Research Paper

Spread of MCR-3 Colistin Resistance in China: An Epidemiological, Genomic and Mechanistic Study

Yongchang Xu a,1, Lan-Lan Zhong b,1, Swaminath Srinivas c,1, Jian Sun d,1, Man Huang a, David L. Paterson e, Sheng Lei a, Jingxia Lin a, Xin Li a, Zichen Tang a,f, Siyuan Feng a, Cong Shen b, Guo-Bao Tian b,⁎, Youjun Feng a,c,d,g,⁎

a Department of Medical Microbiology & Parasitology and Department of General Intensive Care Unit of the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China
b Zhongshan School of Medicine, Key Laboratory of Tropical Diseases Control of Ministry of Education, Sun Yat-sen University, Guangzhou, Guangdong 510080, China
c Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
d National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou 510642, China
e Centre for Clinical Research, Royal Brisbane and Women’s Hospital, University of Queensland, Building 71,918, Brisbane QLD 4029, Australia
f College of Food and Bioengineering, Henan University of Science and Technology, Luoyang, Henan 471023, China
g College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, China

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ABSTRACT

Background: Mobilized resistance to colistin is evolving rapidly and its global dissemination poses a severe threat to human health and safety. Transferable colistin resistance gene, mcr-3, first identified in Shandong, China, has already been found in several countries in multidrug-resistant human infections. Here we track the spread of mcr-3 within 13 provinces in China and provide a complete characterization of its evolution, structure and function.

Methods: A total of 6497 non-duplicate samples were collected from thirteen provinces in China, from 2016 to 2017 and then screened for the presence of mcr-3 gene by PCR amplification. mcr-3-positive isolates were analyzed for antibiotic resistance and by southern blot hybridization, transfer analysis and plasmid typing. We then examined the molecular evolution of MCR-3 through phylogenetic analysis. Furthermore, we also characterized the structure and function of MCR-3 through circular dichroism analyses, inductively coupled plasma mass spectrometry (ICP-MS), liquid chromatography mass spectrometry (LC/MS), confocal microscopy and chemical rescue tests.

Findings: 49 samples (49/6497 = 0.75%) were mcr-3 positive, comprising 40 samples (40/4144 = 0.97%) from 2017 and 9 samples (9/2353 = 0.38%) from 2016. Overall, mcr-3-positive isolates were distributed in animals and humans in 8 of the 13 provinces. Three mcr-3-positive IncP-type and one mcr-1-bearing IncH2-like plasmids were identified and characterized. MCR-3 clusters with PEA transferases from Aeromonas and other bacteria and forms a phylogenetic entity that is distinct from the MCR-1/2/P(M) family, the largest group of transferable colistin resistance determinants. Despite that the two domains of MCR-3 not being exchangeable with their counterparts in MCR-1/2, structure-guided functional mapping of MCR-3 defines a conserved PE-lipid recognizing cavity prerequisite for its enzymatic catalysis and its resultant phenotypic resistance to colistin. We therefore propose that MCR-3 uses a possible “ping-pong” mechanism to transfer the moiety of PEA from its donor PE to the 1(or 4’)-phosphate of lipid A via an adduct of MCR-3-bound PEA. Additionally, the expression of MCR-3 in E. coli prevents the colistin-triggered formation of reactive oxygen species (ROS) and interferes bacterial growth and viability.

Interpretation: Our results provide an evolutionary, structural and functional definition of MCR-3 and its epidemiology in China, paving the way for smarter policies, better surveillance and effective treatments.

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1 Yongchang Xu, Lan-Lan Zhong, Swaminath Srinivas and Jian Sun contribute equally to this work.

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1. Introduction

Antimicrobial resistance (AMR) has become a global public health priority. The accelerated development of multidrug resistance (MDR) is attributed in part (if not completely) to the massive and inappropriate use of antimicrobials in agricultural and clinical settings. Human infections caused by MDR pathogens result in over 70,000 deaths in the United States each year [1, 2]. In fact, a team led by Prof. Lord Jim O’Neil has estimated that AMR could result in 10 million deaths a year worldwide by 2050 [3]. Although the accuracy of this frightening prediction is uncertain, we acknowledge the enormous burden AMR causes at multiple levels (economic, social, clinical and public health) [4]. This highlights the importance and urgency of a coordinated international action to prevent and control the world-wide spread of AMR [4, 5].

Polymyxins refer to an array of non-ribosomally-synthesized, cationic antimicrobial cyclic-peptides (CAMP) [6]. Among the five known subtypes, polymyxin B and polymyxin E (Colistin) are extensively used in agricultural production and clinical therapies [6-8]. Historically, the primary target of colistin is thought to be the negatively charged lipid A moiety of lipopolysaccharides (LPS) on the outer-leafllet of the bacterial outer membrane [9]. Despite its potential nephrotoxicity and neurotoxicity [6, 10-12], colistin is still used for treatment as an ultimate line of defense against critical infections caused by MDR pathogens (esp. carbapenemase-producing Enterobacteriaceae) [7, 13, 14]. However, an acquired resistance to polymyxin has been frequently found in certain species of bacterial pathogens like Klebsiella pneumoniae (K. pneumoniae) [15] and Salmonella enterica (S. enterica) [16, 17]. The chemical mechanism underlying the colistin resistance consistently involves bacterial lipid A-centered surface remodeling, including i) The addition of 4-amino-4-deoxy-L-arabinose in S. enterica [16, 17] and Pseudomonas aeruginosa [18]; ii) The attachment of phosphoethanolamine (PEA) in Neisseria [19], Acinetobacter baumannii [20] and Campylobacter jejuni [21]; and iii) Glycine/diglycine modification in the pandemic Vibrio cholerae biotype El Tor [22-25]. Intrinsic resistance to polymyxin is limited to the originally-resistant population. However, the recent emergence and global discovery of plasmid-borne mobilized colistin resistance determinants (mcr-1) potentially threatens the clinical effectiveness of colistin as a last-resort antibiotic against carbapenem-resistant superbugs [26].

