

In vitro reduction of colistin susceptibility and comparative genomics reveals multiple differences between MCR-positive and MCR-negative colistin-resistant *Escherichia coli*

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Objectives: Although resistance to colistin is increasingly reported from clinical settings, the genetic mechanisms that lead to colistin resistance in *Escherichia coli* have not been fully characterized. Here, we assess the evolution of colistin resistance in clinical isolates of mobilized colistin resistance (MCR)-negative and MCR-positive *Escherichia coli*.

Methods: Spontaneously mutated colistin-resistant progeny were evolved using a step-wise reduction of colistin susceptibility. Resistance phenotypes were confirmed by minimum inhibitory concentration (MIC) determination, and the probable resistance mechanisms were investigated using PCR and reverse transcription-quantitative PCR. Mutated genes of the laboratory-evolved mutants were identified by whole-genome sequencing and comparative genomics. Fitness costs and serum resistance of the mutants were also compared to the corresponding wild types.

Results: MCR-negative isolates displayed higher increases in MICs than did MCR-positive isolates following colistin exposure. Upregulation of *pmrAB* and associated genes was evident among MCR-negative isolates but not MCR-positive isolates. Comparative genomic analysis of mutants and their corresponding wild-types (WTs) revealed numerous mutations in genes encoding membrane transporters and two-component systems. Additionally, MCR-negative mutants exhibited higher fitness costs than MCR-positive mutants compared with their corresponding WTs but displayed similar serum resistance.

Conclusion: Our findings reveal multiple differences between MCR-positive and MCR-negative *E. coli* strains following colistin exposure, which provide reference values for clinical medication.

Keywords: colistin resistance, MCR, comparative genomic, *Escherichia coli*

Introduction

The interest in colistin (polymyxin E) as a treatment option and antibiotic of last resort has been revived, since the increasing incidence of infections caused by multidrug-resistant Gram-negative bacteria in clinical settings.¹ However, the emergence of plasmid-mediated colistin resistance via mobilized colistin resistance (*mcr*) genes has become a challenge to public health worldwide. MCR-positive *Enterobacteriaceae* have been isolated from various sources including humans, other animals, and food, most of which were *Escherichia coli*.²⁻⁵ Except for MCR, colistin resistance in Gram-negative bacteria can also be conferred by

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mutations in chromosomal genes associated with the biosynthesis and modification of lipopolysaccharide (LPS), and include a large array of genes and operons.^{6–8} Two different colistin resistance mechanisms caused by mutations in chromosomal genes have been described; the first involves the loss of LPS due to mutations in the *lpxABCD* genes, as demonstrated in *Acinetobacter baumannii*.^{8,9} The second mechanism entails mutations in genes involved in the regulation of LPS modifications and includes genes involved in PmrAB and PhoPQ two-component systems (2CSs), as well as the negative regulator gene *mgrB* in *Klebsiella pneumoniae*.^{10,11}

Previously described chromosomal mutations responsible for acquired colistin resistance in isolates of *E. coli* have been associated with amino acid changes in PmrAB and PhoPQ.^{1,10,12} In a recent study, we described for the first time a close to equivalent clinical isolation ratio of MCR-negative and MCR-positive colistin-resistant isolates of *E. coli*, with no demonstrable nosocomial transmission.¹³ Five unique amino acid substitutions were identified among these colistin-resistant MCR-negative *E. coli* strains, four in PmrB and one in PhoQ.¹³ However, amino acid changes in PmrAB and PhoPQ might not be essential for colistin resistance in *E. coli*. Mutations in other chromosomal genes and other non-transferable mechanisms conferring colistin resistance in *E. coli* isolates are likely to exist, but have not yet been elucidated; hence further study is warranted.

The clinical breakpoint of colistin is >2 mg/L for Enterobacteriaceae (EUCAST 2018), and *E. coli* producing MCR generally display MICs of 4–8 mg/L for colistin.¹³ However, MCR-negative colistin-resistant *E. coli* generally exhibit higher MICs (16–64 mg/L) than MCR-positive isolates without additional resistance mechanisms.¹³ We hypothesize that acquired colistin resistance due to chromosomal mutations is less likely to evolve in isolates producing MCR than isolates non-producing MCR. In the present study, we assess the evolution of colistin resistance in clinical isolates of MCR-negative and MCR-positive *E. coli*. The results deepen our understanding of colistin resistance mechanisms in MCR-positive and MCR-negative *E. coli* strains.

