wells of a 96-cluster plate (12). After 2 h of incubation at 37°C, serial dilutions were prepared and inoculated onto LB agar plates that were incubated overnight at room temperature prior to colony enumeration.

Lipid A isolation from whole cells. Membrane lipids were extracted, and lipid A isolated, by an optimized small-scale hot ammonium isobutyrate-based protocol (37). Briefly, bacteria were streaked onto LB agar to obtain individual colonies. From these colonies, cultures were prepared in 5 ml of LB medium following overnight incubation at 37°C with continuous shaking. Bacteria were harvested by centrifugation (4,000 relative centrifugal force for 10 min) and pellets were treated with a 5:3 mixture of 70% (vol/vol) isobutyric acid and 1 M ammonium hydroxide (400-μl total volume). Samples were then incubated at 100°C for 30 to 45 min. After centrifugation (2,000 relative centrifugal force for 15 min) to remove cell debris, the supernatants were transferred to fresh tubes, combined in a 1:1 ratio of distilled water, frozen on dry ice, and lyophilized overnight. The resulting dry pellets contained whole-cell extracts of membrane lipids.

MALDI-TOF mass spectrometry. Dry lipid extracts were washed twice with 1 ml of methanol and then resuspended in 200 μl of a 2:1:0.25 chloroform-methanol-water solvent mixture. Aliquots of 1 μl each of norharmane matrix (10 mg/ml in 2:1 chloroform-methanol) and then analyte were spotted directly onto stainless steel target plates in duplicate. Mass spectra were recorded in negative-ion mode with a Bruker Microflex LRIF MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) operated in reflectron mode. The instrument was equipped with a 337-nm nitrogen laser, and analyses were performed at 39.5% global intensity. Typically, 900 laser shots were summed to acquire each spectrum and at least three mass spectra were acquired per spot. Electrospray tuning mix (Agilent, Palo Alto, CA).
was used for mass calibration. Data were acquired and processed with flexControl and flexAnalysis

version 3.4 (Bruker Daltonics Inc.). Mass spectra were smoothed by a Savitzky-Golay filter, and the
baseline was corrected with TopHat. Molecular structures and m/z values of major ions identified in E. coli and K. pneumoniae lipid A mass spectra are presented in Fig. S1. K. pneumoniae lipid A mass spectra not presented here are in Fig. S2.

**Genetic complementation and molecular cloning.** Complementation of isolate MS84 with mgrB

was accomplished as previously reported for genetic complementation in KKBO4 (24). Briefly, native

mgrB and flanking regions were amplified from the genomic DNA of isolate BL12125 by high-fidelity PCR

with primers Aval mgrB_F1 (5′-GACTAGCTCGGGAACAGTGTGGAAACAAATCGATGGATT-3′) and Eco-

RI mgrB_R1 (5′-CTAGTCGAACTCACCACCTCAAGAGAAAGCGGT-3′). PCR mixtures were prepared with

Phusion DNA polymerase and incubated at 98°C for 30 s prior to 32 cycles of 98°C for 10 s, 62°C for 15 s, and

and 72°C for 20 s, followed by a final extension at 72°C for 7 min. The mgrB amplicon was purified with the

MinElute PCR purification kit (Qiagen, Hilden, Germany) and sequence verified. The

mgrB insert was cloned into pACYC184 (New England Biolabs, Ipswich, MA) following double restriction digestion of each

with Aval and EcoRI-HF, and ligation with T4 DNA ligase. The resulting construct, pACYC::mgrB, was electroporated into E. coli DH5α.

Following sequence validation, pACYC::mgrB was transformed into freshly prepared, electrocompetent MS84 cells (1.8 kV, 200 Ω, 25 μF). An empty-vector control strain was also generated by transformation.

The introduction of mcr-1 from isolate FI-4531 into E. coli JS3 (F− met pro A2i [azide resistant]) was accomplished by conjugation at a donor/recipient ratio of 1:10 (38). Bacteria were incubated at 35°C for 16 h, and transconjugants were selected on Mueller-Hinton agar supplemented with CST (2 μg/ml) and sodium azide (150 μg/ml). Transfer of the mcr-1 resistance determinant was confirmed by PCR. For mcr-1 expression by K. pneumoniae ATCC 13883, the mcr-1 gene and its native promoter were amplified by PCR and cloned into pBCSK. The generation of ATCC 13883 harboring pBCSktmc-1 or the empty vector alone has been described previously (15).