The mcr-1 gene product, MCR-1, is a PEA lipid A transferase, belonging to the “YhiW/YjdB/YipP” alkaline phosphatase super-family [26, 27]. MCR-1 catalyzes the transfer of the PEA group from lipid A glucosamine (GlcN) moieties [19, 28, 29]. Structure-guided functional studies have determined this mechanism and demonstrated that the enzymatic activity of MCR-1 renders the recipient strains resistant to polymyxin [27, 30-35]. Intriguingly, the determinants of transferable colistin resistance have extended beyond MCR-1, to a number of new MCR-like members [36] (namely MCR-2 [37-39], MCR-3 [40, 41], MCR-5 [42], MCR-6 [Genbank no.: ASK49942] (Indeed, it is a MCR-1/2 progenitor from Moraxella sp. MSG47-C17 [43], and exhibits high level of homology to ICR-Mo of M. osloensis [44]. Thus, it is supposed to be renamed as ICR-M), MCR-7 [45] and MCR-8 [46]), as well as over a dozen of new heterogeneous MCR-1 variants (e.g., MCR-1.2 [47] and MCR-1.6 [48]). Unlike the predominant MCR-1 which is distributed worldwide [49], both MCR-2 (81% identity to MCR-1 and originally found in Belgium [37, 38], and very recently detected in pigs/poultries [39] and human vaginal swabs [50] from China) and MCR-5 (only detected in Germany [42]) are thought to be two rare members of the MCR-like protein family. This is slowly changing with the discovery of MCR-2 and its variants in countries like China [39]. As for MCR-4, it has been detected in a pig isolate of S. enterica in Italy 2013 [41], swine isolates of E. coli from Spain and Belgium in 2015-2016 [41], and clinical isolates of carbapenemase-producing Enterobacter cloacae from Singapore in 2017 [51]. In terms of epidemiological/geographic distribution, MCR-3 seems to be second only to MCR-1. Phylogenetic analysis indicates that MCR-3 is evolutionarily distinct from MCR-1 and closely clustered with chromosomally-encoded MCR-like proteins in certain species of Aeromonas (Fig. 3) [52, 53]. To the best of our knowledge, the new mcr-3 gene has been discovered in 3 of 7 continents, namely Asia (China [40, 54], Singapore [51], Japan [55], Thailand [40] and Malaysia [40]), Europe (Denmark [56, 57], France [58] and Spain [59]) and North America (the United States [40]). Given that i) In Europe, colistin is used to treat bacterial infections of livestock (such as pigs, cows, and goats) [60]; and ii) Colistin is heavily supplemented as a growth promoter of livestock (pigs and poultries) in Asian countries (e.g., China, Japan, and Vietnam) [61], it is possible that indiscriminate antibiotic use has selected for the emergence of new colistin resistance determinants like mcr-3.
for monitoring of antibiotic resistance. Although two independent research groups recently evaluated the prevalence and clinical risk of mcr-1 carriage in human infections [62, 63], the molecular epidemiology and functional aspects of mcr-3 in China are poorly understood. In this work, we aim to close this knowledge gap and present an overview of the dissemination, comparative genomics, evolution and mechanism of MCR-3-mediated colistin resistance.

2. Methods

2.1. Ethics and Consents

Ethical approval was sought and informed consent was provided by patients, healthy volunteers and farm owners. Individual consent forms were translated into Mandarin Chinese and the study vocally explained to each subject. All documents complete with patient signatures have been retained. All participants held the right to withdraw from the study at any stage.

2.2. Epidemiological Study

We undertook a retrospective cross-sectional study to assess the prevalence of mcr-3-carrying isolates in animal, farmer, inpatient and healthy volunteer in China. A total of 6497 non-duplicate fecal samples were collected from thirteen provinces in China, from Oct. 2016 to Dec. 2017. Specifically, 1951 fecal samples of farmer who came into the hospitals for routine physical examinations. Fecal samples from inpatient and healthy volunteers were recovered from 5 hospitals which contain >6000 beds in Guangdong province. All these samples were isolated from Oct. 2016 to Dec. 2017 (Tables S1 and S2).

Table 1
Prevalence of mcr-3 in samples from swine and farmers in this study.

| Provinces | Farms | No. of swine samples | mcr-3-positive rate of pigs | No. of farmer samples | mcr-3-positive rate of farmers |
|-----------|-------|----------------------|----------------------------|-----------------------|-------------------------------|
| Guangdong | GD1   | 166                  | 0.60% (1)                  | -                     | -                             |
|           | GD2   | 122                  | 0.00% (0)                  | -                     | -                             |
|           | GD3   | 208                  | 3.85% (8)                  | 10                    | 0.00% (0)                     |
|           | GD4   | 15                   | 0.00% (0)                  | -                     | -                             |
|           | GD5   | 38                   | 0.00% (0)                  | -                     | -                             |
|           | GD6   | 97                   | 0.00% (0)                  | 20                    | 0.00% (0)                     |
|           | GD7   | 150                  | 1.33% (2)                  | 30                    | 0.00% (0)                     |
|           | GD8   | 50                   | 0.00% (0)                  | 10                    | 0.00% (0)                     |
|           | GD9   | 50                   | 10.00% (5)                 | 10                    | 2.00% (2)                     |
|           | GD10  | 31                   | 0.00% (0)                  | 6                     | 0.00% (0)                     |
|           | GD11  | 49                   | 4.08% (2)                  | -                     | -                             |
|           | GD12  | 146                  | 6.85% (10)                 | -                     | -                             |
| Anhui     | AH1   | 10                   | 40.00% (4)                 | 2                     | 0.00% (0)                     |
|           | AH2   | 50                   | 10.00% (5)                 | 10                    | 0.00% (0)                     |
|           | AH3   | 49                   | 0.00% (0)                  | 7                     | 0.00% (0)                     |
| Guangxi   | GX1   | 33                   | 0.00% (0)                  | -                     | -                             |
|           | GX2   | 47                   | 0.00% (0)                  | -                     | -                             |
|           | GX3   | 48                   | 0.00% (0)                  | 9                     | 0.00% (0)                     |
|           | GX4   | 41                   | 2.44% (1)                  | -                     | -                             |
| Hunan     | HN1   | 3                    | 0.00% (0)                  | -                     | -                             |
|           | HN2   | 50                   | 2.00% (1)                  | -                     | -                             |
|           | HN3   | 45                   | 0.00% (0)                  | -                     | -                             |
| Jiangxi   | JX1   | 1                    | 0.00% (0)                  | -                     | -                             |
|           | JX2   | 79                   | 1.27% (1)                  | -                     | -                             |
| Hebei     | HB    | 50                   | 0.00% (0)                  | 10                    | 0.00% (0)                     |
| Heilongjiang | HLJ | 50                   | 2.00% (1)                  | 9                     | 0.00% (0)                     |
| Hubei     | HuB   | 51                   | 1.96% (1)                  | 4                     | 0.00% (0)                     |
| Jilin     | JL    | 50                   | 0.00% (0)                  | 1                     | 0.00% (0)                     |
| Liaoning  | LN    | 50                   | 0.00% (0)                  | 7                     | 0.00% (0)                     |
| Sichuan   | SC    | 120                  | 0.83% (1)                  | 29                    | 13.79% (4)                    |
| Shaanxi   | SX    | 1                    | 0.00% (0)                  | -                     | -                             |
| Hainan    | HA    | 1                    | 0.00% (0)                  | -                     | -                             |
| Total     |       | 1951                 | 2.20% (43)                 | 174                   | 3.45% (6)                     |

2.3. Measurement of Bacterial Growth

To address effect exerted on bacterial growth by the eptA and/or mcr-like genes, the E. coli strain MG1655 alone [or with either the pBAD24 empty vector or plasmid-borne eptA/mcr genes] was subjected to the cultivation at 37 °C (180 rpm) in LB broth (Table S3). Totally, three different versions of mcr-like genes used here included mcr-1, mcr-2, and mcr-3, respectively. To generate bacterial growth curves, over-night cultures were inoculated (1:1000) in fresh LB broth (37 °C, 180 rpm) with varied level of arabinose (0.00%, 0.02%, and 0.20%). Spectrophotometer (Spectrum lab S32A) was used to measure the optical density (OD) at 600 nm at a regular interval of one hour for a total of 20 h.

2.4. Measurement of Bacterial Growth

Conjugation experiments were performed to test the transferability of the mcr-3-harboring plasmid, using a Streptomyacin-resistant E. coli C600 as the recipient. Briefly, overnight culture of mcr-3-positive isolates and recipient strain E. coli C600 were mixed (ratio of 1:9) in LB broth, which was subjected to overnight incubation. The mixture was then spread on LB agar plates containing sodium streptomycin (2000 μg/ml) plus colistin (4 μg/ml) to select trans-conjugants that had acquired the mcr-3-harboring plasmid. Carriage of such a plasmid in the parental strain and corresponding trans-conjugants was confirmed by PCR and Sanger sequencing. MLST was determined using BLASTn and publicly available database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli (Table S1).