Methods

Bacterial isolates and antimicrobial susceptibility testing

Clinical *E. coli* isolates were identified using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Isolates evaluated in this study included

clinical isolates and laboratory-evolved colistin-resistant mutants (Table 1). The broth microdilution method was used for colistin MIC determination in accordance with the Clinical and Laboratory Standards Institute (CLSI-2018).

Laboratory evolution of mutants with reduced colistin susceptibility

A step-wise reduction in colistin susceptibility was performed according to a previous study.¹⁴ Briefly, a single colony of each clinical isolate was grown in Muller-Hinton (MH) broth overnight at 37°C, and cultures were diluted 1:100 in MH broth containing serially increasing concentrations of colistin, starting at the one-half MIC value of the respective isolate, and doubling every 24 hrs until bacterial growth was completely inhibited (with no bacteria growth after spreading 100 µL of the culture on MH agar plates supplemented with corresponding concentration of colistin). The concentration of colistin at which bacterial growth was completely inhibited was recorded as the final colistin concentration (FCC). Subsequently, overnight cultures were plated on MH agar plates containing one-half FCC. Twenty mutants of each WT strain were randomly selected from plates containing one-half FCC. The stability of the selected mutants was tested by serial passage in MH broth without colistin, and the colistin resistance phenotype was tested by the MICs. In addition, we measured the frequency of appearance of the colistin-resistant mutants under the first concentration of the step-wise induction, half MICs of the WT strains. Induced cultures were plated on MH agar with or without colistin (2 mg/L for MCR-negative group and 4 mg/L for the MCR-positive group). All the plates were incubated overnight at 37°C, and the colony counts on colistin-containing plates after 24 hrs of growth were regarded as the number of derived mutants.¹⁵

Whole-genome sequencing and bioinformatic analysis

Single colonies of WTs and their two laboratory-evolved mutants were cultured overnight at 37°C in MH broth, and genomic DNA was prepared using a Yeast/Bact. Kit (cat. no. 8,510,633; QIAGEN) according to the manufacturer's instructions. After library preparation, genomes were sequenced using the Illumina HiSeq 2500-PE150 platform (Illumina, San Diego, CA, USA). Quality-trimmed raw sequence data were assembled using Velvet 1.2.7. Annotation was performed by uploading data to the RAST server (rast.nmpdr.org). Sequence data from all isolates were deposited in GenBank

Table 1 Clinical information, minimal inhibitory concentration (MIC), mutated genes, and serum resistance (survival) of mobilized colistin resistance (MCR)-negative group and MCR-positive group isolates

Groups	Isolates	Isolate source	Colistin MIC (mg/L)	Final colistin concentration (FCC; mg/L)	PmrA	PmrB	Survival (%)
MCR-negative group	ATCC25922	Standard strain	0.25	64	–	–	43.10±3.12
	ATCC25922-1	Laboratory-evolved	64	– ^a	–	L17R	39.82±5.10
	ATCC25922-2	Laboratory-evolved	64	–	–	L17R	42.98±2.39
	1,273	Sputum	0.25	32	–	–	58.12±5.72
	1,273-1	Laboratory-evolved	32	–	–	Position 83 insert “IVPGVFMVSL”	49.23±3.59
	1,273-2	Laboratory-evolved	32	–	–	Position 83 insert “IVPGVFMVSL”	55.73±4.98
	1,876	Urine	0.5	32	–	–	45.23±5.54
	1,876-1	Laboratory-evolved	32	–	G53R	VI64M	48.11±2.34
	1,876-2	Laboratory-evolved	32	–	G53R	VI64M	50.38±6.75
	1,910	Fester	0.5	32	–	–	35.75±3.33
	1,910-1	Laboratory-evolved	32	–	G53S	–	33.76±2.35
	1,910-2	Laboratory-evolved	32	–	G53S	–	38.44±6.54
	MCR-positive group	16,802	Ascites	4	16	–	–
16,802-1		Laboratory-evolved	16	–	–	–	38.45±2.53
16,802-2		Laboratory-evolved	16	–	–	–	37.03±1.92
18,017		Urine	4	16	–	–	60.01±3.67
18,017-1		Laboratory-evolved	16	–	–	–	55.35±7.21
18,017-2		Laboratory-evolved	16	–	G53R	–	58.23±6.24
24,990		Urine	4	16	–	–	48.23±5.35
24,990-1		Laboratory-evolved	16	–	–	–	50.66±5.45
24,990-2		Laboratory-evolved	16	–	–	–	52.34±4.98
25,315		Ascites	4	16	–	–	28.35±3.33
25,315-1		Laboratory-evolved	16	–	–	–	32.34±5.38
25,315-2		Laboratory-evolved	16	–	–	–	35.12±6.73

