Mechanistic insights into transferable polymyxin resistance among gut bacteria

Yongchang Xu†1, Jingxia Lin†1, Tao Cui§1, Swaminath Srinivas¶, and Youjun Feng‡1,2

From the †Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China, the §College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, China, the ¶Department of Life Sciences, Northwestern Polytechnical University, Xi’an, Shannxi 710072, China, and the ‡Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

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Polymyxins such as colistin are antibiotics used as a final line of defense in the management of infections with multidrug-resistant Gram-negative bacteria. Although natural resistance to polymyxins is rare, the discovery of a mobilized colistin resistance gene (mcr-1) in gut bacteria has raised significant concern. As an intramembrane enzyme, MCR-1 catalyzes the transfer of phosphoethanolamine (PEA) to the 1 (or 4′)-phosphate group of the lipid A moiety of lipopolysaccharide, thereby conferring colistin resistance. However, the structural and biochemical mechanisms used by this integral membrane enzyme remain poorly understood. Here, we report the modeled structure of the full-length MCR-1 membrane protein. Together with molecular docking, our structural and functional dissection of the complex of MCR-1 with its phosphatidyethanolamine (PE) substrate suggested the presence of a 12 residue-containing cavity for substrate entry, which is critical for both enzymatic activity and its resultant phenotypic resistance to colistin. More importantly, two periplasm-facing helices (PH2 and PH2′) of the trans-membrane domain were essential for MCR-1 activity. MALDI-TOF MS and thin-layer chromatography assays provide both in vivo and in vitro evidence that MCR-1 catalyzes the transfer of PEA from the PE donor substrate to its recipient substrate lipid A. Also, the chemical modification of lipid A species was detected in clinical species of bacteria carrying mcr-1. Our results provide mechanistic insights into transferable MCR-1 polymyxin resistance, raising the prospect of rational design of small molecules that reverse bacterial polymyxin resistance, as a last-resort clinical option to combat pathogens with carbapenem resistance.

Antibiotic resistance is becoming a global public health priority (1–3). Bacterial pathogens with multidrug resistance (MDR)§ are considered to be a leading challenge to global public health in that they result in over 700,000 deaths each year (1, 3). Colistin (referred to polymyxin E), a member of cationic antimicrobial peptides (CAMP), is used as a “last-resort” defense against serious infections caused by MDR-producing Gram-negative pathogens (5). However, it seems that its use as a final-line clinical option might be potentially disrupted by the emergence of transferable colistin resistance determinant MCR-1 (6, 7). Structural alterations of lipid A species anchored on bacterial lipopolysaccharide (LPS) are implicated in the resistance to polymyxins. So far, at least three distinct molecular mechanisms have been identified for the chemical modification of lipid A: first, phosphoethanolamine (PEA) is attached to the 1 (or 4′)-phosphate position of the lipid A glucosamine (GlcN) moieties (8, 9); second, the 1 (or 4′)-phosphate position of the lipid A GlcN moiety is modified with amino-arabinose (8, 10); third, glycine modification occurs at the 3′-linked secondary acyl chain of lipid A (11). This indicates a significant diversity in the machineries responsible for colistin resistance.

Very recently, a new mobilized colistin resistance gene (mcr-1) was discovered from the gut microbiota of human beings and animals in southern China (12). Given the efficient transfer of plasmid-borne mcr-1 via transposon-like genetic elements (13, 14), it is not surprising that mcr-1 has already disseminated into 40 countries covering 5 of the 7 continents (6). More than 10 species of Enterobacteriaceae have been detected to carry the mcr-1 gene, including Escherichia coli (15) and Klebsiella pneumoniae (16). Also, an unexpectedly rich diversity in the mechanisms has been found in the growth niches (and/or host reservoirs) of mcr-1-positive bacteria, like pigs (17, 18), meats

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This article contains Tables S1–S3 and Figs. S1–S12.

1 These authors contributed equally to this work.
2 Recipient of the “Young 1000 Talents” Award. To whom correspondence and requests for materials should be addressed: Dept. of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China. Tel.: 86-571-88208524; Fax: 86-571-88208524; E-mail: fengyj@zju.edu.cn.

3 The abbreviations used are: MDR, multidrug resistance; NBD-glycerol-(mcr-1)-3-PEA, 1-acetyl-2-(12-{(7-nitro-2-1,3-benzoxadiazol-4-yl) amino} dodecanoyl)-sn-glycerol-3-phosphoethanolamine; CAMP, cationic antimicrobial peptides; LPS, lipopolysaccharide; PEA, phosphoethanolamine; PE, phosphatidyethanolamine; GlcN, glucosamine; aa, amino acid(s); PPEA, phosphatethanolamine; TM, transmembrane; NBD, 12-(N-methy1-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); DDM, dodecyl-β-D-maltoside; TLC, thin-layer chromatography; PDB, Protein Data Bank; MIC, minimum inhibitory concentration; ICP-MS, inductively coupled plasma mass spectrometry; CAMHB, cation-adjusted Mueller–Hinton broth; LB, Luria–Bertani; LBA, Luria–Bertani (LB) agar; Ni-NTA, nickel-nitriolactric acid; NBD-PEA, 1-acetyl-2-(12-{(7-nitro-2-1,3-benzoxadiazol-4-yl) amino} dodecanoyl)-sn-glycerol-3-phosphoethanolamine; Kdoα, 3-deoxy-o-manno-octulosonic acid.