2.6. Sequencing, Assembly and Annotation of Plasmid Genomes

The acquired mcr-1/3-positive plasmids were used to construct a sequencing library using the TruePrepTM DNA Library Prep Kit V2
from Illumina® (Vazyme, USA). The qualified libraries were sequenced on an Illumina HiSeq X-ten platform (Illumina, USA), in which 150 bp paired-end reads were generated. Following removal of low-quality reads with software Trimmomatic [65], the high-quality clean reads were assembled into long contigs with the software SPAdes (version 3.11.0) [66]. The assembled contigs with resistance genes were identified and compared to those closely-related plasmids (with known genome sequences deposited in NCBI database) using the program Blastall (version 2.2.22) with an e-value cutoff of 1 × 10−5. The suspected gaps between contigs were closed with Sanger quenching reads with software Trimmomatic [65], the high-quality clean reads paired-end reads were generated. Following removal of low-quality

2.8. S1-PFGE and Southern Blotting

The plasmid and/or chromosome location of mcr-3 and mcr-1 genes was determined by S1-nuclease digestion and pulsed-field gel electrophoresis (S1-PFGE), followed with southern blotting hybridizations. The mcr-3-harboring isolates were suspended in PBS and embedded in gold agarose gel plugs (SeaKem® Gold Agarose, Lonza, USA). The plugs were digested with S1 nuclease (TaKaRa, Dalian, China) and the DNA fragments were separated by PFGE. Southern blotting hybridizations of plasmid DNA were performed with DIG-labelled mcr-3 or mcr-1 probes according to the manufacturer’s instructions (Roche Diagnostics, Germany).

2.9. Antimicrobial Susceptibility Tests

We determined minimum inhibitory concentrations (MICs) of colistin, polymyxin B, tigecycline, ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, cefepime, gentamicin, amikacin, ertapenem, imipenem, meropenem, fosfomycin, nitrofurantoin and ciprofloxacin for all isolates using the agar dilution method in accordance with the Clinical and Laboratory Standards Institute guidelines, and we interpreted the results according to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016) for colistin, polymyxin B, and tigecycline and those of the Clinical and Laboratory Standards Institute (CLSI, 2016) for other antimicrobials (Table S2).

2.10. Structure Modeling and Molecular Docking

The software Swiss-Model (https://swissmodel.expasy.org/interactive/qMEvX5/models/) [69] was used to obtain the modelled structure of MCR-3 using the structure of the Neisseria meningitidis EptA (PDB, SFGN) as the template [70]. Although MCR-3 shows only 37.55% identity to EptA, its modelled structure exhibits the coverage score of 98% (6–540). It was evidently a qualified structural prediction, in which GMQE (Global Model Quality Estimation) is 0.73, and QMEAN (quality estimation on the modelled structure) is −3.68. The ready-to-dock 3D structure of phosphatidylethanolamine (PE) (ID: ZINC32837871) and head group of PE (ID: ZINC02798545) was obtained from ZINC database [71]. As we recently described with EptA/MCR-1 [72], the program DOCK6.7 was used to dock the PE ligand to designated binding pocket into MCR-3 [73].

2.11. Overlap Extension PCR and Site-directed Mutagenesis

Using appropriate primers (Table S3), overlap extension PCR was conducted, generating the hybrid derivatives of MCR-1/3 and Z140 [30]. Site-directed mutagenesis was applied to produce all the point-mutants of mcr-3 [27, 30, 72]. The Mut Express II Fast Mutagenesis Kit V2 (Vazyme Biotech Co., Ltd.) was used along with specific primers for mcr-3 (Table S3).

2.12. Expression and Identification of MCR-3 Membrane Protein

To produce MCR-3 integral membrane protein, the strain FYJ1153 (BL21 with pET21a::mcr-3) was engineered (Table S3). As we described with MCR-1/2 [30, 72], the expression of MCR-3 was induced by the addition of 0.5 mM isopropyl [α-D-1-thiogalactopyranoside (IPTG). The bacterial pellets were harvested and lysed through a French Press (JN-Mini, China) [at 500 p.s.i. once and 1300 p.s.i twice]. Following the routine purification process for the membrane proteins MCR-1/2, MCR-3-containing fraction solubilized in buffer B [20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5% glycerol, 1% detergent dodecyl-[β-D-maltoside (DDM, M/V)] were incubated overnight with pre-equilibrated Ni-NTA agarose beads at 4 °C. Then, the intramembrane protein MCR-3 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 (vol/vol), 0.03% DDM (M/V)]. The acquired protein was concentrated with a 30 kDa cut-off ultra-filter (Millipore, USA), and analyzed with 12% SDS-PAGE. Further for its identity of recombinant MCR-3, the expected protein bands were cut from the SDS-PAGE, digested with Trypsin (G-Biosciences St. Louis, MO), and subjected to the confirmation with A Waters Q-ToF API-US Quad-ToF mass spectrometer [74, 75]. Finally, data analyses were conducted using the Waters Protein Lynx Global Server 2.2.5, Mascot (Matrix Sciences) and BLAST against the NCBI nr database.

2.13. Circular Dichroism Analyses

The protein secondary structure of MCR-3 was determined using circular dichroism (CD) [72]. Prior to CD analyses, MCR-3 protein (0.2 mg/ml) was dissolved in Tris-buffer [20 mM Tris–HCl, 300 mM NaCl, 1% (vol/vol) glycerol, pH 8.0]). For every experiment, ~600 μl protein sample was dropped into a quartz cylindrical cuvette. The CD spectra were recorded on a Jasco Model J-1500 spectrometer (Jasco Corp., Tokyo, Japan) through continuous wavelength scanning (in triplicate) from 200 to 260 nm at a scan rate of 50 nm/min [76] and smoothed with a Savitsky-Golay filter [77].

2.14. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

As recently demonstrated with MCR-1 [72], ICP-MS was adopted to examine the protein-bound zinc in MCR-3. The MCR-3 protein sample was subjected to a NexION™ 300x ICP-MS instrument (PerkinElmer, USA) in which the mass-to-charge ratio (m/z) was calculated [78]. The carrier gas was helium.

2.15. Assays for Enzymatic Reaction of MCR-3

As recently showed for EptA [79] and MCR-1/2 [72, 79] and ICR-Mo [44], the reaction catalyzed by MCR-3 in vitro was conducted. In brief, the 1-acyl-2-{12-{[(7-nitro-2-1,3-benzoazadiazol-4-yl) amino]dodecanoyl}-sn-glycero-3-phosphoethanolamine (Avanti Lipids, USA), acted as the substrate for MCR-3, which is abbreviated as NBD-PeA. The reaction system (50 μl in total) consisted of the following components [50 mM HEPES (pH 7.50), 100 mM NaCl, 0.03% of DDM, 0.2 mM NBD-PeA and 40 μM MCR-3] and kept for 20 h at room temperature. Subsequently, the reaction products were separated with thin layer chromatography (TLC) in a mobile phase consisting of ethyl acetate:
methanol; water (7:2:1, vol/vol). The fluorescent signal on the TLC plate was visualized under Epi blue light (455–485 nm) with a gel imaging system (Bio-Rad) as Anandan et al. described [70].