**Examination of chromosomal loci associated with CST resistance.** PCR amplification of chromosomal loci was performed by using primer pairs and cycling conditions previously reported for phoP, phoQ, pmrA, and pmrB (21) or for mcrB (19). PCR amplicons were visualized by gel electrophoresis, and the nucleotide sequences determined were compared to those of K. pneumoniae reference strains of the same sequence type to identify mutations/disruptions. The status of phoP/phoQ, pmrA/pmrb, and mcrB maintained by the 10 MDR K. pneumoniae isolates from Pakistan was also examined by using available draft whole-genome sequences available under accession numbers MAG000000000 MAGE0000000000 MAMG0000000000 (20). The genome of K. pneumoniae HS11286 was used for comparison (39). Where appropriate, the absence of mcr variants was confirmed on assembled genomes with CLC Genomics Workbench 9.5.4 by using the reference sequences NG_050417.1

and MAG0000000000 (mcr1). Isolates from Pakistan was also examined by using available draft whole-genome sequences available under accession numbers MAG0000000000 MAG0000000000 (20). The genome of K. pneumoniae HS11286 was used for comparison (39). Where appropriate, the absence of mcr variants was confirmed on assembled genomes with CLC Genomics Workbench 9.5.4 by using the reference sequences NG_050417.1

and MAG0000000000 (mcr1). Insertion sequences were identified with ISfinder (40). For K. pneumoniae isolate BL849, no mcrB sequence or flanking regions could be identified by either in silico

PCR or read mapping.

**Statistical analysis.** Statistical analysis and graphing were performed with GraphPad Prism 7

software; a P value of <0.05 was considered significant. Statistically significant differences among

treatment groups were determined by one-way analysis of variance with Bonferroni’s multiple-
comparison test or, when only two groups were experimentally assayed, an unpaired Student’s t test. The

EC50 of CXCL10-mediated bactericidal activity was determined by using the sigmoidal dose-response

equation of nonlinear regression and is presented as the EC50 and the 95% confidence interval.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01549-17.

**FIG S1, TIF file, 0.8 MB.**

**FIG S2, TIF file, 0.7 MB.**

**FIG S3, TIF file, 1.6 MB.**

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REFERENCES

1. Zilberberg MD, Shorr AF, Micek ST, Vazquez-Guillamet C, Kollef MH. 2014. Multi-drug resistance, inappropriate initial antibiotic therapy and mortality in Gram-negative severe sepsis and septic shock: a retrospective cohort study. Crit Care 18:596. https://doi.org/10.1186/s13054-014-0056-8.

2. Ventola CL. 2015. The antibiotic resistance crisis: part 1: causes and threats. P T 40:277–283.

3. Liu Y-Y, Wang Y, Walsh TR, Yi I-L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, 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.

4. 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.

5. Crawford MA, Zou Y, Green CS, Burdick MD, Sanz P, Alem F, O’Brien AD, Mehrad B, Strieter RM, Hughes MA. 2009. Antimicrobial effects of interferon-inducible CXC chemokines against Bacillus anthracis spores and bacilli. Infect Immun 77:1664–1678. https://doi.org/10.1128/IAI.01208-08.

6. Yung SC, Murphy PM. 2012. Antimicrobial chemokines. Front Microbiol 3:276. https://doi.org/10.3389/fmicb.2012.00276.

7. Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, Oppenheim JJ. 2003. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. J Leukoc Biol 74:448–453. https://doi.org/10.1189/jlb.1013024.

8. Crawford MA, Burdick MD, Glomski IJ, Boyer AE, Barr JR, Mehrad B, Strieter RM, Hughes MA. 2010. Interferon-inducible CXC chemokines directly contribute to host defense against inhalational anthrax in a murine model of infection. PLoS Pathog 6:e1001199. https://doi.org/10.1371/journal.ppat.1001199.