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To determine its enzymatic activity in vitro (Fig. 1A), the recombinant form of MCR-1 integral membrane protein was overexpressed in an E. coli expression system and purified by nickel-affinity chromatography in the presence of 1% detergent dodecyl-β-D-maltoside (DDM). Following gel filtration, the protein of interest was visualized by SDS-PAGE (Fig. 1B) and verified by MALDI-TOF mass spectrometry shows in vivo evidence that MCR-1 transfers the PEA moiety to its recipient, LPS-lipid A (34). Collectively, these results give us a more complete picture of the structure and mechanism for MCR-1 polymyxin resistance.

Results

Biochemical insights into MCR-1 catalysis

To further detect the in vivo transfer of PEA moiety from glycerol-3-PEA to the acceptor substrate LPS-lipid A (Fig. 1E), LPS-lipid A was prepared and purified from E. coli strains with/without the expression of mcr-1 (Fig. 1F). Unlike the negative control, MG1655, which has a single lipid A peak (m/z = 1797.356), MALDI-TOF mass spectrometry reveals two unique peaks in the mcr-1–harboring E. coli strain: a bis-phosphorylated hexa-acylated lipid A (m/z = 1797.416) and PPEA-1 (or 4′)-lipid A (m/z = 1920.501), a modified form with an additional PEA (m/z = 123) (Fig. 1G). This in vitro evidence when taken with the in vivo data demonstrates that MCR-1 catalyses proceeds by PEA transfer from the donor PE lipid substrate to
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Mechanism for transferable polymyxin resistance

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the receiver Kdo₂-lipid A, giving the two final products PEA-Kdo₂-lipid A and diacylglycerol (Fig. 1H). Given that observation of the Thr₁⁰⁰⁰-PEA enzyme adduct in EptA (34, 44) is quite similar to scenarios in alkaline phosphatase-type phosphate transferase (45), we thus speculate that a PEA-enzyme intermediate is probably released from the PE lipid molecule in the first half-reaction of MCR-1 catalysis (Fig. S1B). In the second half-reaction, PEA is transferred from a MCR-1-bound PEA adduct to the 1 (or 4’)-phosphate position of Kdo₂-lipid A GlcN moieties, generating PEA-Kdo₂-lipid A (Fig. S1C). It seems likely that MCR-1 might adopt a possible “ping-pong” reaction mechanism for enzymatic catalysis, similar to the proposal for EptA (34).

**Structure-guided functional dissection of MCR-1**

Overall structure of MCR-1 in full length was modeled using the structure of *N. meningitidis* EptA (Protein Data Bank entry 5FGN) as a template (34). The architecture consists of two discretely folded domains: an N-terminal TM region and a periplasm-facing catalytic domain (PEA transferase) at the C terminus (Fig. 2, A and B). These two domains are linked by an extended periplasmic loop and bridging helix (Fig. 2, A and B).

The TM domain—spanning bacterial inner membrane includes six α-helices (designated as TMH1, TMH1’, TMH2, TMH3, TMH4, and TMH5; Fig. 2A), oriented approximately in parallel to one another (Fig. 2B). Similar to NmEptA, the longest helix, TMH5, is the only one of six TM helices completely spanning the membrane, whereas all of the other five helices (TMH1, TMH1’, TMH2, TMH3, and TMH4) are buried in the membrane in that the length of helices seems to be less than the average width of the membrane bilayer (30 Å) (Fig. 2B). It is possible that the clustered positively charged residues on TMH5 (e.g. lysine) might be involved in cross-talking with the negatively charged headgroup of phospholipid on the surface of the inner membrane. Interestingly, two short periplasm-facing helices (PH2 and PH2’) are positioned on a long loop that connects TMH3 with TMH4 (Fig. 2, A and B), suggesting that they might be partially embedded in the membrane. Also, the linker between TMH4 and TMH5 is a short loop (Fig. 2A). Two more periplasmic helices that are amphipathic in nature (PH3 and PH4; Fig. 2B) are localized on a coiled loop between TMH5 and a bridge helix (Fig. 2A). Similar to the folding mode of NmEptA (34), the soluble catalytic domain also exhibits a hydrolase-like configuration comprising 10 α-helices (referred to as H1, H2, . . . H10) and 7 β-sheets (designated as S1, S2 . . . S7) (Fig. 2, A and B). Also, the presence of a long-coiled loop adjacent to the catalytic motif probably ensures better capture/binding of the lipid substrate via its flexible rotation/movement centering on the TM region fixed on the inner membrane.

To test whether the two domains of MCR-1 can be functionally replaced with their counterparts in EptA, we employed domain swapping to generate two hybrid versions of MCR-1/ EptA (referred to as TM1-EptA, a modified EptA whose TM region is replaced with TM1 of MCR-1, and TM-MCR-1, a chimeric version of MCR-1 in which the TM of EptA is present) (Fig. 3A). In agreement with our observation on LBA plates (31), the minimum inhibitory concentration (MIC) of colistin due to EptA (2 µg/ml) is less than that of MCR-1 (4 µg/ml). However, the two hybrid versions of MCR-1/EptA consistently exhibited a 0.25 µg/ml colistin MIC (identical to that of the colistin-susceptible strain MG1655, which served as a negative control) (Fig. 3B). This is in contrast to the results of domain swapping between MCR-1 and MCR-2 (Fig. 3S). It is possible that no expression or misfolding of the hybrid proteins (TM1-EptA and TM-MCR-1) occurs in *E. coli*. Indeed, not only did we employ Western blotting to validate appreciable expression of all of the four membrane proteins (Fig. 3C), but we also utilized MS to verify them (Fig. 3C and Fig. 5S (A–D)). Similar to observations with the two parent versions of EptA (Fig. 4A) and MCR-1 (Fig. 4B), the two chimeric proteins of TM1-EptA (Fig. 4C) and TM-MCR-1 (Fig. 4D) exhibit typical CD spectra with the hallmark of being rich in α-helix. These results ruled out the aforementioned two possibilities. As expected, MCR-1 and EptA could hydrolyze the substrate NBD-glycerol-3-PEA, whereas the two chimeric versions (TM-MCR-1 and TM1-EptA) could not (Fig. 3D). This is opposite to the scenarios seen with domain swapping between MCR-1 and MCR-2 (Fig. 3S) (36), validating that the domains between MCR-1 and EptA are not functionally exchangeable (Fig. S4).