2.16. Preparation and Structural Determination of Lipopolysaccharide-Lipid A

The crude lipopolysaccharide (LPS) was extracted as described by Caroff et al. [26, 80] with modifications. The resulting LPS species were freeze-dried, and then dissolved in the buffer of 30 mM Tris-HCl (pH 8.0) containing 0.2% SDS, DNase I (25 μg/ml) and RNase A (100 μg/ml) were used to remove the nucleic acid contamination. The removal of protein contaminants was preceded via 1 h of treatment with proteinase K (500 μg/ml). To release the Kdo linkage from lipid A, the crude LPS was heated at 100 °C for 1 h in the sodium acetate buffer (10 mM, pH 4.5) supplemented with aqueous 0.2% SDS. Then, the residual SDS was cleaned through the precipitation with acidic ethanol [81].

SDS-PAGE coupled with silver staining was utilized to detect the purity of lipid A pools from different bacterial strains [82]. The qualified lipid A species were subjected to MALDI-TOF/TOF-MS (Bruker, ultraflexIII) in negative-ion mode with the linear detector [23, 83]. Briefly, the samples of lipid A were dissolved in 20 ul of chloroform: methanol (2:1) solution and then loaded onto the MALDI sample plate, giving MS spectrum. In general, each spectrum was collected from an average of 500 shots and 50% laser power [26, 80, 84].

2.17. Liquid Chromatography Mass Spectrometry (LC/MS)

The LC/MS system (Agilent technologies 6460 Triple Quad LC/MS) was applied in structural measurement of the alternative lipid substrate, NBD-Glycerol-3-PEA [85]. Similarly, the reaction mixture catalyzed by MCR-3 was assayed. In the trials of GC/MS, the analytical chromatographic column (Zorbax SB C18 (2.1*50 mm, 3.5 μm)) was utilized. The samples were eluted with methanol/0.1% methanolic acid (95:5) at 0.3 ml/min. Of particular note, an electrospray ionization (ESI) source was connected with mass spectrometry (MS), and the positive ion scanning appeared in the mode of neutral loss ion (m/z, 141).

2.18. Confocal Microscopy

Confocal microscopy was applied in both the determination of ROS level and the differentiation of ALIVE/DEAD status of E. coli. First, to evaluate the potential effects of MCR-3 (and/or EptA) on colistin-induced ROS production, the E. coli strains with or without the gene of mcr-3/epa were subjected to confocal microscopy analyses. All the E. coli strains in mid-log phase were sub-cultured at a ratio of 1: 500 into LB medium for ~5 h to 1800 rpm, gently removing the supernatant, and the dissolved pellets were used to reduce the word size to 2 and to return 500 target sequences. Sequences of distantly related MCR proteins including those in the MCR-1/2/4/5 family were manually included to obtain a comprehensive analysis. Redundant sequences were eliminated with the Uniref seq server (https://www.ncbi.nlm.nih.gov/ReferenceSeq/) and aligned using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). In total, 511 unique amino acid sequences were utilized for the subsequent phylogenetic analysis.

JModeltest (via MEGA 7) was used to identify the best-fit protein substitution model and the best model was used to generate a maximum-likelihood tree with 1000 bootstrap replicates. A LG model with Gamma distribution and Invariant sites was used. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The results are presented as a radial phylogram. A subset of the sequences was re-analyzed using the above method to obtain a smaller, more detailed phylogenetic tree.

2.21. Role of the Funding Source

The funder had no role in the study design, data collection, data analysis, data interpretation, or writing of the article. The corresponding
authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

3. Results

3.1. Molecular Epidemiology of mcr-3 in China

Of the 6497 unique samples collected, a total of 6497 non-duplicate samples were collected from thirteen provinces in China, from Oct 2016 to Dec 2017. Of them, 49 samples (49/6497 = 0.75%) were mcr-3 positive when verified by PCR and Sanger sequencing. Specifically, 3.45% (6/174) of samples from farmers were mcr-3 positive, followed by 2.20% (43/1951) from swine-derived samples. None of the mcr-3-positive samples were from patients or other healthy individuals. In addition, 14.02% (911/6497) samples were mcr-1-positive. In particular, mcr-3-positive samples were identified in 8 out of 13 provinces, namely Guangdong, Guangxi, Heilongjiang, Anhui, Hebei, Jiangxi, Hunan, and Sichuan (Fig. 1 and Table 1). The rate of incidence of mcr-3 was as low as 0.59% in the isolates from Guangxi to as high as 8.26% from Anhui (Fig. 1). No mcr-3 was detected in the neighboring provinces of Hebei, Jilin, Liaoning, Shaanxi, and Hainan (Fig. 1).

We randomly chose 8 isolates out of 49 mcr-3-positive samples for microbial analyses (Table S1). All the 8 mcr-3-carrying isolates were resistant to colistin and polymyxin B. Most of them were susceptible to cefotaxime (6 of 8 isolates), ceftazidime (7 of 8 isolates), cefepime (8 of 8 isolates), gentamicin (5 of 8 isolates), amikacin (8 of 8 isolates), ertapenem (7 of 8 isolates), imipenem (8 of 8 isolates), meropenem (8 of 8 isolates), fosfomycin (7 of 8 isolates), tigecycline (8 of 8 isolates), only some of the isolates remained susceptible to amoxicillin-clavulanate (1 of 8 isolates), nitrofurantoin (4 of 8 isolates), ciprofloxacin (4 of 8 isolates) and one isolate was susceptible to ampicillin (1 of 8 isolates) (Table S2). We further investigated the plasmids harboring mcr-3. In conjugation experiments, mcr-3-bearing plasmids were successfully transferred to E. coli C600 in 4 out of 8 mcr-3-carrying isolates, and 2 of 8 exhibited high conjugation rates ($10^{-3}$) (Table S1). Five isolates co-carried mcr-1, 2 of which were on plasmids and successfully transferred to E. coli C600. S1-PFGE and southern blotting of 8 mcr-3-positive isolates and their trans-conjugants showed that mcr-3 genes were located on plasmids with sizes of ~35-kb, ~48-kb, ~210-kb and ~240-kb, whereas mcr-3 genes of GDZJ005, GDZJ006, GDZJ007 and GDZJ008 were not successfully transferred to E. coli C600. The mcr-3 genes of GDZJ005, GDZJ006, GDZJ007 and GDZJ008 were located on plasmids, which is based on S1-PFGE and Southern blot (Fig. S1). In addition, we identified two novel variants of mcr-3 from 3 isolates, namely mcr-3.4 and mcr-3.7 (Table S2 and Fig. S4). This brings the number of mcr-3 variants up to eleven (Table S2).