Note:^aThe dashes in this table mean not mentioned or no mutations.

under the accession numbers indicated in Supplementary Table S1. Mapping and single-nucleotide polymorphism (SNP) detection were performed by breseq¹⁶ using *E. coli* ATCC 25,922 (GenBank: CP009072.1) as a reference. Regions containing the detected mutations were amplified by PCR using primers *EC-gene-F/R* and verified by Sanger sequencing where necessary (Supplementary Table S2). SMART analysis (<http://smart.embl-heidelberg.de/>) was performed to determine the domain architecture of target proteins. Sorting Intolerant From Tolerant (SIFT) scores (<http://sift.jcvi.org>) were calculated to predict whether amino acid changes affected protein function.

Reverse transcription-quantitative PCR (RT-qPCR)

Overnight cultures of WTs and their corresponding mutants were diluted 1:100 and subcultured in MH medium for ~3 hrs

at 37°C (absorbance at 600 nm [OD₆₀₀] ~0.6) without colistin. Cells were collected at 4°C by centrifuging at 10,000 rpm for 1 min, and RNA was extracted using TRIzol Reagent (Invitrogen). DNase I-treated RNA was obtained using an RNeasy Mini Kit (QIAGEN, No. 75,142), and mRNA expression levels of genes involved in colistin resistance were examined using real-time PCR primers (*EC-gene-RT-F/R*) listed in Supplementary Table S2. RT-qPCR was performed using an ABI 7300 96-well RT-qPCR system (Applied Biosystems) with SYBR Premix Ex Taq II (cat. no. RR820A; TaKaRa). Experiments were repeated three times. Expression levels of target genes were normalized against the 16S rRNA gene of *E. coli* using the standard curve method.

Fitness cost measurement

We determined the growth of WTs and mutants in MH broth respectively, before fitness measurement, to exclude the effect

of original growth. Fresh medium was inoculated with overnight cultures grown from a single colony by 1:100 dilution, and growth was determined by recording the optical density (OD) of cultures at OD₆₀₀. Fitness costs of mutants compared with their respective WT were determined by competition experiment as described previously.¹⁷ Briefly, overnight cultures of WT and their corresponding mutants were diluted 1:100 into fresh MH broth and cultivated to an OD₆₀₀ of 0.5. A 1:1 volumetric mixture of WT and corresponding mutants was diluted 1:400 into 10 mL of fresh MH medium with no colistin addition, and 200 µL of the diluted culture was removed and used for viable colony counting on MH agar. Viable colony counting was performed on MH agar both with and without colistin (2 mg/L in MCR-negative groups and 8 mg/L in MCR-positive groups). The remaining culture was incubated at 37°C for 20 hrs with shaking at 200 rpm, after which viable colony counting was performed again. Relative fitness was calculated as described previously.¹⁸

Serum resistance assay

Serum resistance assay was measured according to the previous study.¹⁹ Briefly, after cultured in MH broth for 14 hrs, the bacteria were washed and resuspended in phosphate-buffered saline to an OD of 1.0. Human serum from healthy individuals was mixed, and bacterial suspensions were then added to human serum to obtain a bacterial cell concentration of 1×10^7 CFU/mL. Then, samples were incubated at 37°C for 2 hrs. Viable counts were determined at 0 and 2 hrs time points. Three independent experiments were performed.

Statistical analysis

For statistical analysis, values are presented as means \pm standard deviation (SD). Rank-sum tests were performed for pair-wise comparisons of groups, and $p < 0.05$ (two-tailed) was considered significant.