**Discovery of a cavity of MCR-1 for PE substrate binding**

In general agreement with the description of the truncated MCR-1 by different research groups using X-ray crystallography (31, 37 – 40), the results of inductively coupled plasma mass spectrometry (ICP-MS) proved that zinc is occupied inside the full-length MCR-1 protein as well as its mosaic derivatives TM-
MCR-1 and TM1-EptA (Fig. 4E). Earlier studies suggested that Zn$^{2+}$/H11001 might be surrounded with five conserved catalytic residues. They correspond to Glu246, Thr285, His390, Asp465, and His466, respectively (Fig. 4A and Fig. S1). Among them, Thr$^{285}$ acts as a putative nucleophile site for MCR-1 activity (Fig. 5A). It is noteworthy that our recent site-directed mutagenesis...
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Assays demonstrate critical roles of these active sites in MCR-1 catalytic function and its resultant colistin resistance (31). However, we are not aware how the two lipid substrates (PE and lipid A) enter into (and/or bind to) MCR-1 enzyme.

The re-analyses of complex structure of DDM-EptA illustrate a cavity for DDM binding. Structural superposition of MCR-1 onto EptA allowed us to observe a similar DDM-binding cavity in MCR-1. Considering that DDM detergent is structurally similar to the real PE lipid substrate of EptA (Fig. S7A), it is reasonable to define a potential cavity for PE substrate entry (Fig. S7, B and C) by employing the approach of molecular docking (and/or molecular replacement). As expected, molecular docking of the PE substrate to NmEptA revealed six known active sites (Asn108, Thr112, Glu116, Ser330, Lys333, His395, and His378), which is consistent with the scenarios seen in the X-ray crystal structure of NmEptA (44) (Fig. S8). Among them, four residues (Thr112, Glu116, Thr280, and His378) were also detected in trials of molecular docking of NmEptA to the PE headgroup alone (Fig. S9). This partially validates the feasibility of molecular docking in this situation. Because the full-length PE with flexible acyl chains was hard to dock into MCR-1, the PE molecule with acyl chains removed (and only the headgroup retained) was subjected to molecular docking, which also in turn gave four possible crucial residues (Gln114, Glu116, Thr285, and His378) (Fig. S10), most of which have been verified in our earlier experiments (31). On the basis of the complex structure of the lipid substrate PE-EptA protein modeled by molecular docking, we replaced EptA with MCR-1 in the trials of structural superposition, which gave a modeled complex structure of MCR-1–PE (Fig. 2C). Although the complex structure might not be the best one with minimum energy, it does show clearly a potential cavity for PE substrate binding/entry into MCR-1 enzyme (Fig. 2D).

Fine analyses of the MCR-1–PE complex structure allows us to better define this cavity (Fig. 5A). This cavity has the following three elements: (i) TM region; (ii) PH2 plus PH2′; and (iii) parts of the catalytic domain (Figs. 2 C and D and 4A). In addition to the five zinc-interacting sites (Glu246, Thr285, His390, Asp465, and His466) (Fig. 5A), we discovered seven additional residues (Asn108, Thr112, Glu116, Ser330, Lys333, His395, and His378) from this cavity, which may be implicated in potential interaction with the headgroup of PE lipid substrate (Fig. 5A). It seems likely that the two periplasm-facing helices (PH2 and PH2′) contributed significantly to the recognition and occupation of the cavity by PE lipid substrate in that (i) each of the two PH helices has an essential residue (Glu116 in PH2 and Thr112 in PH2′) and (ii) the interspace between PH2 and PH2′ harbors a critical site, Asn108. However, the structure-guided discovery of the cavity requires experimental evidence.

Functional evidence for PE lipid substrate-binding sites

To test our interpretation of this cavity, we systemically performed site-directed mutagenesis analyses. In total, 12 MCR-1 derivatives having one point mutation each were assayed in this study and were categorized into two groups: (i) five mutants with zinc-binding/catalytic site inactivated (E246A, T285A, H390A, D465A, and H466A) (31, 36) and (ii) seven mutants with functional impairment of the PE lipid substrate-binding site (N108A, T112A, E116A, S330A, K333A, H395A, and H478A). The expression of the aforementioned MCR-1 mutants was verified with Western blotting (Fig. 6A). Subsequently, all of the 12 MCR-1 derivatives were purified to homogeneity (Fig. 6B) and subjected to the in vitro enzymatic assays (Fig. 6C). Among them, only three point mutants (N108A, T112A, and S330A) were detected to retain the partial activity of hydrolyzing of NBD-glycerol-3-PEA into NBD-glycerol (Fig. 6C). To further determine levels of colistin resistance conferred by different MCR-1 mutants, two different approaches were applied: one is visualization of bacterial growth on LBA solid plates with varied colistin level (Fig. S11), and the other is measurement of colistin MIC in liquid broth dilution tests (Fig. 5). In the LBA plate assay, bacterial growth of negative control (MG1655 with/without the pBAD24 vector) is visualized only under 0.5 μg/ml colistin. Intriguingly, the remaining three MCR-1 versions with a point mutation retained partial
activity on the colistin LBA plates (i.e. 8 μg/ml for N108A, 4 μg/ml for S330A, and 1 μg/ml for T112A). In general agreement with the scenarios we very recently observed (31, 36), none of the five MCR-1 mutants lacking a full set of catalytic sites could allow the recipient strain MG1655 to grow with ≥0.5 μg/ml colistin (Fig. 5B). Of note, the discrepancy in the maximal level of colistin tolerance on the LBA plates is in part due to different brands and batches of agar used here.