3.2. Comparative Genomics of mcr-3-Harboring Plasmids

We succeeded in sequencing of MCR-3-producing plasmids pGDZJ002-1, pGDZJ003-2 and pGDZJ004 from GDZJ002, GDZJ003 and GDZJ004, respectively, and mcr-1-encoding plasmid pGDZJ003-1 from the GDZJ003. Of the two plasmids isolated from GDZJ003, whole-genome sequencing shows that plasmid pGDZJ003-2 is a 50.537 kb plasmid that contains 73 ORFs including the mcr-3.7 gene. MCR-3.7 has 18 amino acid substitutions when compared to MCR-3. This IncP-type plasmid is also seen to possess the tra and trb conjugal regions, partitioning modules par, replication initiator trfA, host-lethal protein encoding genes klc and their regulators kor and a toxin-antitoxin system higA-B (Fig. 2B). It shows very high sequence identity to another IncP-type mcr-3.7-bearing plasmid pMCR3_WCHEC-LL123 [54] (Accession no.: MF489760) isolated from human isolates of E. coli. However, unlike pMCR3_WCHEC-LL123, pGDZJ003-2 is missing the insertion sequence IS1294 (Fig. 2C) and does not have additional antibiotic resistance genes like blayam (conferring ampicillin resistance). When compared to another IncP type mcr-1-carrying plasmid, pMCR_1511 [93], pGDZJ003-2 also lacks the transposition unit of mcr-1 (ISAPl1-mcr-1-hp) on pMCR_1511 (Fig. 2D). The other plasmid from GDZJ003 is

![Fig. 1. Dissemination of mcr-3 (and/or its variants) in swines of China. Shandong Province where mcr-3 is initially detected [54] is highlighted with green boundary. Those with mcr-3-positive isolates in our study are labelled with red boundaries, whereas those without mcr-3-positive isolates are indicated with black boundaries. Of note, a mcr-3 variant, mcr-3.7, was recently detected in Sichuan Province of China [54].](image-url)
Fig. 2. Comparative genomics of the plasmids carrying mcr-3 and/or mcr-1 genes. A. Genomic map of the mcr-1-bearing plasmid pGDZJ003_mcr-1 that coexists with the mcr-3.7-harboring plasmid pGDZJ003_mcr-3.7 in E. coli. B. Schematic representation for circular genome of the mcr-3.7-harboring plasmid pGDZJ003_mcr-3.7. Circles (from inside to outside) separately denote the GC screw, GC content and the open-reading frames in both DNA strands [27]. The plasmid sequences were annotated by RAST, and the maps were generated using Circos program [27]. C. Colinear genomic analyses of three plasmids pGDZJ003_mcr-1, pSH146-32 and pCSZ4. D. Linear genome alignment of the mcr-3-positive plasmid pGDZJ003_mcr-3.7 with the other two plasmids (pMCR3_WCHEC-LL123 and pMCR_1511).
pGDZJ003-1 which harbors the mcr-1 gene and is 33,522 kb in length with a GC content of 42%. This plasmid contains 43 predicted ORFs, with 17 associated with formation of the type IV pilus (Fig. 2A). Comparative analysis shows that its backbone is very closely related to two narrow-host-range IncX4-type plasmids [94] pSH146-32 (Accession no.: JX258655) and the mcr-1-harboring plasmid pCSZ4 [95] (Accession no.: KX711706) (Fig. 2C). Like most IncX4 plasmids, both pCSZ4 and pGDZJ003–1 are missing the ISAp1 insertion sequence in front of the mcr-1 gene. Similarly, genome sequences for two other mcr-3-carrying plasmids, pGDZJ002-1 (50,818 kb; Fig. S2) and pGDZJ004 (50,519 kb; Fig. S3) were obtained. Both plasmids have a GC content of 47% and contain similar elements for replication, conjugation, and partitioning (Figs S2 and S3). Colinear genome analysis of pGDZJ003–1 and pMCr3_WCHEC-L123 shows that they possess similar genetic arrangements (Fig. S4) and belong to the IncP family of plasmids. Except for little bite of SNPs and deletions of short fragments, both pGDZJ002-1 and pGDZJ004 carry the mcr-3 gene within the same context as pGDZJ003–2 and pMCr3_WCHEC-L123, which carry the mcr-3 variant, mcr-3.7 instead. However, unlike pMCr3_WCHEC-L123, the other 3 plasmids do not contain IS1294 (Fig. S4). Together, these plasmids are additive members of the mcr-1-carrying (pGDZJ003-1) and mcr-3-carrying family of plasmids (pGDZJ002-1, pGDZJ003–2 and pGDZJ004). mcr-like genes have been increasingly identified on plasmids of multiple incompatibility groups within a wide range of bacterial hosts. Though these plasmids were isolated from diverse hosts, their frequent sharing of similar genetic elements suggests a common evolutionary origin.

3.3. Evolution of MCR-3

We utilized phylogenetic analysis to try and understand the evolution of MCR-3, which shares a closer homology to Neisseria EptA (53.1%) than MCR-1 (44.1%) using MCR-3 as a template, >500 homologs with >95% sequence coverage and 60% identity were identified via blastp and compared with representative protein sequences from the MCR-1/2/P families. The radial phylogram shows three broad groups within the MCR-3 family and their homologs and a fourth distinct and detached group consisting of the MCR 1/2 proteins and their progenitors. Paraphyletic branches within each subclade are indicated in different colors, MCR-3 variants (in light blue), MCR-1-like proteins (in blue), MCR-1/2 (in light green) and MCR-1/2 progenitors (in dark green). MCR-3 and MCR-1 is highlighted in bold font. Asterisk denotes >4 amino acid substitutions in comparison to MCR-3. The evolutionary history of MCR-3 was inferred using the maximum likelihood method. The trees presented here have been inferred from 1000 bootstrap replicates using a LG amino acid substitution model. The percentages of replicate trees in which the associated taxa are clustered in the bootstrap test (1000 replicates) are shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites with some evolutionarily invariant sites. Protein accession numbers of individual members have been indicated in the figure. Given that it is a putative member of PEA lipid transferase without detectable activity conferring colistin resistance, Z1140 of the E. coli O157:H7 EDL933 is used here as an internal reference for phylogeny.

![Phylogeny of MCR-3](image-url)
This branch is surrounded by other MCR-3-like genes that encode putative PE-transferases from *Aeromonas* species (Fig. 3A), primarily found in fresh and brackish water but also implicated in human infections. The second group consists of paraphyletic branches with PE-lipid A transferases from several bacterial genera including *Thalassospira*, *Rahnella*, *Hafnia*, *Edwardsiella*, *Buttiauxella* and *Enterobacter*, some of which are normal human commensals that have been reported to be resistant to a number of antibiotics (e.g., *Hafnia* spp.), while others (e.g., *Edwardsiella* spp.) are naturally resistant to colistin. The third group consists of other putative PE-lipid A transferases from *E. coli* and *Vibrio/Photobacterium* species. The marine bacterium *Photobacterium damselae* is a human and fish pathogen with colistin resistance [96]. The *E. coli* members of this group form a very tight subclade. One of these members, *E. coli* O157:H7 strain EDL933 containing a putative PEA-transferase Z1140 was tested experimentally and conferred no appreciable colistin resistance on LB agar plates (<0.5 µg/ml) (Fig. 5J). Swapping the transmembrane domain of MCR-3 with that from Z1140 still maintained activity (8 µg/ml) (Fig. 5J). The fourth subclade is further organized into 2 sub-groups: i) MCR-1/2 and their point mutant variants; ii) MCR-P (M) and other transitional intermediates of the MCR-1/2 family from *Moraxella* spp. (Fig. 3B). MCR-1/MCR-2 represent a group undergoing rapid evolution with the MCR-P (M) family serving as a potential reservoir of chromosomally-encoded transitional intermediates in the evolution of the MCR-1/2 family (Fig. 3B). Surprisingly, *Neisseria* EptA, a chromosomal colistin resistance determinant (Fig. 3A) is an evolutionarily-distant member.