9. González Lí J, Bahr G, Nakashige TG, Nolan EM, Bonomo RA, Vila AJ. 2016. Membrane anchoring stabilizes and favors secretion of New Delhi metallo-β-lactamase. Nat Chem Biol 12:516–522. https://doi.org/10.1038/nchembio.2083.

10. Paul D, Bhattacharjee A, Bhattacharjee D, Dhar M, Mauya AP, Chakravarty A. 2017. Transcriptional analysis of bioN-DNM-1 and copy number alteration under carbapenem stress. Antimicrob Resist Infect Control 6:26. https://doi.org/10.1186/s41376-017-0183-2.

11. Dorrert L, Poirel L, Nordmann P. 2014. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. Biomed Res Int 2014:249856. https://doi.org/10.1155/2014/249856.

12. Schutte KM, Fisher DJ, Burdick MD, Mehrad B, Mathers AJ, Mann BJ, Nakamoto RK, Hughes MA. 2015. Escherichia coli pyruvate dehydrogenase complex is an important component of CXCL10-mediated antimicrobial activity. Infect Immun 83:320–328. https://doi.org/10.1128/IAI.00552-15.

13. Hieshima K, Ohntani H, Shibano M, Izawa D, Nakayama T, Kawasaki Y, Shiba F, Shiota M, Katou F, Saito T, Yoshie O. 2003. CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. J Immunol 170:1452–1461. https://doi.org/10.4049/jimmunol.170.3.1452.

14. Yu Z, Qin W, Lin J, Fang S, Qiu J. 2015. Antibacterial mechanisms of polymyxin and bacterial resistance. Biomed Res Int 2015:679109. https://doi.org/10.1155/2015/679109.

15. Liu Y, Chandler CE, Leung LM, McElheny CL, Mettus RT, Shankis RMQ, Liu JH, Goodlett DR, Ernst RK, Doi Y. 2017. Structural modification of lipopolysaccharide conferred by mcr-1 in Gram-negative bacteria. Antimicrob Agents Chemother 61:e00580-17. https://doi.org/10.1128/AAC.00580-17.

16. Gunn JS. 2008. The Salmonella PmrA/PmrB regulon: Lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol 16:284–290. https://doi.org/10.1016/j.tim.2008.03.007.

17. Kato A, Groisman EA. 2004. Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. Genes Dev 18:2302–2313. https://doi.org/10.1101/gad.1230804.

18. Lippa AM, Goulian M. 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet 5:e1000798. https://doi.org/10.1371/journal.pgen.1000798.

19. Cannatelli A, Giani T, D’Andrea MM, DI Pilato V, Arena F, Conte V, Tryfino-poulou K, Vatopoulos A, Rossolini GM, COLGIRIT Study Group. 2014. mcr-1 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.

20. Crawford MA, Timme R, Lomonaco S, Lascols C, Fisher DJ, Sharma SK, Strain E, Allard MW, Brown EW, McFarland MA, Croley T, Hammad M, Weigel LM, Anderson K, Hodge DR, Pillai SP, Morse SA, Khan E, Hughes MA. 2016. Genome sequences of multidrug-resistant, colistin-susceptible and -resistant Klebsiella pneumoniae clinical isolates from Pakistan. Genome Announc 4:e01419-16. https://doi.org/10.1128/genomeA.01419-16.

21. Jayal A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrA among Klebsiella pneumoniae isolates of worldwide origin. Antimicrob Agents Chemother 58:4762–4766. https://doi.org/10.1128/AAC.00084-14.

22. Mitropohanov AY, Jevew MW, Hadley TJ, Groisman EA. 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. PLoS Genet 4:e1000233. https://doi.org/10.1371/journal.pgen.1000233.

23. Zwill J, Shin D, Kato A, Nishino K, Latifi T, Solomon F, Hare HM, Huang C, Groisman EA. 2005. Dissecting the PhoP regulatory network of Escherichia coli and Salmonella enterica. Proc Natl Acad Sci U S A 102:2862–2867. https://doi.org/10.1073/pnas.0408238102.