Using the liquid broth dilution tests as recommended by EUCAST with cation-adjusted Mueller–Hinton broth (CAMHB) (31, 38), we further quantified the MIC of colistin in the E. coli MG1655 strain expressing the wildtype mcr-1 (and/or its mutants). In this case, the colistin MIC of the strain MG1655 with/without vector alone (negative control) is around 0.25 μg/ml, whereas for the positive control strain MG1655 expressing the wildtype MCR-1, it is about 4 μg/ml (Fig. 5C). Similar to MG1655, the negative control, 9 of 12 MCR-1 point mutants (four substrate binding–deficient mutants (E116A, K333A, H395A, and H478A) and five catalytically inactivated mutants (E246A, T285A, H390A, D465A, and H466A)) cannot confer a significant increment in colistin MIC (Fig. 5C). The MIC of colistin for the remaining three MCR-1 mutants (N108A, T112A, and S330A) was 2.0, 0.5, and 2.0 μg/ml, respectively (Fig. 5C). Before MALDI-TOF MS detection of altered structure of lipid A pools, we carried out Western blotting to examine expression of the aforementioned mcr-1 and its 12 point mutants. As expected, all of the MCR-1 and its derivatives were well expressed in E. coli (Fig. 6A). Moreover, all of the versions of integral membrane enzyme MCR-1 (the wildtype and 12 mutants) were successfully extracted from
Evidently, the of the other nine mutants of MCR-1 (Fig. 7, with the enzymatic hydrolysis of PE by MCR-1. The PE molecule is shown with blue sticks, and the zinc ion is presented as a pink sphere. Molecular docking and structural analyses allow us to anticipate that 12 residues might be critical for roles of this cavity in MCR-1 catalytic mechanism. Five residues (Glu^{246}, Thr^{285}, His^{390}, Asp^{465}, and His^{466} in blue) are essential for the zinc ion, and the remaining seven amino acids (Asn^{108}, Thr^{112}, Glu^{116}, Ser^{330}, Lys^{333}, His^{395}, and His^{478}, in red) are implicated into crosstalk with the head of PE substrate molecule. It seems likely that the two periplasm-facing helices (highlighted in orange) are critical for MCR-1 activity in that this short region has three crucial residues (namely Asn^{108}, Thr^{112}, and Glu^{116} with the involvement of its binding to PE substrate. A site-directed mutagenesis assay for the role of the 12 critical residue-containing cavity in the MCR-1 colistin resistance. Structure-guided site-directed mutagenesis was routinely conducted as recommended by the manufacturer. All of the strains tested here are listed in Table S1. The experiment of colistin susceptibility was conducted using the LBA plates containing with colistin in a series of dilutions. The value of resistance to colistin was extremely consistent in our five independent experiments of solid plate dilution; thus, a representative result is given. Vec, an empty pBAD24 vector. C, MIC comparison of colistin in the engineered E. coli strains with expression of the wildtype mcr-1 and its point mutants. Colistin MIC trials were determined using the microbroth dilution method, and the breakpoints were set as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015, version 5.0) (24). All of the experiments of MIC determination were conducted more than three times, and the results were very consistent. As a result, colistin MIC is presented as recommended by EUCAST.

**Figure 5. Structure-guided functional dissection of the PE substrate-recognizable cavity.** A, fine structural illustration of the cavity for the entry of PE substrate. B, the enzymatic hydrolysis of PE by MCR-1. The PE molecule is shown with blue sticks, and the zinc ion is presented as a pink sphere. Molecular docking and structural analyses allow us to anticipate that 12 residues might be critical for roles of this cavity in MCR-1 catalytic mechanism. Five residues (Glu^{246}, Thr^{285}, His^{390}, Asp^{465}, and His^{466}, in green) are essential for the zinc ion, and the remaining seven amino acids (Asn^{108}, Thr^{112}, Glu^{116}, Ser^{330}, Lys^{333}, His^{395}, and His^{478}, in red) are implicated into crosstalk with the head of PE substrate molecule. It seems likely that the two periplasm-facing helices (highlighted in orange) are critical for MCR-1 activity in that this short region has three crucial residues (namely Asn^{108}, Thr^{112}, and Glu^{116} with the involvement of its binding to PE substrate. B, site-directed mutagenesis assays for the role of the 12 critical residue-containing cavity in the MCR-1 colistin resistance. Structure-guided site-directed mutagenesis was routinely conducted as recommended by the manufacturer. All of the strains tested here are listed in Table S1. The experiment of colistin susceptibility was conducted using the LBA plates containing with colistin in a series of dilutions. The value of resistance to colistin was extremely consistent in our five independent experiments of solid plate dilution; thus, a representative result is given. Vec, an empty pBAD24 vector. C, MIC comparison of colistin in the engineered E. coli strains with expression of the wildtype mcr-1 and its point mutants. Colistin MIC trials were determined using the microbroth dilution method, and the breakpoints were set as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015, version 5.0) (24). All of the experiments of MIC determination were conducted more than three times, and the results were very consistent. As a result, colistin MIC is presented as recommended by EUCAST.