Ethics statement

Serum was obtained from four healthy volunteers who gave their written informed consent to the study, which was approved by the medical ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

Results

MCR-negative isolates could be induced to higher FCCs than MCR-positive isolates

Two groups of *E. coli* were involved in this study; the colistin-susceptible, MCR-negative group includes ATCC

25,922 and three randomly selected clinical isolates (1,273, 1,876, and 1,910); the colistin-resistant, MCR-positive (harboring MCR-1) group includes four randomly selected clinical isolates (16,802, 18,017, 24,990, and 25,315). Colistin MICs of these eight isolates were measured (Table 1). In vitro reduction of colistin susceptibility was conducted. We measured the frequency of appearance of the resistant mutants under the first concentration of the step-wise induction and discovered no significant differences between MCR-positive group and MCR-negative group. Twenty mutants of each WT strain were randomly selected from the one-half FCC plates. Mutants from the same WT strain displayed the same MIC value after measuring in triplicate. The MICs of mutants from the MCR-negative group ranged from 32 to 64 mg/L, whereas MICs for mutants from the MCR-positive group were 8–16 mg/L. Thus, MICs of mutants from the MCR-negative group were higher than those from the MCR-positive group ($p < 0.01$). Isolates from the MCR-negative group displayed higher FCCs than isolates from the MCR-positive group (Table 1). We then randomly selected two mutants (labeled -1 and -2) of each WT strain (Table 1) for further study.

MCR-negative mutants possess mutations in *pmrAB*

Mutations in the 2CS genes *pmrAB* and *phoPQ*, and the PhoPQ negative regulator gene *mgrB*, which are known to engender colistin resistance via their role in LPS modification, were analyzed. WT and mutant sequences were compared, and mutations were confirmed by PCR using primers described previously¹³ (Supplementary Table S2). Most of the mutations were synonymous. Surprisingly, amino acid alterations were observed not in PhoP, PhoQ, or MgrB, but in PmrA and PmrB. Specifically, four out of five mutations in PmrA and all six mutations in PmrB were found in the MCR-negative group, but only one mutation in PmrA was found in the MCR-positive group (Table 1). Two amino acid alterations, G53R (isolates 1,876-1, 1,876-2, and 1,8017-2) and G53S (isolates 1,910-1 and 1,910-2) were observed in PmrA (Table 1). SMART analysis indicated that position 53 of the transcriptional regulator PmrA is within the receiver domain (REC), which is important for the recognition of its regulons. Two amino acid alterations, L17R (ATCC 25,922-1 and ATCC 25,922-2) and V164M (1,876-1 and 1,876-2), were found in PmrB (Table 1). These two positions lie within a transmembrane region and the histidine kinase (HisK) domain, respectively. L17R and V164M were predicted to

affect the function of PmrB based on SIFT score (<0.05). Moreover, a 30 bp intragenic tandem repeat (TTGTCCC CGGCGTCTTTATGGTCAGCCTGA) was observed in the *pmrB* gene in isolates 1,273–1 and 1,273–2. This repeat generates a 10 amino acid peptide (IVPGVFMVSL) insert from position 83 of PmrB.

Expression of *pmrB* and several *pmrB* regulated genes are elevated in MCR-negative mutants, but not in all MCR-positive mutants

Expression levels of *pmrAB*, *phoPQ*, and *mgrB* in MCR-negative and MCR-positive mutants were compared with those in their corresponding WT. No discernable changes in expression levels of *phoPQ* and *mgrB* were observed in any pairs. However, differences between mutants and WT isolates were observed for *pmrA* and *pmrB* (Figure 1A–B); relative expression in MCR-negative mutants was higher than in WT. By contrast, no regular pattern in the expression of *pmrA* and *pmrB* was observed for MCR-positive isolates.

The PmrAB 2CS regulates a set of genes associated with the chemical alteration of LPS, which in turn affects colistin susceptibility in *E. coli*. These regulated genes including *eptA* (phosphoethanolamine transferase), the *arnB* operon (polymyxin resistance-associated operon), and *yibD* (galactosyltransferase). As expected, expression of PmrB regulated genes was increased when the *pmrB* expression was increased (Figure 1C–E). Expression levels of *eptA*, *arnB*, and *yibD* were significantly higher in MCR-negative mutants than in their corresponding WT ($p < 0.05$). This was not the case for the MCR-positive group.

Comparative genomics reveals the importance of membrane transporters and 2CSs for acquired, chromosomal colistin resistance

In addition to known colistin resistance-related genes in *E. coli*, other chromosomal genes associated with colistin resistance can be explored using comparative genomics. All 16 selected mutants and their WT strains were whole genome-sequenced. Nucleotide sequences of other genes encoding proteins associated with LPS modifications (*arnC*, *arnT*, and *pmrDGJML*) were analyzed, but no point mutations or sequence inserts were found.

Furthermore, nucleotide sequences of genes associated with colistin resistance via loss of LPS (*lpxA*, *lpxB*, *lpxC*, and *lpxD*) were also analyzed, but amino acid alterations were not present in mutants.