The findings indicate that MCR-3 and its variants share a common ancestor with MCR-like proteins from *Aeromonas* species and constitute a rapidly evolving branch of transferable colistin resistance genes that are more closely related to chromosomally encoded intrinsic determinants found in commensal and environmental bacteria. *Aeromonas* species might occupy an unexplored niche in the marine ecosystem that is yet to be examined for colistin resistance. A complete biochemical and physiological study of MCR-3 is missing and would provide a better understanding of its evolutionary pattern.

3.4. Characterization of MCR-3 Intramembrane Protein

Predictions with the TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM) suggest that MCR-3 is an integral membrane protein possessing five N-terminal helices (Fig. 5A), which is almost identical to those of MCR-1 [27], MCR-2 [30] and EptA [19]. The hexa-histidine-tagged MCR-3 protein was tracked throughout the purification process (via Ni affinity chromatography) by Western blot with an anti-6× His antibody (Fig. 5A). The purified MCR-3 protein was visualized by 12% SDS-PAGE and its mass was estimated to be around 60 kDa (Fig. 5B). Its exact mass was then determined to be 61.3 kDa (Fig. 5C) by MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry) and its sequence identity was verified by peptide mass fingerprinting followed by MALDI-TOF MS with a 56.4% fragment coverage (Fig. 5D). Further, the secondary structure and folding properties of MCR-3 were determined using circular dichroism (CD). The spectrum obtained had negative dips at 208 nm and 222 nm, characteristic of alpha helices (Fig. S6E) and consistent with earlier observations made for MCR-1 [27] and MCR-2 [30]. More importantly, consistent with crystallographic evidence [27, 31–34], our results indicate the presence of protein bound zinc in full-length MCR-3 (Fig. S6F). This was measured using inductively coupled plasma mass spectrometry (ICP-MS). In agreement with our recent biophysical description of MCR-1 and its derivatives [72], MCR-3 seemed to be well expressed and purified to homogeneity while maintaining its structural integrity.

MCR-3 was modelled (Fig. S6G) against the structure of full-length EptA (PDB: 5FGN) [70] using Swiss-Model (https://swissmodel.expasy.org/interactive/qMEvX5/models/) [69] with a coverage of 98%. The model was found to retain a similar topology [70], despite possessing only 37.55% identity to EptA (Fig. S5B). As observed with EptA [70], the overall architecture of MCR-3 contains an N-terminal trans-membrane (TM) domain and a periplasm-facing catalytic domain at C-terminus (Fig. S6G). The catalytic domain has a hydrolase-fold (Fig. S6G) comprising 10 α-helices and 7 β-sheets (Figs S5B and S6G). This is connected to a TM domain containing six α-helices (Fig. S5A) by four short periplasmic loops (PH1, PH2, PH3 and PH4), a bridge helix (BH) and a long-coiled loop (Figs S5B and S6G). Certain residues (Figs S5B) within these structural elements are found to be deeply conserved via amino acid sequence alignments between MCR-1, MCR-2, EptA and MCR-3. These include five potential zinc-interacting residues (E238, T277, H375, D450 and H451, Fig 6A) and seven possible PE substrate-binding residues (N103, T107, E111, G322, K325, H380, and H463, Fig 6C–D), consistent with experimental evidence from EptA [70] and MCR-1 [72]. The roles of these residues in MCR-3 catalytic activity require further biochemical analysis.

3.5. Enzymology of MCR-3 Catalysis

In an in vitro reaction with a fluorescent substrate NBD-glycerol-3-PEA, (Fig. 5A–B), MCR-3 catalyzes the production of NBD-glycerol (Fig. 5C) from the substrate. NBD-glycerol-3-PEA (Fig. 5B) is observed as a faster migrating product when separated on a TLC. Hence, the presence of product was further confirmed by liquid chromatography mass spectrometry (LC/MS). This clearly demonstrated that MCR-3 can enzymatically catalyze the removal of the PE moiety from the lipid substrate in vitro like other well studied PE transferases such as MCR-1 and MCR-2. Through MALDI-TOF MS, this PEA-glycerol-3-PEA peak [m/z 1919.806 (Fig. 5E)] or 1919.832 (Fig. 5F) in addition to the unmodified lipid A peak [m/z 1796.665 (Fig. 5E) and 1796.541 (Fig. 5F)] demonstrates...
that the PEA moiety is enzymatically cleaved from the PE lipid substrate and is transferred to lipid A.

To unequivocally demonstrate that MCR-3 is responsible for the modification of lipid A in vivo, we engineered an E. coli MG1655 strain carrying an arabinose-inducible plasmid pBAD24-borne mcr-3. Through MALDI-TOF MS, lipid A isolates from MG1655 or MG1655 with an empty pBAD24 vector showed the presence of a single peak \([m/z 1796.46 \text{ or } 1796.377]\) corresponding to bis-phosphorylated hexa-acylated lipid A, similar to the unmodified peak observed in the clinical strains above (Fig 5E–F). Minor strain to strain variations were observed in the mass of unmodified lipid A. The expression of mcr-3 lead to the production of an additional peak at \(m/z 1920.803\) (Fig. 7C) corresponding to a single addition of a PEA group (mass difference of 123) to lipid A (Fig. 5D). The position of this addition may be at the 1 or 4′ position.

Given the similarity between the Thr280-PEA adduct seen in EptA \([70, 97]\) and its counterparts in reactions involving alkaline phosphatase-type phosphate transferases \([98]\) along with the in vivo

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Fig. 5. Enzymology of MCR-3. A. Scheme for the removal of PEA from an alternative substrate NBD-glycerol-3-PEA by MCR-3 to give the product of NBD-glycerol and an intermediate of MCR-3-bound PEA. PEA is an abbreviation of phosphoethanolamine. NBD was indicated in blue, and PEA was labelled in red. B. LC/MS determination of the alternative substrate of MCR-3, NBD-glycerol-3-PEA. C. LC/MS-based detection of the NBD-glycerol product from the hydrolysis reaction of the NBD-glycerol-3-PEA substrate by MCR-3. The inside gels denote the TLC assays of the NBD-glycerol-3-PEA substrate (in Panel B) and the MCR-3-catalyzed hydrolysis product, NBD-glycerol (in Panel C). NBD-glycerol-3-PEA is eluted at \(m/z\) of 814.1, whereas the resultant product NBD-glycerol is present at \(m/z\) of 691.5. Abbreviation: TLC, thin layer chromatography. D. Schematic illustration for generation of PPEA-4′-lipid A product from lipid A by transferring of PEA from MCR-3-bound PEA MALDI-TOF MS evidence for the chemical modification of the lipid A moieties of lipopolysaccharide (LPS) in GDZJ002, a clinical strain of mcr-3-positive E. coli strain (E) and its conjugant C600-GDZJ002 (F). The peak of the bis-phosphorylated hexa-acylated lipid A varies at \(m/z\) (1796.541–1796.665), whereas resultant derivative with PEA modification (PPEA-4′-lipid A) is at \(m/z\) (1919.632–1919.806).
and in vitro evidence presented before, it can be hypothesized that in the first half of the reaction, MCR-3 binds to the PE lipid substrate and releases diacyl glycerol, resulting in a MCR-3 bound PEA adduct (Fig. 5A). In the second half-reaction, PEA is transferred from this adduct to the 1(4′)-phosphate position of lipid A GlcN moieties, generating PPEA-4′-lipid A (Fig. 5D). Thus, MCR-3 might utilize a “ping-pong” mechanism of catalysis like that observed in EptA [70].