**A role of MCR-1 in colistin resistance of gut microbiota**

To assess contribution of mcr-1 to colistin resistance in clinical pathogens, we selected three representative clinical species (E. coli, Salmonella enterica, and K. pneumoniae) from gut microbiota. First, a PCR screen allowed us to determine the presence of mcr-1 (Fig. 8A). As expected, expression of MCR-1 conferred an appreciable level of colistin resistance to clinical strains (Fig. 8B and Fig. S12). The colistin MIC of the mcr-1–bearing E. coli E15017 (4.0 μg/ml) was found to be 16-fold that of the mcr-1–negative E. coli MG1655 (0.25 μg/ml) (Fig. 8B). Relative to this, the mcr-1–harboring S. enterica strain S14018 has a relatively higher basal level of intrinsic resistance to colistin (2.0 μg/ml) (Fig. 8B). As expected, expression of MCR-1 conferred an appreciable level of colistin resistance to clinical strains (Fig. 8B and Fig. S12). The colistin MIC of the mcr-1–bearing E. coli E15017 (4.0 μg/ml) was found to be 16-fold that of the mcr-1–negative E. coli MG1655 (0.25 μg/ml) (Fig. 8B). Relative to this, the mcr-1–harboring S. enterica strain S14018 has a relatively higher basal level of intrinsic resistance to colistin (2.0 μg/ml) (Fig. 8B). As expected, expression of MCR-1 conferred an appreciable level of colistin resistance to clinical strains (Fig. 8B and Fig. S12). The colistin MIC of the mcr-1–bearing E. coli E15017 (4.0 μg/ml) was found to be 16-fold that of the mcr-1–negative E. coli MG1655 (0.25 μg/ml) (Fig. 8B). Relative to this, the mcr-1–harboring S. enterica strain S14018 has a relatively higher basal level of intrinsic resistance to colistin (2.0 μg/ml) (Fig. 8B). As expected, expression of MCR-1 conferred an appreciable level of colistin resistance to clinical strains (Fig. 8B and Fig. S12). The colistin MIC of the mcr-1–bearing E. coli E15017 (4.0 μg/ml) was found to be 16-fold that of the mcr-1–negative E. coli MG1655 (0.25 μg/ml) (Fig. 8B). Relative to this, the mcr-1–harboring S. enterica strain S14018 has a relatively higher basal level of intrinsic resistance to colistin (2.0 μg/ml) (Fig. 8B).
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MS peak appeared at m/z 1920.546 in the clinical strain E15017, mcr-1–carrying E. coli (Fig. 8E), which corresponds to either PPEA-1–lipid A or PPEA-4′–lipid A, the lipid A (m/z 1796.565–1797.216; Fig. 8, D and E) with chemical modification of a PEA (m/z = 123 units). The MS spectrum illustrates that (i) the lipid A peak is present at the m/z position of 1796.970 in the mcr-1–lacking S. enterica serovar Typhimurium strain ATCC 14028s (Fig. 8F) and (ii) two specific peaks consistently appear (one is m/z 1797.374 for lipid A, and the other is m/z 1919.199 for PPEA-1 (or 4′)–lipid A) in the mcr-1–harboring S. enterica strain S10 (Fig. 8G). Of note, two peak forms (m/z = 1824.049 and/or 1840.117) of the lipid A consistently occurred in K. pneumoniae, which is due to the variable length of acyl chains (Fig. 8, H and I). In the mcr-1–positive strain Kp253 of K. pneumoniae, PPEA-1 (or 4′)–lipid A was shown at the position of mass (m/z = 1947.624, i.e., 1824.049 + 123) (Fig. 8F). Collectively, MCR-1 modifies the chemical structure of LPS-lipid A with the addition of PEA at either the 1 or 4′ position, which consequently leads to colistin resistance.

Discussion

The metabolic mechanism by which Gram-negative bacteria developed resistance to the CAMP-type antibiotic colistin mainly relies on the reduction of net negative charge of bacte-
Taken together, these findings might represent a full mechanistic glimpse of transferable MCR-1 colistin resistance, providing a structural and functional basis for the rational design of small molecule compounds targeted at reversing resistance to colistin, a final line of defense antibiotic against lethal infections with MDR superbugs.

**Experimental procedures**

**Bacterial strains, plasmids, and growth conditions**

The bacterial strains used here include *E. coli*, *S. enterica*, and *K. pneumoniae*, respectively (Table S1) (12). PCR assays were conducted to screen for the presence of the *mcr-1* gene in clin-

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**Figure 7. MS-based functional analyses of the 12 MCR-1 mutants.**