To investigate the diversity of mutations potentially responsible for increasing colistin MICs in the laboratory-evolved mutants, pair-wise genetic relatedness among mutants and their corresponding WT was analyzed using whole-genome SNP data. A total of 333 genes carrying non-synonymous SNPs among the 16 mutants were identified (Supplementary Table S3). 213 and 202 non-synonymous SNPs were detected in mutants from MCR-negative and MCR-positive groups, respectively. Eighty-two genes have non-synonymous SNPs in both groups. No overlapped genes were discovered in the top 10% mutation ratio (number of non-synonymous SNPs/nucleotide length) genes in these two groups (Supplementary Table S4), indicating diversity and differentiation of SNPs between MCR-negative and MCR-positive mutants. One hundred and forty genes with non-synonymous SNPs were identified using Kyoto Encyclopedia of Genes and Genomes classification (Supplementary Table S5). The most common were metabolism-associated genes ($n=41$), followed by membrane transporters ($n=18$), DNA replication and repair genes ($n=15$), and toxin-antitoxin (TA) system genes ($n=13$; Supplementary Table S5). In proportion to the number of genes in these categories in the *E. coli* genome, mutations in metabolism-associated genes, DNA replication and repair genes, and TA system genes were much less prevalent ($<10\%$ according to the UniProt database), while mutations in membrane transporter genes were more abundant ($>10\%$). Notably, 18 membrane transporter genes were classified, including OmpA family proteins and efflux pump systems such as CusC and NepI (Table 2).

Due to the important contributions of 2CSs in colistin resistance, we paid particular attention to other mutated 2CSs in the mutants. In addition to PmrA/PmrB, there were non-synonymous mutations in three other 2CSs: EvgS/EvgA (regulation of MDR transporters), AtoS/AtoC 2CS (associated with calcium channels), and KdpE/KdpD (regulation of potassium transporters; Table 2). The four 2CSs mutated in this study account for more than 10% of all 30 2CSs present in *E. coli* (<http://www.p2cs.org/>). These data manifest an important role of membrane transporters and 2CSs for acquired, chromosomal colistin resistance, especially proteins mutated in both groups, including FadL, OmpX, and EvgS (Table 2), which deserves further study.