24. Cannatelli A, D’Andrea MM, Giani T, Di Pilato V, Arena F, Abbrecht S, Gailbani P, Rossolini GM. 2013. In vivo emergence of colistin resistance in Klebsiella pneumoniae producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgb8 regulator. Antimicrob Agents Chemother 57:5521–5526. https://doi.org/10.1128/AAC.01480-13.

25. Needham BD, Trent MS. 2013. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol 11:467–481. https://doi.org/10.1038/nrmicro3047.

26. Llobet E, Martinez-Molina V, Moranda D, Dahlström KM, Regueiro V, Tomás A, Cano V, Pérez-Gutierrez C, Frank CG, Fernández-Carrasco A, Insua JL, Salmena TA, Garmedia J, Bongoeea JA. 2015. Deciphering tissue-induced Klebsiella pneumoniae lipid A structure. Proc Natl Acad Sci U S A 112:E6369–E6378. https://doi.org/10.1073/pnas.1508831112.

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27. Guo L, Lim KB, Poduje CM, Daniel M, Gunn JS, Hackett M, Miller SI. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95:189–198. https://doi.org/10.1016/S0092-8674(00)81750-X.

28. Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389–395. https://doi.org/10.1038/415389a.

29. Ageitos JM, Sánchez-Pérez A, Calo-Mata P, Villa TG. 2017. Antimicrobial peptides (AMPs): ancient compounds that represent novel weapons in the fight against bacteria. Biochem Pharmacol 133:117–138. https://doi.org/10.1016/j.bcp.2016.09.018.

30. Crawford MA, Lowe DE, Fisher DJ, Stibitz S, Plaut RD, Beaber JW, Zemansky J, Mehrad B, Glomski IJ, Strieter RM, Hughes MA. 2011. Identification of the bacterial protein FtsX as a unique target of chemokine-mediated antimicrobial activity against Bacillus anthracis. Proc Natl Acad Sci USA 108:17159–17164. https://doi.org/10.1073/pnas.1108495108.

31. Margulieux KR, Fox JW, Nakamoto RK, Hughes MA. 2016. CXCL10 acts as a bifunctional antimicrobial molecule against Bacillus anthracis. mBio 7:e00334-16. https://doi.org/10.1128/mBio.00334-16.

32. Cannatelli A, Giani T, Antonelli A, Principe L, Luzzaro F, Rossolini GM. 2016. First detection of the mcr-1 colistin resistance gene in Escherichia coli in Italy. Antimicrob Agents Chemother 60:3257–3258. https://doi.org/10.1128/AAC.00246-16.

33. CLSI. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standards, 10th ed. CLSI document m07-A10. CLSI, Wayne, PA.

34. CLSI. 2016. Performance standards for antimicrobial susceptibility testing; 26th informational supplement. CLSI document M100-S. CLSI, Wayne, PA.

35. EUCAST. 2016. Breakpoint tables for interpretation of MICs and zone diameters, version 6.0. EUCAST, Basel, Switzerland. http://www.eucast.org.

36. Lascols C, Hackel M, Marshall SH, Hujer AM, Bouchillon S, Badal R, Hoban D, Bonomo RA. 2011. Increasing prevalence and dissemination of NDM-1 metallo-β-lactamase in India: data from the SMART study (2009). J Antimicrob Chemother 66:1992–1997. https://doi.org/10.1093/jac/dkr240.

37. 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.

38. Di Pilato V, Arena F, Tascini C, Cannatelli A, Henrici De Angelis L, Fortunato S, Giani T, Menichetti F, Rossolini GM. 2016. mcr-1,2, a new mcr variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing Klebsiella pneumoniae strain of sequence type 512. Antimicrob Agents Chemother 60:5612–5615. https://doi.org/10.1128/AAC.01075-16.

39. Liu P, Li P, Jiang X, Bi D, Xie Y, Tai C, Deng Z, Rajakumar K, Ou HY. 2012. Complete genome sequence of Klebsiella pneumoniae subsp. pneumoniae HS11286, a multidrug-resistant strain isolated from human sputum. J Bacteriol 194:1841–1842. https://doi.org/10.1128/JB.00043-12.

40. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequence. Nucleic Acids Res 34:D32–D36. https://doi.org/10.1093/nar/gkj014.