A, MALDI-TOF MS analyses for the LPS-lipid A isolated from the colistin-susceptible strain MG1655. B, MALDI-TOF MS profile of the LPS-lipid A extracted from the negative strain MG1655 carrying the empty vector pBAD24. C, MALDI-TOF mass spectrometry suggested that a unique peak of the modified lipid A, PPEA-lipid A, is present in the positive strain, MG1655, expressing the wildtype of *mcr-1*. The modified position indicated is only a suggestion. D, the mutation T112A does not fully inactivate the enzymatic activity of MCR-1. E, the E246A mutation impairs the function of MCR-1. F, the mutation T285A of MCR-1 is nonfunctional. G, the E116A mutation impairs the function of MCR-1. H, the T330A mutation of MCR-1 still possesses partial ability to catalyze the transfer of PPEA to the 4'-phosphate group of lipid A. I, the point mutation of MCR-1 (S330A) inactivates MCR-1. J, the point mutant of MCR-1 (K333A) is inactive in the enzymatic activity of PEA transferase. K, the residue His466 is critical for the activity of MCR-1. L, no detectable activity in the H395A mutant of MCR-1. M, the mutation H478A plays an important role in MCR-1 catalysis. N, the residue His466 is critical for the activity of MCR-1. O, the D465A mutation of MCR-1 is lethal. P, the residue His466 is critical for the activity of MCR-1.
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ical strains like E15017, using specific primers (Table S2). The two strains of *E. coli* (DH5α and BL21 (DE3)) separately acted as the gene cloning host and the protein expression host, respectively (Table S1). The *E. coli* MG1655, a colistin-susceptible strain, functioned as a recipient strain of the *mcr-1* gene and/or its mutants (Table S1). All bacterial cultures were maintained in Luria–Bertani (LB) broth. Solid LB agar plates supplemented with appropriate antibiotics were used to either screen the *mcr-1*–containing clones or determine the level of bacterial colistin resistance.

Expression and purification of MCR-1 membrane protein

To express the MCR-1 integral membrane protein, the strain FYJ915 (BL21 with pET21a::mcr-1, a new construct that results
A suggestion. A representative result from over three independent experiments is given. Of note, two peaks (I) were centrifuged at 16,800 rpm for 1 h at 4 °C to collect the supernatant. China) (at 500 p.s.i. once and 1,300 p.s.i twice), and then centrifuged at 16,800 rpm for 1 h at 4 °C to collect the supernatant. Cells were grown overnight and harvested by centrifugation (5,000 rpm for 20 min) at 4 °C, washed once with 1× PBS, and stored at −80 °C until needed (48).

For large-scale purification of the MCR-1 protein, the bacterial pellets we harvested were resuspended in buffer A (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM DNase I, 1 mM phenylmethylsulfonyl fluoride, 2 mM MgCl2) to 20% (m/v), lysed by a single passage through a French press (JN-Mini, Guangzhou, China) (at 500 p.s.i. once and 1,300 p.s.i twice), and then centrifuged at 16,800 rpm for 1 h at 4 °C to collect the supernatant (31, 36). This was further spun at 38,000 rpm for 1 h at 4 °C to collect the precipitate. The pellet was then dissolved in buffer B (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5% glycerol, 1% detergent DDM (m/v)) and subjected to further centrifugation at 38,000 rpm for 1.5 h at 4 °C, giving MCR-1−containing supernatants that were incubated overnight with pre-equilibrated Ni-NTA–agarose beads at 4 °C.

As we established recently (31, 36) with minor improvement, Ni-NTA–agarose beads were loaded onto a column and rinsed with the wash buffer (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 30 mM imidazole, 5% glycerol (v/v), 0.03% DDM (m/v)). The target membrane protein MCR-1 was eluted from the Ni-NTA–agarose beads using the elution buffer (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 100 mM imidazole, 5% glycerol (v/v), 0.03% DDM (m/v)). The eluted protein was concentrated with a 30-kDa cutoff ultrafilter (Millipore), cleaned with a Resource-Q column (GE Healthcare), and analyzed by gel filtration with a Superdex 75/100GL size exclusion column (GE Healthcare) that was pre-equilibrated with 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.03% DDM. The peak fractions were pooled, analyzed with 12% SDS-PAGE, and concentrated to ~20 mg/ml.

Circular dichroism analyses

To test the protein secondary structure and folding properties, MCR-1/EptA and its derivatives (TM1-EptA and TM-MCR-1) were subjected to routine CD assays. In each trial, 600 μl of protein (~0.2 mg/ml) in Tris buffer (20 mM Tris–HCl, 300 mM NaCl, 0.03% DDM, 10% (v/v) glycerol, pH 8.0) was placed into a quartz cylindrical cuvette with a path length of 2 mm. The CD spectra were collected on a Jasco model J-1500 spectrometer (Jasco Corp., Tokyo, Japan) by continuous wavelength scanning (in triplicate) from 200 to 260 nm at a scan rate of 50 nm/min (49) and smoothed with a Savitsky–Golay filter (50).

ICP-MS

To determine whether or not Zn2+ is bound to MCR-1 and its derivatives, ICP-MS was applied. Briefly, the protein samples were loaded into an NexIONTM 300× ICP-MS instrument (PerkinElmer Life Sciences) switched to Collision-Cell mode, and then the mass-to-charge ratio (m/z) was monitored using the kinetic energy discrimination mode with helium as the carrier gas (51).

Liquid chromatography quadrupole time-of-flight mass spectrometry

The identities of two chimeric MCR-1 versions (TM-MCR-1 and TM1-EptA) were determined using a Waters Q-ToF API-US Quad-Tof mass spectrometer connected to a Waters nano Acquity UPLC as described earlier (52, 53). The expected protein bands were cut from the SDS-PAGE and digested with trypsin (G-Biosciences, St. Louis, MO). The resultant peptides were loaded on a Waters Atlantis C-18 column (0.03-mm particle, 0.075 × 150 mm) and subjected to MS/MS analyses (54). Finally, data analyses were conducted using the Waters Protein Lynx Global Server 2.2.5, Mascot (Matrix Sciences), and BLAST against the NCBI non-redundant database.