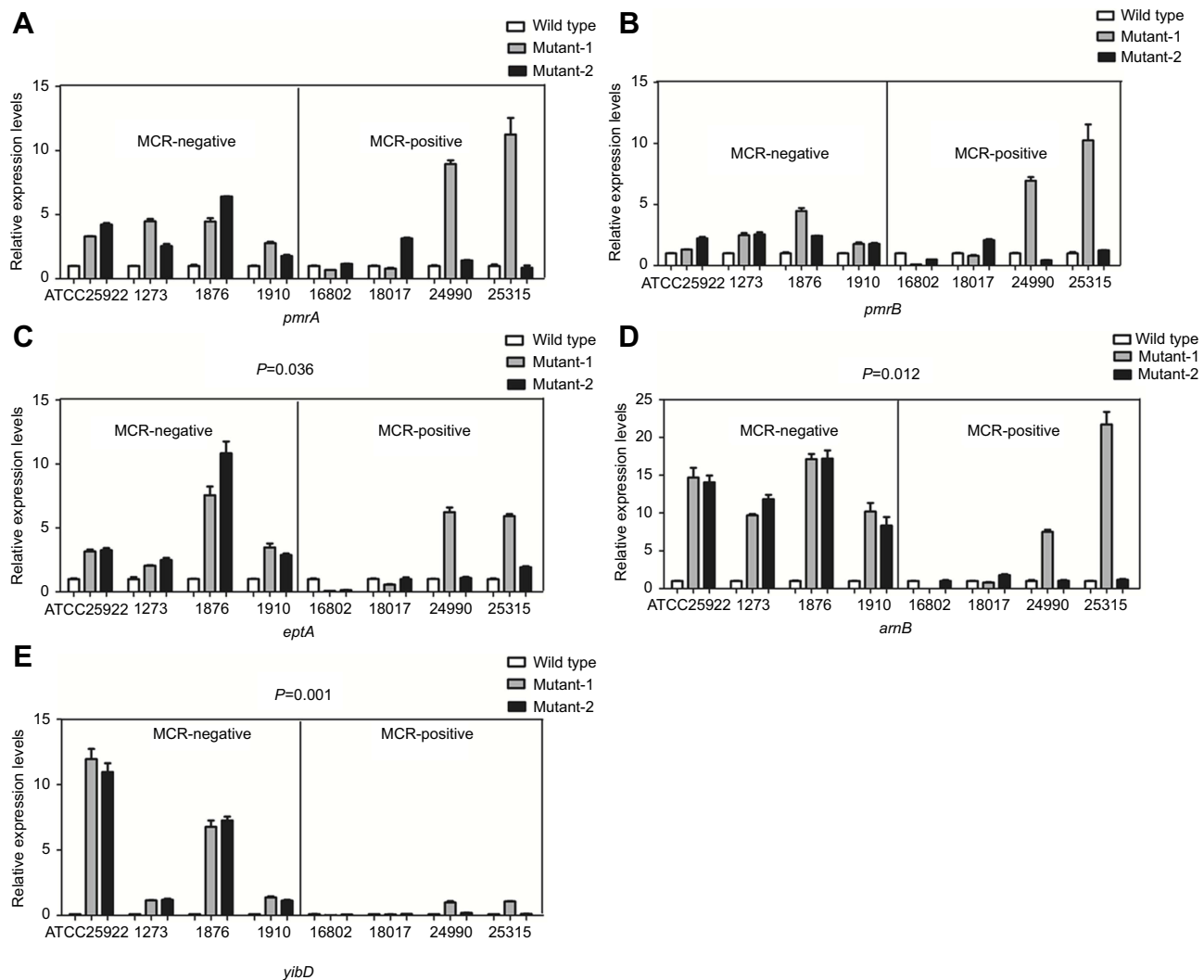


Figure 1 Relative expression levels of *pmrA* (A), *pmrB* (B), *eptA* (C), *arnB* (D), and *yibD* (E) in wild-type (WT) *E. coli* and their corresponding laboratory-evolved mutants. Gene expression levels of WTs served as controls, and expression levels of laboratory-evolved mutants were compared with those of their respective WTs. Mobilized colistin resistance (MCR)-negative and MCR-positive groups were compared using *p*-values calculated by Statistical Package for the Social Sciences (SPSS) using rank-sum tests (shown in the figure). All experiments were performed in triplicate.

MCR-positive mutants exhibit lower fitness costs than MCR-negative mutants, but display similar serum resistance

To investigate the effects of the mutations on *E. coli* fitness, growth competition experiments between mutant and WTs were conducted after confirmation of no original growth differences between WTs and their corresponding mutants. The fitness costs of MCR-negative mutants vs corresponding WTs were higher than those of MCR-positive mutants vs corresponding WTs ($p < 0.01$), and the relative fitness ranged from 0.42 to 1.09 among isolates (Figure 2). Furthermore, mutants of ATCC 25,922 displayed more severe fitness costs than other mutants from the MCR-negative group ($p < 0.05$; Figure 2).

To analyze whether the survival in human blood of the mutants is different from the WTs, serum resistance analysis was conducted. The results of serum resistance assays are shown in Table 1 as survival of mutants compared with WTs. There were no obvious differences in survival between WTs and mutants in both MCR-negative and MCR-positive groups. In conclusion, mutants from the MCR-positive group exhibited lower fitness costs than mutants from the MCR-negative group, and displayed similar serum resistance.

Discussion

In this study, colistin MICs of in vitro mutants of *E. coli* are lower than those of other bacteria such as *A. baumannii* (>256 mg/L). A plausible explanation for