3.6. Mechanistic Insights into MCR-3 Polymyxin Resistance

To understand the relationship between the two domains of MCR-3 observed in the modelled structure and the evolution of its function from MCR-like enzymes such as EptA and MCR-1, we utilized domain swapping analysis to engineer four hybrid proteins as denoted below: i) TM1-MCR-3, MCR-3 catalytic domain and TM region of MCR-1; ii)
Fig. 7. Metabolic evidence that the PE lipid substrate-interactive cavity of MCR-3 participates in chemical modification of the lipid A moieties of lipopolysaccharides in E. coli. MALDI-TOF MS spectrum of the LPS-lipid A species isolated from the two negative controls, the E. coli strain MG1655 alone (A) and with the empty vector pBAD24 (B). C. Expression of MCR-3 in E. coli leads to the appearance of an additional peak of PPEA-4′-lipid A, the modified form of lipid A. The substitution of N103A (D) and T107A (E) in MCR-3 cannot completely impair the enzymatic activity in the structural modification of lipid A moieties. MALDI-TOF MS analyses confirm that the three point-mutants of MCR-3 [namely E111A (F), E238A (G) and T277A (H)] are nonfunctional in the transfer of PEA to lipid A species. I. The point mutation of MCR-3 (G322A) does not influence its enzymatic activity in the addition of PPEA to the 4′-phosphate group of lipid A moieties. The six point-mutants of MCR-3 whose enzymatic activities are fully inactivated include K325A (J), H375A (K), H380A (L), D450A (M), H451 (N) and H463A (O), respectively. The MS peak of lipid A species in E. coli is shown at m/z of 1796.305–1797.630, whereas its modified form occurs at m/z of 1919.585–1920.803, upon the presence of functional (and/or partial active) versions of mcr-3 in E. coli.
Fig. 8. A working model for MCR-3-mediated impairment of the hydroxyl radical death pathway in E. coli. A. Scheme for membrane disruption by the cationic antimicrobial polypeptide colistin and the resultant ROS production in E. coli. B. Functional expression of MCR-3 prevents the penetration of colistin into bacterial membrane and thereby attenuates the production of ROS in E. coli. It was modified appropriately from Wei et al. [44] with permission. C. Chemical rescue experiments reveal that Fenton reaction involves colistin-triggered hydroxyl radical killing pathway in E. coli. The LPS-lipid A moiety denotes the first target for colistin treatment. Bipyridine is a well-known ferric chelator, and L-cysteine is the ROS scavenger. D. Exposure to colistin boosts the accumulation of hydrogen peroxides in the negative control, the strain MG1655 of E. coli with the empty vector pBAD24. E. The expression of plasmid-borne MCR-3 attenuates the production of hydrogen peroxides in E. coli, regardless of the presence of colistin. An oxidant-sensitive dye, DCFH2-DA, was used to detect the level of intracellular ROS, which was oxidized by hydrogen peroxides into the fluorescent product of DCF. The fluorescence intensity of DCF was measured with a Zeiss LSM 510 Meta confocal laser scanning microscope (100× oil immersion objective). Hydrogen peroxides appear in green. F. Quantitative comparison of colistin-promoted ROS levels in E. coli with or without expression of mcr-3. Ratio of fluorescent cells was calculated through counting the number of cells with/without fluorescence. In each group, no <500 cells were counted from 4 individual photographs. The data was evaluated through one-way analysis of variance (ANOVA) as well as Tukey–Kramer multiple comparisons post hoc test.
TM3-MCR-1, MCR-1 catalytic domain with TM from MCR-3; iii) TM-MCR-3, MCR-3 catalytic domain with TM of EptA; iv) TM3-EptA, catalytic domain of EptA fused to TM3 of MCR-3 (Fig. 4A). The chimeric proteins expressed as well as the full-length proteins, as evaluated by western blot (Fig. 4B). Further, functional assays for resistance to colistin indicated that in E. coli MG1655, all four hybrids were susceptible to colistin in MIC assays [0.25 μg/ml] (Fig. 4C), indistinguishable from the negative controls (strain with either no plasmid or empty vector). In contrast, expression of the full-length proteins, MCR-1, EptA and MCR-3, allowed the E. coli strain to tolerate 4 μg/ml 2 μg/ml and 2 μg/ml of colistin, respectively (Fig. 4C). The incompatibility of the TM and PEA transferase domains of MCR-3 and EptA or MCR-1/2 (Fig. 4A) hints at an evolutionary distance between them (Fig. 3).

To obtain detailed experimental evidence regarding the specific residues responsible for PE substrate binding, we utilized molecular docking (MD) to analyze the modelled structure of MCR-3 with its physiological substrate, PE (Fig. 6A and C). By comparing similarly docked models of MCR-1, EptA and MCR-3, the catalytic role of these sites was experimentally confirmed upon the expression of MCR-3 (Figs 8A and B) through chemical rescue experiments, where the presence of the ferric chelator bipyridine significantly bypasses the effect of colistin treatment on mcr-3-negative E. coli (Fig. 8C). This is also true in the presence of the ROS scavenger, L-cysteine (Fig. 8C). Surprisingly, E. coli is prevented from entering the hydroxy radical death pathway during the expression of mcr-3 (or mcr-1/mcr-2) in the presence of colistin, independent of bipyridine and L-cysteine (Fig. 8C). To monitor the intracellular production of H2O2 species in E. coli with/without MCR-3 (or MCR-1/MCR-2), an oxidant-susceptible dye DCFH2-DA (2′,7′-dichlorodihydrofluorescin diacetate) was utilized and cells were monitored in a fluorescent field (Fig. 8D). As expected, treatment with colistin induced ROS production in E. coli MG1655 with an empty vector (Fig. 8D), which was significantly alleviated upon the expression of MCR-3 (Figs 8E and 9). MCR-3 expression, thus, seems to prevent the entry of colistin into the cells thereby quenching/alleviating ROS production in vivo, and consequently bypassing antibiotic killing by colistin (Fig. 8B).

3.7. MCR-3 Quenches Hydroxyl Radical Death Pathway

Colistin is known to activate a downstream hydroxyl radical mediated cellular death pathway [89, 99]. Recently, the intracellular level of ROS was found to be prevented by the presence of MCR-1/2 and its progenitor IC-IMo [44]. Thereby, we utilized two methods [chemical rescue assay (Fig. 8B) and confocal microscopy (Fig. 8C)] to evaluate the effect of MCR-3, another lipid A modifier on colistin-induced ROS formation. We show the involvement of the Fenton reaction in the production of free hydroxyl radicals (Fig. 8A and B) through chemical rescue experiments, where the presence of the ferric chelator bipyridine significantly bypasses the effect of colistin treatment on mcr-3-negative E. coli (Fig. 8C). This is also true in the presence of the ROS scavenger, L-cysteine (Fig. 8C). Surprisingly, E. coli is prevented from entering the hydroxy radical death pathway during the expression of mcr-3 (or mcr-1/mcr-2) in the presence of colistin, independent of bipyridine and L-cysteine (Fig. 8C). To monitor the intracellular production of H2O2 species in E. coli with/without MCR-3 (or MCR-1/MCR-2), an oxidant-susceptible dye DCFH2-DA (2′,7′-dichlorodihydrofluorescin diacetate) was utilized and cells were monitored in a fluorescent field (Fig. 8D).