In vitro assay for MCR-1 enzymatic activity

The in vitro reaction catalyzed by MCR-1 was performed as described by Anandan et al. (34) with minor modifications. In this biochemical assay, the fluorescently labeled substrate for MCR-1 enzyme is 1-acetyl-2-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl−sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids), referred to as NBD-PEA. The reaction system (50 μl in total) was kept for 20 h at room temperature and contained 50 mM HEPES (pH 7.50), 100 mM NaCl, 0.03% DDM, 0.2 mM NBD-PEA, and 40 μM MCR-1. The reaction products were subjected to TLC in a mobile phase consisting of ethyl acetate/methanol/water (7:2:1, v/v/v). The fluorescent

Figure 8. mcr-1−positive clinical species of Enterobacteriaceae have altered structure of LPS-lipid A and exhibit an appreciable level of colistin resistance. A, PCR screen for the presence of mcr-1 in clinical strains of Enterobacteriaceae. B, determination of the MIC of colistin on the clinical strains of Enterobacteriaceae. C, silver staining analyses for the lipid A of the LPS isolated from the clinical strains with or without the mcr-1 gene. Three types of clinical species of Enterobacteriaceae used here include E. coli, S. enterica, and K. pneuniae, respectively. −, absence of the mcr-1 gene; +, presence of the mcr-1 gene. Colistin MIC assays were performed using the micro-broth dilution method, and the breakpoints were set as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015, version 5.0) (24). M, DNA marker. The mcr-1−negative E. coli strain is MG1655, whereas the mcr-1−positive one is E15017 (21). The mcr-1−negative S. enterica is S14018 in our laboratory stock, whereas the mcr-1−positive S. enterica refers to S10. The mcr-1−negative K. pneuniae is RK14011, whereas the mcr-1−positive K. pneuniae is Kp253. D and E, MALDI-TOF MS profile of the LPS-A isolated from the mcr-1−negative E. coli (D) and the mcr-1−positive E. coli (E). The MS peak of lipid A species in E. coli is shown at m/z of 1796.565–1797.216, whereas it appears at m/z 1920.546 in the mcr-1−harboring E. coli because the phosphoethanolamine-modified species (PPEA-lipid A) is given. Here, the PEA mass is 123 units. F and G, MALDI-TOF MS-based analyses for the lipid A isolated from the mcr-1−negative S. enterica (F) and the mcr-1−positive S. enterica (G). The MS peak of the lipid A species is shown at m/z 1796.970 in the mcr-1−negative S. enterica serovar Typhimurium strain ATCC 14028s and m/z 1797.374 in the mcr-1−positive S. enterica strain S10, respectively. The PPEA-lipid A is shown at m/z 1919.199 in that the addition of PEA mass is 123 units in the mcr-1−carrying strain S10. Shown is a MALDI-TOF MS−based comparison of the LPS-lipid A extracted from the mcr-1−negative K. pneuniae (F) and the mcr-1−positive K. pneuniae (G). Of note, two peaks (m/z = 1824.049 and/or 1840.117) correspond to the lipid A in K. pneuniae, which is due to the variable length of acyl chains. In the mcr-1−positive K. pneuniae, PPEA-lipid A is present at the position of mass m/z = 1947.624 (i.e. 1824.049 + 123). The position of PEA modifications is only a suggestion. A representative result from over three independent experiments is given.

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signal on the TLC plate was detected under Epi blue light (455–485 nm) with a gel imaging system (Bio-Rad) (34).

LC/MS

In addition to the pure lipid substrate of NBD-glycerol-3-PEA, the mixture of the MCR-1–catalyzed reaction was subjected to further analyses with the LC/MS system (Agilent Technologies 6460 Triple Quad LC/MS) (55). The analytical chromatographic column was Zorbax SB C18 (2.1 × 50 mm, 3.5 μm), and it was eluted with methanol, 0.1% methanoic acid (95:5) at 0.3 ml/min. MS was coupled with an electrospray ionization source, in which neutral loss ion (m/z 141) mode was used for the positive ion scanning.

Overlapping PCR and site-directed mutagenesis

To generate the chimeric EptA/MCR-1 protein, overlapping PCR was performed with appropriate primers (Table S2) (36). Site-directed mutagenesis was conducted to give the point mutants of mcr-1 (31). The PCR system was the Mut Express II fast mutagenesis kit V2 (Vazyme Biotech Co., Ltd.) with an array of specific primers for mcr-1 (Table S2).

Measurement of colistin resistance

To quantify the MIC of colistin in different mcr-1–carrying clinical strains and/or the E. coli MG1655 strain expressing the wildtype mcr-1 (and/or its mutants), liquid broth dilution tests were carried out as recommended by EUCAST with CAMHB (31, 38). Briefly, overnight cultures were diluted 100-fold in fresh CAMHB medium and grown until A600 reached 0.5. To measure MIC value, these cultures were diluted again to A600 0.05 in CAMHB containing varied levels of colistin (ranging from 0 to 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 μg/ml).

The level of colistin resistance was also determined using a solid LBA broth dilution test (21, 31). Bacterial survival ability was detected as follows. Mid-log phase cultures diluted appropriately were spotted on LBA plates supplemented with colistin at different levels (ranging from 0 to 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 μg/ml) and kept at 37 °C overnight. Of note, the pBAD24-borne expression of mcr-1 and its derivatives required the addition of 0.2% arabinose as an inducer into either CAMHB medium or LBA plates.