Table 2 Mutated membrane transporter genes and two-component system genes

Genes	Classification	Gene function	Non-synonymous SNPs	
			MCR-negative group	MCR-positive group
AIL14573.1	Transporters	OmpA-like transmembrane domain protein	0	5
AIL15489.1	Transporters	FadL; long-chain fatty acid transport protein	5	10
AIL15800.1	Transporters	YfbK; Ca-activated chloride channel homolog	0	4
AIL15843.1	Transporters	Autotransporter beta-domain protein	22	0
AIL16156.1	Transporters	Nepl; MFS transporter, DHAI family, purine ribonucleoside efflux pump	2	0
AIL16412.1	Transporters	TonB-dependent siderophore receptor family protein	0	4
AIL16812.1	Transporters	YgiS; peptide/nickel transport system substrate-binding protein	0	2
AIL16890.1	Transporters	Autotransporter beta-domain protein	7	0
AIL16901.1	Transporters	Autotransporter beta-domain protein	4	0
AIL17136.1	Transporters	Outer membrane autotransporter barrel domain protein	2	0
AIL17178.1	Transporters	LamB; maltoporin	2	0
AIL17436.1	Transporters	DsdX; D-serine transporter	4	0
AIL17627.1	Transporters	CusC; cation efflux system protein CusC	0	2
AIL17645.1	Transporters	OmpX; outer membrane protein X	6	2
AIL18234.1	Transporters	Outer membrane autotransporter barrel domain protein	0	13
AIL18250.1	Transporters	OmpD; outer membrane porin protein ompD	0	4
AIL18257.1	Transporters	OmpA family protein	2	0
AIL18453.1	Transporters	GspJ; general secretion pathway protein J	0	2
AIL14041.1	Two-component system	KdpE; OmpR family, KDP operon response regulator KdpE	0	7
AIL14161.1	Two-component system	AtoC; NtrC family, response regulator AtoC	0	2
AIL15737.1	Two-component system	EvgS, NarL family, sensor histidine kinase EvgS	6	4
AIL15941.1	Two-component system	PmrA; antimicrobial peptide resistance response regulator	4	1
AIL18507.1	Two-component system	PmrB; OmpR family, sensor histidine kinase BasS	4	0

Abbreviation: MCR, mobilized colistin resistance.

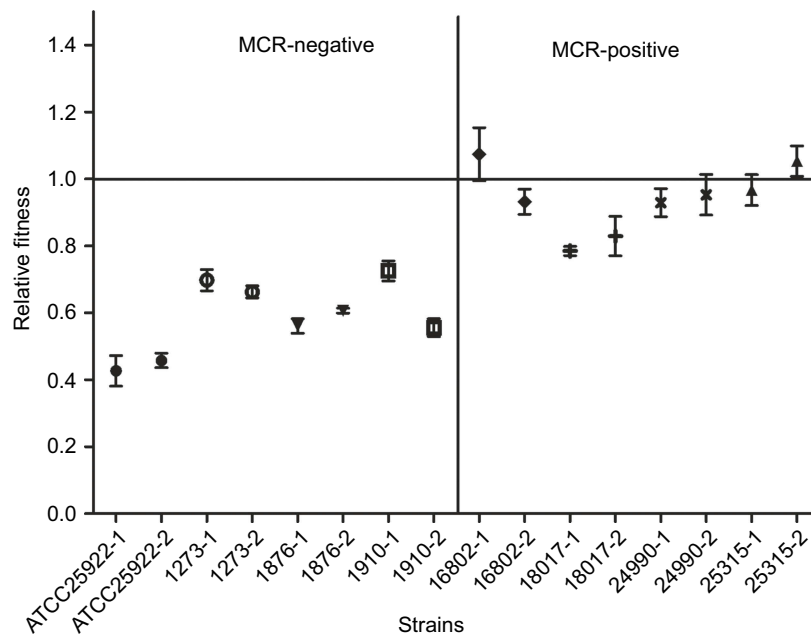


Figure 2 Relative fitness of wild-types (WTs) and corresponding laboratory-evolved mutants. The initial ratio between WTs and corresponding mutants was 1:1. Growth competition between WTs and mutants was measured as described in the Materials and Methods, and all experiments were performed in triplicate.

Abbreviation: MCR, mobilized colistin resistance.

this may be that loss of LPS in *E. coli* could lead to cell death, whereas this is not the case for *A. baumannii*.²⁰ In other words, loss of LPS may be the mechanism engendering high colistin MICs, whereas modification of LPS leads to lower MICs. In addition, MCR-negative isolates could be induced to higher FCCs than MCR-positive isolates, which indicates that MCR has the capacity to protect bacteria to be induced to high-level colistin resistant mutants from exposure to colistin in vitro.

Isolates in the MCR-negative group tended to possess amino acid changes in chromosomal genes related to colistin resistance, particularly *pmrA* and *pmrB*. The lack of such mutations in MCR-positive group indicates that isolates producing MCR are less likely to evolve chromosomally mediated colistin resistance, resulting in lower MICs compared with MCR-negative group. This could explain why MCR-positive isolates tend to have lower MICs in the previous study.¹³ Mutations observed in PmrB in this study have not been described in the previous studies.