To systematically compare the metabolic fitness of gut bacteria in response of expression of intrinsic and/or transferable colistin resistance gene, we engineered an array of derivatives of E. coli MG1655 [with the pBAD24 empty vector alone or harboring eptA/mcr-like gene, in Table S3]. The five strains used here separately included the negative control FY796 [MG1655/pBAD24] [72], the neisserial intrinsic colistin resistance gene epta-expressing strain FY832 [MG1655/pBAD24::epta] [79], and three mcr-like gene-bearing strains [FY795 [MG1655/pBAD24::mcr-1] [72], FY855 [MG1655/pBAD24::mcr-2] [79] & FY1125 [MG1655/pBAD24::mcr-3]]. As expected, the following four points are recorded: i) the addition of arabinose (an inducer of the arabino promoter of pBAD24) failed to exert significant effect on growth curves of MG1655 alone (Fig. 10A) or with the empty vector pBAD24 (Fig. 10B); ii) the expression of epta obviously interferes the growth of E. coli MG1655 even on the condition of induction with as low as 0.02% arabinose (Fig. 10C); iii) in general agreement with the observation of mcr-1 in TOP10 strain [87], the expression of MCR-1 (only induced by 0.2% arabinose) can greatly cause growth retardation of the recipient strain MG1655 (Fig. 10D); iv) the suppressed growth of gut bacterium MG1655 harboring either mcr-2 (Fig. 10E) or mcr-3 (Fig. 10F) is indistinguishable from that seen with mcr-1 (Fig. 10D).
Despite that its ability is comparable with that of eptA [72], BUT weaker than those of mcr-1/2 in rendering the recipient strain MG1655 resistant to polymyxin [79], the cellular effect of mcr-3 on bacterial growth is surprisingly seen to be similar to those of mcr-1/2 (Fig. 10D & E), but somewhat appreciably-less than that of eptA, an intrinsic determinant of colistin resistance (Fig. 10C).

To further address the unexpected scenarios aforementioned amongst MCR-1/2/3 and EptA, we conducted confocal microscopy-based investigation on these gut bacteria using the “LIVE/DEAD” assays of bacterial viability (Fig. 11). As predicted, low percentage of dead cells was visualized for the log-phase culture of MG1655 carrying pBAD24 (Fig. 11A and F), whereas appreciably-increased level of killed cells was observed upon the induced expression of EptA with 0.2% arabinose (Fig. 11B and G). As for every one of mcr-like genes (Fig. 11C–E) in MG1655, activation of its expression apparently produced metabolic pressure featuring with the decrement of bacterial viability (Fig. 11H–J). In general agreement with scenarios seen in the experiments of growth curves (Fig. 10), the side effects exerted by MCR-1/2/3 on bacterial viability are consistently less profound than that of EptA (Fig. 11K), BUT clearly stronger than that of the negative control strain with the empty vector alone (Fig. 11K). In summary, cellular effects of the three resistance proteins MCR-1/2/3 on bacterial metabolic fitness (at least bacterial growth and viability, if not all) are relatively comparable, despite they are in quite differentiation of gene sequence (Fig. S5) as well as its resultant phenotypic resistance level (Figs 4 and 6).

4. Discussion

More than 11 genetic variants of MCR-3 have been identified since its original discovery in the Shandong province of China. This study has now identified mcr-3 in bacterial isolates (Fig. 1) from 8 of the 13 provinces examined in China. This suggests a reasonable level of control in the spread of mcr partly caused by the ban of colistin use in
animal feedstock in China and by improved animal husbandry practices, though further studies would be necessary. A previous study reported a significantly higher prevalence of mcr-3 (>10%) from animals in 9 provinces in China, while the prevalence of mcr-3 is lower in this study [39]. The reasons could attribute to the different sampling time or/and the conditions of antibiotic resistance could be various in different farms.

All four randomly-selected mcr-3-positive clinical strains for comparative genomics of mcr-3-harboring plasmids also harbored the mcr-1 gene. In our study, all the three mcr-3-positive plasmids were identified through whole genome sequencing to be IncP type conjugable plasmids, while mcr-1 was on a non-conjugable IncX4-type plasmid. In fact, these 3 plasmids lack the IS1294 insertion element found on other mcr-3-bearing plasmids like pMCR3_ECHEC-LL123 or the ISApI insertion element found on mcr-1 plasmids like pMCR_1511. These insertion elements have been proposed to be involved in the initial mobilization of these genes through transposition. It has been shown that mcr-1 genes lacking insertion elements on either side must have lost them by abortive transposition and without at least a single downstream insertion element incapable of mobilization. Phylogenetically, MCR-3 is very distinct from MCR-1 and its variants (Fig 3A–B) and clusters within a larger family of MCR-3 like putative PE-lipid A transferases exclusively from the Aeromonas species. Interestingly, the neighboring sub-clades consist of putative PE-lipid A transferases from human commensal and naturally colistin-resistant bacteria from Hafnia and Edwardsiella species. Another neighboring sub-clade predominantly consists of PE-lipid A transferases from Vibrio species where a chromosomal system that attaches glycine to LPS-lipid A GlcN moieties and provides ‘intrinsic’ resistance to colistin has been described in Vibrio cholerae.

Taken together, we can be reasonably confident that MCR-3 and its variants might have a parallel evolutionary path to MCR-1 from a yet to be identified chromosomal progenitor. Despite that the co-occurrence of mcr-1 and mcr-3 on certain plasmids [59] raises the possibility of mcr-1/3 evolved through gene duplication events, the poor homology between MCR-1 and MCR-3 argues the aforementioned hypothesis. The divergence of these two groups is probably a distant event and hence, the current source of genetic diversity for each of these species might be different. Recently, Moraxella species have been proposed as reservoirs for MCR-1/2 species [43, 100]. A similar reservoir might exist in the Aeromonas (Fig. 3B), though little is known about the functionality of their MCR-like genes. Unique selection pressures in the environment might have triggered a recent mobilization of some of these intrinsic genes. Z1140 might be an excellent example of a protein caught in the act of evolving either through gain or loss of function over time (Fig. S9). This idea is further strengthened by the biochemical and physiological characterization of MCR-3 which shows that

![Fig. 11. Alteration in bacterial viability of E. coli by the expression of eptA and/or mcr-like genes.](image-url)
the two distinct protein domains of MCR-1 and MCR-2 are functionally exchangeable, while those in MCR-3 and MCR-1/EptA are not (Fig. 4). However, we show that MCR-3 and MCR-1/MCR-2/EptA share a common PE substrate binding cavity, which is essential for its activity and for conferring resistance to colistin (Fig S7). This indicates an evolutionarily conserved mechanism for both intrinsic and transferable polymyxin resistance wherein they share the same catalytic scheme involving a non-sequential or ‘ping-pong’ reaction mechanism. Further, MCR-3 displays similar abilities in both modifying lipid A structure and myxins like colistin have been shown to activate a downstream hydroxyl radical mediated cellular death pathway [89, 99], MCR-3 also displays similar abilities in both modifying lipid A structure and polymyxin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Antimicrob Agents Chemother 55 (7), 3730–3739.

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Author Contributions

YF and GT designed and supervised this project; YF, YX, LZ, JS, SS, JL and ZT performed experiments; YF, YX, LZ, MH, DP, JS, SS, JL, XL and ZT analyzed the data and prepared figures; YF, SS and GT drafted this manuscript.

Conflict of Interest

We declare that no interest conflict is present.

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