Extraction and purification of LPS-lipid A

The crude LPS was prepared as described by Caroff et al. (56) with modifications. Briefly, overnight cultures on the LBA plates plates with supplied 8 μg/ml colistin were collected for LPS isolation. Bacterial cells (~20 mg) were washed with 10 ml of Tris-HCl (30 mM, pH 8.0), centrifuged at 6,000 rpm for 10 min, and resuspended in 0.4 ml of Tris-HCl (30 mM, pH 8.0) containing 20% sucrose. After a 0.5-h incubation with 40 μl of lysozyme (1 mg/ml, 100 mM EDTA, pH 7.3) on ice, the bacterial samples were kept at −80 °C for 0.5 h and then thawed at room temperature. Following two rounds of the processes described above, the sample was resuspended in 3 ml of EDTA (3 mM, pH 7.3) and subjected to sonication-aided lysis using a probe tip sonicator at a constant duty cycle for 2 min at 50% output (0.5 s/burst). Bacterial lysate was spun for 15 min at 6,000 rpm, and the resultant supernatant was further centrifuged at 16,000 rpm for 1 h to precipitate the crude LPS.

Then the crude LPS was freeze-dried and redissolved in the solution of 30 mM Tris–HCl (pH 8.0) with 0.2% SDS. To remove nucleic acid contamination, DNase I (25 μg/ml) and RNase A (100 μg/ml) were added to the solution and incubated for 2 h at 37 °C, which was followed by the removal of protein contaminants via treatment with proteinase at 37 °C for 1 h. To cleave the Kdo linkage, the crude LPS was heated in 10 mM sodium acetate buffer (pH 4.5) with aqueous 0.2% SDS at 100 °C for 1 h and then precipitated with acidified ethanol (100 μl) for the removal of SDS (56), which was followed by centrifugation (5,000 rpm, 5 min). Finally, the precipitate was subjected to two rounds of washing with 100 μl of 95% ethanol and a final round of wash with 1 ml of ethanol, giving purified lipid A (44).

The purity of lipid A was judged by the method of sensitive sliver staining following separation with SDS-PAGE (57). The brief protocol was described as follows: First, the lipid A sample was dissolved in LUG buffer and separated with a SDS-PAGE (10%) gel under a constant current (10 mA) until the blue front was in the middle of the gel. Second, the gel was soaked in fixing solution consisting of 25% methanol and 7.5% acetic acid for 2 h and washed twice in 7.5% acetic acid (30 min each). Third, the gel was washed three times (30 min each), after a 5-min soak in periodic acid solution containing 7.5% acetic acid. Finally, the gel was soaked in AgNO3 solution for 10 min, rinsed with water three times (5 min each), and developed in fresh developing solution until bands were appropriately visible. Development was terminated by rinsing for 1 h in a 1% acetic acid solution.

MALDI-TOF mass spectrometry

The chemical structures of lipid A were analyzed by MALDI-TOF/TOF MS (Bruker, ultraflexXtreme) in negative-ion mode with the linear detector (11, 58). Lipid A fractions were dissolved in 20 μl of chloroform/methanol (2:1) solution and mixed with 2,5-dihydroxybenzoic acid matrix in the solution of chloroform/methanol/water (3:1.5:0.25) (20 mg/ml) in a ratio of 1:1 (44). Finally, 1 μl of lipid A solution was loaded onto the MALDI sample plate, giving the MS spectrum. In this case, each spectrum was derived from an average of 500 shots and 50% laser power (12, 46, 59).

Structure modeling and molecular docking

The 3D structure of lipid A phosphoethanolamine transferase (EptA) from N. meningitidis (PDB entry 5FGN) (34) was obtained from the RCSB PDB database (60). The structure of MCR-1 was modeled by software Swiss-Model (https://swissmodel.expasy.org/interactive) (61), using the structure of EptA (PDB accession number 5FGN) as the template. Although MCR-1 shows 35.65% identity to EptA, its modeled structure possesses a coverage score of 96% (aa 11–541) relative to EptA. In this case, the score of GMQE (global model quality estimation) is 0.7, and the value of QMEAN (which provides a global and local absolute quality estimate on the modeled
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Multiple-sequence alignment of PEA lipid A transferases was conducted using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (67, 76), and its resultant output of phylogeny was given with the program TreeView (68). In addition to EptA, the MCR-1-like proteins included MCR-1 (and its eight variants, namely MCR-1.2 (accession number WP_065274078) (41), MCR-1.3 (accession number WP_077064885) (42), MCR-1.4 (accession number WP_076611062), MCR-1.5 (accession number ARX60875) (69), MCR-1.6 (accession number WP_077248208) (43), MCR-1.7 (accession number WP_085562392), MCR-1.8 (accession number WP_085562407), and MCR-1.9 (accession number KY964067) (12); MCR-2 (accession number NG_051171) (70) plus MCR-2.1 (accession number ASK49941) and MCR-2.2 (accession number ASK49942) (71); MCR-3 (accession number NG_055505) (72) and MCR-3.2 (accession number NZ_FLW0100034) (72), MCR-3.3 (accession number NZ_FLXAO1000011) (72), MCR-3.4 (accession number NG_055497), MCR-3.5 (accession number ERR1971735) (73), and MCR-3.7 (accession number MF489760) (74); MCR-4 (accession number MF543359) (75); and MCR-5 (accession number KY807921) (4), respectively. Of note, the Z1140 (accession number AAG55285) of E. coli O157:H7 strain EDL 933, which is a putative PEA transferase with unknown function, acted as an internal reference for the phylogenetic tree.

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