In the DNA level, we discovered that the mutated position of the mutants focuses on PmrAB in the MCR-negative group, while the MCR-positive group possessed less PmrAB mutations (Table 1). In the expression level, we found that expression of *pmrAB* and *pmrAB* regulated genes was elevated in all MCR-negative mutants, but it was not always the case in the MCR-positive group. These results suggest that mutations in the sensor kinase PmrB, which might lead to altered expression of the PmrB-regulated genes, could be a common colistin resistance mechanism in *E. coli*. Furthermore, we hypothesize that the differences of these two groups might be because of the expression of MCR, a phosphoethanolamine transferase, could feedback to PmrAB, and affect the expression of *pmrAB* (PmrB regulates the chromosomal phosphoethanolamine transferase in *E. coli*). The differences expression pattern of *pmrAB* and its regulated genes in these two groups indicated that MCR might affect the regulation mechanism of colistin resistance. Further study is needed, especially focusing on the role of MCR in the colistin resistant regulation. The significant discordance in the expression of each gene (Figure 1 A–E) between mutant –1 and –2 for *mcr-1* isolates 24,990 and 25,315 was observed. The mechanisms for this phenomenon may be because of the different gene mutations other than the known genes between these mutants.

In addition to mutations in genes known to be associated with colistin resistance, mutations in several other genes were also observed in the comparative genomic

analysis. Herein, we paid more attention to genes potentially encoding outer membrane components, the target site of polymyxins. Several studies suggest that membrane transporters are involved in colistin resistance,^{21,22,23,24} but their roles remain poorly understood. In this study, the high frequency of mutations in membrane transporter genes among mutants indicated there may be an important role for membrane transporters in acquired, chromosomal colistin resistance. It is likely that resistance via chromosomal mutations often results from a combination of resistance mechanisms, such as defects in outer membrane proteins combined with a structural modification of LPS. Additionally, the frequency of mutations in genes associated with 2CSs was observed, including EvgS/EvgA, AtoS/AtoC, and KdpE/KdpD. EvgS/EvgA regulates acid resistance genes²⁵ and multidrug efflux pump genes.²⁶ AtoS/AtoC and KdpE/KdpD are associated with cationic iron transporters. When colistin binds to the cell membrane of bacteria, the negatively charged outer membrane is disrupted through electrostatic binding of positively charged regions of colistin.²⁷ Malfunction of cationic iron transporters may increase the concentration of cationic iron around the cell, potentially shielding the outer membrane from neutralization by colistin, and thus reducing the cell's susceptibility to colistin. These results indicate that membrane transporters and 2CSs play important roles in acquired, chromosomal colistin resistance in *E. coli*, and provide a potentially interesting area for further study.

Mutations in MCR-negative mutants resulted in a considerably higher cost in terms of fitness than those in MCR-positive mutants compared with their corresponding WTs, a phenomenon that may be associated with the higher colistin MICs of the MCR-negative group mutants. Mutations with higher colistin MICs possessed higher fitness costs were also found in other bacteria, such as *A. baumannii*.²⁸ Extracellular polysaccharides such as LPS and capsule (K antigen) contribute to virulence in many bacterial pathogens by providing resistance to phagocytosis and protecting against complement-mediated killing, which is often measured by serum resistance. However, mutants in both MCR-negative and MCR-positive groups displayed similar serum resistance to their corresponding WTs, suggesting similar immune defense systems in response to infection.

As the last-line therapy against Gram-negative infection, polymyxins are used when all other treatment modalities have failed. In our study, the colistin susceptibility

of MCR-negative and MCR-positive *E. coli* isolates could be reduced easily in vitro. These results may to some degree, explain the previously reported fact that the mortality was high with polymyxin monotherapy.²⁹ For another, our in vitro data revealed a tendency of induced colistin resistant mutants of MCR-negative *E. coli* to display high colistin MICs, which might provide reference values for polymyxin dosing in clinical medication, because current polymyxin dosing recommended are largely empirical.³⁰

Conclusion

Our experimental data reveal multiple differences between colistin susceptibility-reduced mutants of MCR-positive and MCR-negative *E. coli*, including a tendency for mutants of MCR-negative *E. coli* to display higher colistin MICs, which provide reference values for clinical medication. Until now, the colistin resistance mechanisms of MCR-positive and MCR-negative *E. coli* still need further studies.

Nucleotide Sequence Accession Numbers

The whole genome sequences described in this paper have been deposited in DDBJ/ENA/GenBank under the accession numbers QYJU00000000-QYKR00000000, (BioProject: PRJNA488336). The accession numbers and the corresponding isolates are listed in supplementary Table S1.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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